The Immune System Of The Female Genital Tract

The Effects Of Human Immunodeficiency Virus Infection

A Thesis Submitted For The Degree Of Doctor In Medicine

By

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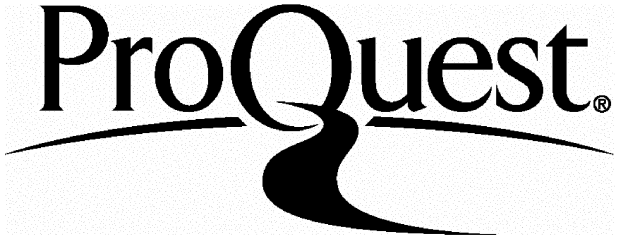

Erratum

The area refered to in this thesis as the 'Squamo-columnar junction' should more properly be described as the 'Transformation zone.'

ABSTRACT

This project was designed to investigate the immune function of the healthy female cervix and to determine how this may be affected in human immunodeficiency virus (HIV) disease.

Immunohistochemical, in situ hybridisation and polymerase chain reaction techniques were applied to determine the distribution of immunocompetent cells, the cytokine profile and HIV load in cervical biopsies obtained at colposcopy from 40 HIV-positive and 20 HIV-negative women. The findings were correlated with peripheral immune status, as determined by peripheral CD4 lymphocyte count and HIV load.

Cervical biopsy sections from HIV-positive women showed significantly decreased Langerhans' cell counts in the epithelium and significantly increased T lymphocytes in the sub-epithelial stroma compared with HIV-negative women. There was an increase in CD8+ lymphocytes in sections from HIV-positive women, leading to an inversion of the CD4/CD8 ratio compared with HIV-negative women. The majority of these CD8+ cells were 'primed' (CD45ro+) but they showed a reduced expression of cytolytic granules (perforin negative, low TIA-1) and impaired survival ability (Bcl-2 low). These changes occurred in advance of systemic immunosuppression.

The cervical biopsy sections from HIV-infected women had decreased mRNA for the Th-1 cytokine, Interleukin-2 (IL-2) and increased IL-4, IL-5, IL-10 mRNA (Th-2 cytokines) compared with HIV-negative women, but there was no significant

difference in Interferon-γ mRNA. Viral load studies showed a higher HIV in cervical tissue than in serum. There was no correlation between HIV disease stage and cervical cytokine mRNA or viral load.

These observations suggest that HIV-infected women mount an impaired cytotoxic lymphocyte response at a local level which may affect their ability to resist genital tract infections and cervical neoplastic change. Increased production of inhibitory cytokines (IL-4 and IL-10) may, in part, account for the chronicity of the virus in cervical tissues. High viral load in cervical tissues may contribute to the high risk of vertical transmission in the peripartum period.

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Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
BAL	Bronchoalveolar lavage
BSA	Bovine Serum Albumin
CIN	Cervical intraepithelial neoplasia
CMI	Cell Mediated Immunity
DAB	Diamino-benzidine
DNA	Deoxy-ribonucleic acid
GC	Gonococcus
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
IVDU	Intravenous Drug Users
HSV	Herpes Simplex Virus
MALT	Mucosal-Associated Lymphoid Tissue
MHC	Major Histocompatibility Complex
MoAb	Monoclonal Antibody
NHS	Normal Human Serum
NRS	Normal Rabbit Serum
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
RNA	Ribonucleic Acid
s-IgA	Surface Immunoglobulin A
SIV	Simian Immunodeficiency Virus

Abbreviations (continued)

STD	Sexually Transmitted Disease
TBS	Tris Buffered Saline

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CHAPTER 1: INTRODUCTION

Part 1: Background & epidemiology of HIV in women.

The acquired immune deficiency syndrome (AIDS) was first recognised in male homosexuals in 1981(Morbidity & Mortality Weekly Reporting, 1981). Although in the early years of the epidemic, the majority of cases were diagnosed among this patient group, it was soon realised that the disease also affected intravenous drug users, haemophiliacs and other recipients of blood products, heterosexual men and women, and children born to infected mothers. The discovery of the causative organism, the human immunodeficiency virus (HIV), and of a reliable antibody test to detect infected individuals, has allowed a clearer view of the pandemic.

The global spread of AIDS has now been recognised and, as of December 1995, a cumulative total of 1,291,810 AIDS cases had been reported to the World Health Organisation (WHO) from nearly 200 countries. The United Kingdom had reported 11,494 cases. It is recognised that AIDS case diagnosis and reporting is relatively weak in most developing countries and it is estimated from mathematical modelling that the 1,291,810 reported cases are derived from a global cumulative total of approximately 6 million AIDS cases in adults and children, of which 75% are estimated to have occurred in Sub-Saharan Africa (Nicoll & Simms, 1996). The dynamic nature of the HIV epidemic and the long period from HIV infection to the development of AIDS

mean that the distribution of prevalent HIV infection is different from that of cumulative AIDS cases. The WHO estimates that globally, by the end of 1994, there were 17 million adults living with HIV infection, with the largest number of cases again coming from Africa (11.2 million, 66%). Europe is estimated to have a prevalence of 450,000 HIV infections, 3% of the global total.

In developed countries, HIV infection has remained relatively confined to defined risk groups with a marked male predominance but in sub-Saharan Africa, it is more widespread, with an equal number of men and women being infected. However, heterosexually-acquired HIV infection has become increasingly important in developed countries and AIDS has become a major cause of illness and death among women and children. In the USA in 1993, AIDS was the fourth leading cause of death among women 25-44 years of age (National centre for health statistics, 1993), and the 7th leading cause of death in 1992 among children aged one to four years, nearly all of whom acquired infection perinatally (National centre for health statistics, 1992).

In the UK, data from the anonymous unlinked antenatal screening program has shown a rise in the prevalence of HIV infection between 1990 and 1993, particularly in the inner London area, with the highest prevalence (0.30%) among women aged 20 to 30 years (Nicoll *et al,* 1994). A recent cross-sectional study of HIV infection in women in Britain and Ireland showed that 65% of infected women were white and 29% black African. The majority were infected through heterosexual intercourse (Study group for

the MRC collaborative study, 1996). The realisation that the epidemic is no longer confined to homosexual men has led to greater interest in the natural history of HIV in women, and the heterosexual and vertical transmission of the virus.

Part 2: Natural History of HIV in women

Much of what is known of the natural history of HIV has been learned through the prospective study of large cohorts of gay men, as they still form the largest group of HIV-infected individuals in the developed world, and also account for the majority of AIDS patients. Since the development of the HIV epidemic in women is still recent, the description of HIV disease in women consists largely of cross-sectional or retrospective reports (Sha *et al,* 1995, Mulcahy *et al,* 1994).The mean incubation period for AIDS is estimated to be about 8-10 years from time of seroconversion (Rutherford *et al*, 1990), and no differences in rate of progression have been observed between men and women. The dynamic CD4 lymphocyte count decrease is similar, whatever the gender (von Overbeck *et al,* 1994, Melnick *et al*, 1994).

With the exception of Kaposi's sarcoma, which occurs more frequently in homosexual men (23%), and rarely in Caucasian women (1%), there is no difference in AIDS defining conditions between men and women (Saltzman *et al*, 1988). Older age at seroconversion is the only consistent adverse prognostic factor (Medley *et al*, 1987, Downs *et al,* 1991) and this is common to both sexes. The areas specific to women are

pregnancy, childbirth and gynaecological disorders, including cervical intraepithelial neoplasia, and these are now considered in turn.

Pregnancy, childbirth and vertical transmission

The WHO estimates that by the end of the decade 10 million children world-wide will be infected with the human immunodeficiency virus, with the majority acquiring their infection from HIV infected mothers (Chin, 1990). The largest follow-up study of pregnancy with HIV infection has reported a vertical transmission rate of 14.4% in the western world (European Collaborative Study, 1994). Higher transmission rates have been reported in Africa (Datta *et al*, 1994). While the ideal approach to reducing childhood HIV infection is to eliminate maternal HIV infection, it is unlikely that this will be feasible in the near future. Attention has therefore been focused on strategies to eliminate vertical transmission.

There is a wealth of circumstantial evidence to suggest that perinatal transmission occurs principally during the peripartum period, and most obstetric interventions are being targeted to this stage. There is some evidence, however, that intrapartum transmission does occur. HIV RNA has been detected by polymerase chain reaction (PCR) in aborted foetuses from HIV infected mothers as early as 16 weeks gestation (Courgnand *et al*, 1991). However, Brossard *et al* (1995) were able to detect HIV in only two of 100 aborted foetuses from HIV infected mothers. The first foetus, whose

mother had advanced AIDS, had died in utero and the second died following extremely premature delivery in a pregnancy complicated by repeated bleeding. They therefore concluded that the frequency of early in-utero HIV transmission is low and that specific risk factors may have implications in the occurrence of early, as opposed to late transmission.

Most of the evidence currently available supports the theory that early intrauterine transmission of HIV occurs rarely. The absence of HIV-specific congenital malformations (Qazi *et al,* 1988), and the relative infrequency of other clinical stigmata consistent with intrapartum infection such as intrauterine growth retardation (Italian multicentre study, 1988) would indicate that HIV transmission occurs primarily in the peripartum period. Retrospective evidence from the European collaborative study (1994) indicating that Caesarean Section may reduce the risk of vertical transmission (odds ratio:0.56) is consistent with intrapartum transmission of HIV. Perhaps the most striking evidence comes from the International Registry of HIV-Exposed Twins which showed that in 115 twin sets delivered vaginally, the first twin had a 2.8-fold greater risk of infection than the second twin (Goedert *et al,* 1991). The greater risk for the first twin may be related to more prolonged exposure to maternal fluids during labour.

The theory of intrapartum transmission of HIV would imply that the infectious viral agent is present in genital tract secretions or blood. Cervico-vaginal secretions from

non-menstruating women have been found to contain both cell-free and cell-associated virus (Voght *et al,* 1986; Clemetson *et al,* 1993) and studies indicate that the presence and amount of HIV in genital tract secretions may be higher in pregnant than in non-pregnant HIV-infected women (Henin *et al,* 1993). Although recent data suggest a correlation between peripheral viral load and perinatal transmission, the exact relationship between viral load in the genital tract and in the peripheral blood is unknown and yet may be more relevant to our understanding of vertical transmission of HIV.

The risk of vertical transmission is increased by advancing maternal disease (p24 antigenaemia, CD4 count<700/μm), and this may reflect increased systemic viral load (European Collaborative Study, 1992). HIV seroconversion during pregnancy is associated with a higher risk of vertical transmission, again probably because of a high viral load (Roques *et al,* 1993). Prophylactic administration of the antiretroviral agent zidovudine to HIV infected mothers has been shown to reduce HIV transmission to the off-spring by two-thirds (Connor *et al,* 1994). More direct evidence comes from recent studies (Dickover *et al,* 1996) in which maternal serum viral load was measured sequentially during pregnancy and a positive correlation was demonstrated between viral load and maternally-acquired HIV infection in the off-spring. The administration of antiretroviral medication led to a measurable reduction in viral load and diminished the risk of vertical transmission (Melvin *et al,* 1995; Weiser *et al,* 1994).

It would be expected that a high peripheral viral load will correlate with high viral loads in the genital tract but this may not be the case. Viral load may vary in different body compartments and may be influenced by systemic and local immunological factors. For instance, Wofsy *et al* (1986) found that the recovery of HIV from the vagina and endocervical canal did not seem to correlate with the degree of virus production from cultured peripheral blood mononuclear cells. A study by Zorr *et al* (1994) suggests that the presence of HIV in genital tract secretions may be related to the mode of acquisition, with virus being recovered from a greater proportion of heterosexually infected women than from those acquiring HIV through intravenous drug use. Nielson *et al* (1996) found no correlation between HIV shedding in cervico-vaginal secretions and plasma HIV RNA levels in a group of 19 pregnant and seven non-pregnant HIV infected women. In addition, it has been shown that the presence of high levels of HIV neutralising antibodies in the maternal plasma reduce the risk of vertical transmission (Goedert *et al,* 1989; Devash *et al*, 1990) but the effect of this on local and peripheral HIV load has not been directly investigated. Chorio-amnionitis has been shown to increase the vertical transmission rate (St Louis *et al*, 1993), implying that local immune reactivity can influence viral transmission. From this it is evident that a better understanding of the local immune system of the genital tract and its effect on viral transmission is of critical importance in the design of strategies to reduce the rate of vertical transmission.

Gynaecological problems including cervical intraepithelial neoplasia

Studies examining the clinical features of HIV disease in women have frequently identified fungal and viral infections of the genital tract as the most prevalent disease manifestations preceding AIDS. Recurrent vaginal candidiasis may be difficult to treat and the severity related to the peripheral CD4 lymphocyte count. Imam *et al* (1990) described vaginal candida in women with normal CD4 counts, oral candida in those with declining counts, and oesophageal involvement in those with AIDS. A large proportion of asymptomatic HIV-infected women attending the Royal Free describe repeated attacks of vaginal candidiasis and genital herpes prior to formal HIV diagnosis (Olaitan *et al,* 1997). The fact that recurrent genital tract infections precede systemic immunosuppression may imply a disturbance in local immunity.

An association has also been established between cervical intraepithelial neoplasia (CIN) and HIV infection, and this is related to the degree of immunosuppression (Schafer *et al,* 1991; Smith *et al,* 1993). CIN, when it occurs, may be extensive and more rapidly progressive than in HIV-negative women. The prevalence of human papilloma virus (HPV) types 16 and 18 is also increased (Agarossi *et al,* 1992), and the proposed oncogenic effects are likely to be enhanced by immunosuppression. Iatrogenic immunosuppression, as found in transplant recipients, is also associated with an increased risk of CIN (Porecco *et al,* 1975) and there is some evidence that circulating T lymphocytes from women with cervical neoplasia show an impaired

response to mitogens (Sawanobori *et al,* 1977), supporting the theory that the host immune response influences the development of cervical neoplasia. However, changes in the local cervical immune response are likely to be of greater importance than systemic changes in the development of cervical carcinoma as failure of local immunosurveillance may permit the proliferation of neoplastic cells.

In the cervix, HIV infection has been associated with a reduction in the number of Langerhans' cells (Barton *et al,* 1990). Depletion in these antigen-presenting cells may facilitate cervical oncogenesis, either independently or in conjunction with HPV infection. Wart virus infection itself is associated with Langerhans' cell depletion (Morris *et al,* 1983a). Similar reduction in cervical Langerhans' cells has been found in cigarette smokers (Barton *et al,* 1988; Poppe *et al,* 1995) and cigarette smoking is known to be an independent risk factor for CIN (Wiggle and Grace, 1980, Trevathan *et al,* 1983, La Vecchia *et al,* 1986). Fukuda *et al* (1993) found that in a population of women with non-HIV associated CIN, persistent dysplasia was associated with decreased numbers of Langerhans' cells and helper-inducer T-lymphocytes in the cervix, indicating that there is a decreased local immune response in persistent, as opposed to spontaneously regressing CIN. These observations imply that an understanding of local immune mechanisms may enhance the approach to the management and possible prevention of gynaecological conditions associated with HIV disease.

Part 3: Horizontal Transmission of HIV

Sexually acquired HIV infection is expected to reach 30 million cumulative cases by the year 2000 (Mann, 1992). Several studies have examined the risk of sexual transmission of HIV from infected men to their female partners (European Study Group on heterosexual transmission of HIV, 1992; Haverkos & Battjes, 1992; Rockstroh *et al,* 1995; Pandian *et al,* 1987). HIV prevalence in female sexual partners ranges from 10-30% in most studies from Europe & the USA. In addition to unprotected vaginal sexual intercourse, anal sex and advanced clinical and immunological stage of HIV infection in the male partner have been shown to significantly increase the risk of transmission. Treatment of the infected partner with antiretroviral medication has been shown to reduce the rate of transmission (Mussicco *et al,* 1994). Other factors which have been reported as increasing the susceptibility of women to HIV infection include peri-menopausal status, defloration, cervical ectopy, use of oral contraceptives and the intra-uterine contraceptive device (Clemetson *et al,* 1993; Kreiss *et al,* 1994).

The role of previous genital lesions was initially confounded by several factors. The fact that all sexually transmitted diseases (STDs), including HIV infection have the same mode of transmission, and that the prevalence of certain STDs may be increased in HIV-infected individuals due to immunosuppression have made it difficult to establish a cause-effect relationship. However, several studies, mainly from African

groups, have provided strong arguments to support the hypothesis that genital infections, particularly ulcerative infections are co-factors for transmission (Weir *et al,* 1994; Laga *et al,* 1993; Piot & Laga, 1989).

Several factors determining the spread of the virus are probably acting simultaneously. These include demographic, behavioural, biological and probably, as these impact on social behaviour, political and economic factors (Ancelle-Parke & De Vincenzi, 1993). General sexual behaviour that involves contact with a small but highly infected core group is associated with the most rapid spread of HIV. Studies among prostitutes have been developed in the frame of research programmes or programmes for sentinel surveillance. Prostitutes and patients attending STD clinics have consistently higher seroprevalence rates than sample groups taken from the general population, such as pregnant women (Nkowane, 1991). A randomised controlled trial in Mwanza, Tanzania demonstrated that HIV transmission could be significantly reduced by enhancing detection and treatment of sexually transmitted diseases, lending weight to the role of other STDs in the spread of HIV(Grosskurth *et al,* 1995).

The various co-factors for male to female transmission of HIV imply that, besides the immunological status of the male partner, all factors (traumatic, hormonal, infectious and inflammatory) able to compromise the integrity of the genital mucosa might be considered as factors which increase the risk of sexual transmission. Development of

strategies to prevent sexual transmission requires an understanding of factors that influence both susceptibility and infectivity.

Mechanism of horizontal transmission

The mechanism of male to female sexual transmission of HIV has not been fully elucidated. Knowledge of the immune components that act in the female genital tract and the transport of macro-molecules in this area would appear to be critically important in terms of understanding the sexual transmission of HIV. After coitus with an HIV-infected man, HIV is deposited into the vagina where, by still unknown mechanisms, it infects the woman's lymphoid cells. The risk of HIV transmission via seminal fluid may be accounted for by the fact that cell-free seminal fluid may contain as much as 10^8 free virus particles/ml (Borzy *et al,* 1988), a level higher than that found in serum. Semen samples in men with acute STDs have been shown to have increased HIV-load which declined with treatment (Atkins *et al,* 1996). However, it is not yet known if cell-associated or cell-free HIV is responsible for infection of the female partner. Artificial insemination of HIV-negative women with sperm from HIV-positive men which had been subjected to gradient centrifugation, repeated washings and a swim-up procedure has been successfully performed without leading to HIV infection in the sperm recipients (Semprini *et al,* 1992). This would imply that cell-free HIV particles are the infective fraction in ejaculates. However, contrary to previous observations (Mermin *et al,* 1991), HIV pro-viral DNA sequences have been detected

in sperm and seminal fluid mononuclear cells but their role in horizontal transmission has not been fully elucidated. The fact that HIV pro-viral DNA occurs more frequently in the mid-piece rather than in the head section of sperm cells which contains highly condensed DNA might explain the apparent inability for HIV-infected sperm to infect ova leading to the presence of integrated DNA in the cells of the offspring (Bagasra *et al*, 1994).

Ejaculated sperm induce a large increase in the concentration of T-lymphocytes, macrophages and neutrophils in the cervix (Thompson *et al*, 1991). These lymphoid cells migrate into the vagina after coitus. Semen also contains CD4+ and CD8+ T lymphocytes (Witkin *et al,* 1988). Exposure to these allogenic immune cells probably activates the cell-mediated immune system and increases the local concentration of cytokines and vaginal immune cells, facilitating HIV access into the female genital tract. The effects of semen on vaginal immunity are important as they may have a direct effect on heterosexual transmission of HIV.

The envelope complex of the HIV virion facilitates viral entry into cells by binding to the CD4 receptor, via the outer envelope protein, gp120. Langerhans' cells, CD4+ bone-marrow derived epithelial cells, and related dendritic cells appear to be the cells most readily infected by the HIV virus (Macatonia *et al,* 1991) and it has been suggested that HIV-infected seminal fluid may be transmitted to Langerhans' cells

within the lower genital tract mucosa. The migration of Langerhans' cells to local lymph nodes further provides a mechanism for the progression of HIV from a localised to a systemic infection. However the fact that there is no evidence that HIV particles ejaculated during oral sex gain entry through the oral mucosa, which has similar concentrations of Langerhans' cells, has led researchers (Lehner *et al*, 1991; Hussain *et al,* 1992) to postulate that there must be another mechanism for HIV transmission. They identified Fc receptors for IgG in the cervical transformation zone epithelia, but not in oral epithelium and suggested that Fc receptors enable HIV-antibody complexes within the seminal fluid to bind to CD4-negative cervical cells which then move to the basement membrane and infect CD4+ T cells, dendritic cells, or macrophages in connective tissue.

Antibodies to HIV have been detected in cervico-vaginal secretions of non-menstruating women at risk of HIV infection (Archibald *et al,*1987; Belec *et al,*1989), with some evidence of compartmentalisation. Belec *et al* 1994, found antibodies to HIV in genital tract secretions of four HIV seronegative women and suggested that immune response to HIV, restricted to the vaginal mucosa, could occur in rare cases after sexual contact with an HIV-positive man. It is conceivable that repeated challenges with small inocula of infected material could initiate local protective immunity. This may in part explain why some women do not become infected with HIV despite repeated sexual exposure. It would appear therefore that susceptibility or

resistance to sexual infection with HIV depends on an interplay between the quantity and possibly the virulence of virus in the inoculum, and host immune factors, probably at a local as well as systemic level.

Part 4: The Immune Response To Viral Infection

Cell-mediated Systemic Immune Response

Antibodies are effective against soluble toxins and some bacteria but immunity to infectious organisms, such as viruses, which have developed a capacity for living and multiplying within host cells is mediated by T lymphocytes. When viruses first gain entry into the human body they are taken up by macrophages and other antigen-presenting cells, leading to the release of the soluble mediator interleukin 1 (IL-1). IL-1 acts via specific receptors to activate T lymphocytes. There are two distinct sub-populations of T lymphocytes, the CD4 receptor-bearing T helper cells which recognise major histocompatability complex (MHC) class II antigens and the CD8+ cytotoxic, suppressor T lymphocytes which recognise MHC class I antigens. Under normal circumstances, both subsets act in synergy to eliminate virus. The CD4+ cells seem to respond by recognising MHC class II antigens on the surface of macrophages and releasing IL-2 and other soluble chemotactic agents. Cytotoxic T cells then express IL-2 receptors (IL-2R) and under the influence of IL-2, proliferate and acquire cytolytic cytoplasmic granules (TIA-1+, perforin+) which confer the ability to destroy virally infected cells. Other cytokines such as interferon-γ (IFN-γ) which inhibits viral

multiplication and IL-10 which suppresses CD4+ IL-2 secretion, are released, serving to enhance and regulate the cell-mediated immune response. Resting T cells and those that have been 'primed' by pathogens can be recognised immunologically. 'Naive' or unprimed T cells express CD45ra and, after activation by antigens, become 'primed' or memory cells and switch to display CD45ro (Janossy *et al,* 1992).

The proliferation of selected T cells thus generated can lead to a 20-30-fold increase in the proportions of virus-specific cytotoxic T lymphocyte percussor cells (Doherty, 1993). This expansion manifests itself as a lymphocytosis and lymphadenopathy which is largely accounted for by an increase in CD8+,CD45ro+ cells, and to a lesser extent CD4+,CD45ro+ cells. After the resolution of an acute viral reaction, T cell numbers return to normal levels. This appears to be due to apoptosis of the expanded population. It has been shown that the lack of the Bcl-2 oncogene is associated with increased programmed cell death (Korsmeyer, 1992). Thus cells destined for apoptosis down-regulate their Bcl-2 and these apoptotic cells are recognised and ingested by macrophages (Akbar *et al*, 1994). It has been postulated that this is the mechanism by which T cell proportions are restored to normal after resolution of an acute viral infection. This T cell homeostasis is again under cytokine control, with Bcl-2 down-regulation being associated with a loss of signals such as IL-2 (Akbar *et al,* 1993) from viral-infected cells as resolution occurs. A small proportion of memory T-cells is retained which can recognise and rapidly respond if the same pathogen is encountered

again. From this it is obvious that the cellular immune response is a finely balanced, complicated mechanism, and failure of any one of its steps can lead to an impairment of the cell-mediated immune response.

The Cell-mediated Immune Response in HIV Infection

AIDS results from a profound defect in cell-mediated immunity (CMI). Infection with HIV is characterised by a reversed CD4+/CD8 lymphocyte ratio in the peripheral blood (Gottlieb *et al,* 1981; Fahey *et al,* 1984) which results from a loss of CD4+ cells (Phillips *et al,* 1989) and an initial increase in CD8+ cells (Gottlieb *et al,* 1981; Fahey *et al,* 1984). CD8+ expansion also occurs in lymph nodes and is responsible for persistent generalised lymphadenopathy in HIV-1 infected individuals. This pattern of CD8+ lymphocytosis contrasts with that associated with an acute viral reaction where the CD8+ numbers return to normal after resolution of the acute infection, as described above.

The mechanism for, and control of CD8 T cell persistence in HIV infection is poorly understood but possible explanations include interference with differentiation and apoptotic pathways, or aberrant cytokine production (Navikas *et al*, 1995; Cayota *et al,* 1992; Lai *et al,* 1991). It has been suggested that T lymphocytes in HIV infection switch from producing T helper cell type 1 (Th-1) cytokines (IL-2, IFN-γ) to Th-2 cytokines (IL-4, IL-5, IL-10) (Del Prete *et al,* 1995; Clerici *et al,* 1994) and that HIV

appears to replicate preferentially in Th-2 rather than Th-1 cells (Clerici & Shearer, 1993). Some of the Th-2 cytokine activities have been implicated in the pathogenesis of HIV disease. For example, high levels of IL-4 and IL-5 (Mossman and Moore, 1991; Xu-Amano *et al,* 1992) are associated with high antibody levels and may be responsible for the hypergammaglobulinaemia seen in patients with advanced HIV disease. However, evidence for a Th-1/Th-2 shift in HIV infection has often been contradictory (Romagnani *et al,* 1994) and in-vitro experiments may not necessarily reflect in-vivo conditions. Bofill *et al* (1995) have suggested that the persistence of CD8+ populations in HIV-1 infection is not a result of the presence of an abnormal CD8+ population but rather a result of an inappropriate stimulation of the CD8+ cells. This is consistent with the fact that despite an apparent cytotoxic lymphocyte reaction, the virus is never cleared and the high CD8+ numbers may be a reflection of the immune response to the constant production of HIV-1. Further studies are clearly needed to determine how the immune system fails to control this on-going infection.

Mucosal Immune Response

The existence of a mucosal-associated immune system (MALT) has been acknowledged for several decades. Its role is to prevent the penetration of microbial and food antigens from the environment to the internal milieu. Mucosal surfaces contain the largest accumulation of lymphoid tissue, more than 85% of the total body lymphoid tissue. Immunocytes are either present diffusely in the mucosa or aggregated

into patches of lymphoid tissue, of which Peyer's patches in the gastrointestinal tract, and human palatine tonsil are examples. Mucosal lymphoid cells are distinguished from immune cells in the blood by specific phenotypic markers and restricted migration patterns (Janossy *et al,* 1989).

Secretory IgA (s-IgA), produced by mucosal plasma cells, is quantitatively the most important immunoglobulin of the humoral immune system in the body (Wolf and Bye, 1984). S-IgA is characterised by a polypeptide protein known as the secretory piece which is thought to be instrumental in the transfer of s-IgA into secretions. Specific s-IgA has been detected in the gastrointestinal tract and other mucosal surfaces after exposure to antigens (reviewed by Mestecky, 1987). In addition to B lymphocytes and plasma cells, mucosal tissue also contains T cells capable of mounting cytotoxic responses against invading pathogens.

Macrophages have also been identified in mucosal tissue and studies have shown that several different cell types exist within the macrophage pool, which can be distinguished by their expression of surface antigens (Poulter *et al,* 1986, Mahida *et al,* 1988). It has also been established that different phenotype is related to different functional status. For instance, there is a sub-population of macrophages which exhibit interdigitating morphology. These macrophages which have the phenotype

RFD1+,RFD7- are efficient antigen-presenting cells in comparison to the RFD1-, RFD7+ macrophages which have poor antigen-presenting capacity though possessing good phagocytic capacity (Poulter *et al,* 1986). Cells displaying both RFD1 and RFD7 antigens have been shown to suppress T lymphocytes in mixed lymphocyte reactions (Spiteri & Poulter, 1991).

Most studies of MALT have been based on the immune system of the gastro intestinal and respiratory tract. Although the existence of a mucosal immune system in the female genital tract has been demonstrated, its role in the prevention of infection through this route in humans has largely been neglected. It is only recently that its potential role in the prevention of vertical and horizontal transmission of HIV and the design of vaccines has attracted the attention of researchers. Lehner and colleagues (1995) have reported experimental evidence of the existence of genital tract associated lymphoid tissue in non-human primates. Observations in mice have suggested a critical role for CMI in effecting the resolution of Chlamydia trachomatis (Cain and Rank, 1995) and herpes simplex virus (Parr *et al,* 1994) genital infections. In humans, s-IgA against Chlamydia trachomatis has been isolated from cervico-vaginal secretions (Thejls *et al,* 1995). The presence of T cells with cytolytic activity, macrophages and dendritic cells has been demonstrated in the lower genital tract mucosa of normal females (White *et al*, 1997). Studies in women with neoplastic disease of the cervix have demonstrated that CD8+ cells are recruited to neoplastic cervix. Analysis of

immunocompetent cells in the general circulation showed little correlation with immunocytes in the cervix (Bell *et al,* 1995), implying a locally mediated immune reaction.

The recognition that mucosal IgA producing plasma cells can be detected, not only at the site of exposure to the antigen, but at other distant mucosal sites has led to the concept of a common mucosal immune system. For example, s-IgA from human colostrum and tears contains antibodies to the oral bacterium Streptococcus mutans (Arnold *et al,* 1976). This response is achieved through the selective uptake of antigen by mucosal epithelial cells, and its delivery to underlying lymphoid tissue which stimulates immunoglobulin-producing percussor cells (Wolf & Bye, 1984). These cells migrate into the blood circulation through the lymphatics, mature, and seed the lamina propria of assorted mucosal surfaces where they secrete specific immunoglobulin, in most cases, s-IgA (reviewed by Mestecky, 1987). T lymphocytes are similarly capable of migrating and this may provide a mechanism for the systemic distribution of HIV after inoculation at mucosal surface such as the genital tract.

Mucosal immune response to HIV infection

Despite the fact that the majority of HIV-positive women world-wide are infected through heterosexual intercourse, the mucosal immune response to HIV in the genital tract is poorly understood. Specific s-IgA (Belec *et al,* 1989) and IgG (Belec *et al,* 1995a) to HIV has been detected in the genital tract of infected women but intermittent HIV shedding seems to occur despite the presence of these antibodies (Neilsen *et al,* 1996). Abnormal cytokine secretion patterns have also been described in cervico-vaginal secretions (Belec *et al,* 1995b) but very little attempt has been made to correlate these observations with function, or to study the cell-mediated response at a local level. Anti-HIV cytotoxic lymphocyte activity has been observed in macaques monkeys intra-vaginally inoculated with HIV (Lohman *et al*, 1995).

In humans, the picture is complicated by the fact that most reports on CD4+/CD8+ T lymphocyte subset proportions in the normal cervix have been contradictory or imprecise (Pomerantz *et al,* 1988; Morris *et al,* 1983b) yet a knowledge of these cell proportions is essential to any study of immunological function of the genital tract mucosa. There has been considerable success at this centre at using double immunofluorescence techniques to delineate these cell subsets at other mucosal surfaces (Lim *et al,* 1993a). Functional macrophage subsets have also been successfully defined (Spiteri *et al,* 1991), and as there is evidence that these may be altered in HIV disease (Lipman *et al,* 1995; Lim *et al,* 1993b), it is evidently important

to establish the proportions in the normal cervix in order to observe what effect HIV disease may have.

Abnormal cytokine production has been implicated in the aberrant systemic immune response to HIV and there is some evidence that cytokine profiles may be altered in genital tract secretions of HIV-infected women (Belec *et al,* 1995b). A study of cytokine secretion may help to explain the functional significance of any alterations in mucosal immunocyte proportions and may be important in the pathogenesis of recurrent genital tract infections observed in HIV-infected women. Finally, as there is evidence that genital tract viral load may not correspond to systemic viral load, and may be related to rates of horizontal and vertical transmission, it is important to correlate any observed changes in immunocompetent cell distribution and function with local viral load.

Part 5: Rationale for techniques applied in this project

Specimen Collection For Studies: Smear & lavage v biopsy

Most previous studies investigating the mucosal immune response in HIV infection have focused on secretory antibody and cytokines in cervico-vaginal secretions. Various techniques have been used to obtain these secretions. Archibald *et al* (1987) sampled the cervix with a wooden blade scraped over the cervical mucosa. The secretions obtained were then diluted and filtered to remove cellular debris before

being analysed for anti-HIV antibodies. The inherent flaw with this technique is that the samples obtained may consist mainly of cervical mucosal cells which become detached by the wooden blade. The trauma caused by the blade may also lead to inflammatory changes which confound the results. Finally, and most importantly, it would be difficult to ensure that the same site and surface area is sampled in each subject, making inter-subject comparisons potentially invalid.

Belec *et al* (1989) collected cervical mucus by washing the vagina with three millilitres of normal saline which was subsequently filtered. Again there are inherent difficulties with establishing that the whole of the vagina is uniformly lavaged. We were initially interested in this technique for studying cytokine profiles but a pilot study of 5 women showed that specimens thus obtained were too dilute and the cytokine content was below the detection limit for Quantokine (R&D, Oxon, UK) cytokine assays for IFN-γ, IL-2 and IL-4. Similar attempts to quantify viral load by polymerase chain amplification of nucleic acid yielded results below the detection threshold of the kit (Amplicor, Roche diagnostic systems, Hertfordshire, UK). Attempting to concentrate the samples yielded volumes too small for analysis.

For the reasons illustrated in the section above (Part 4), we felt that a study of CMI was more important than a study of the secretory response alone in the study of genital tract mucosal immune response to HIV infection. We chose to study the squamo-columnar

junction of the cervix rather than the vagina or upper genital tract for several reasons. Previous studies have shown that immunocompetent cells appear to be chiefly concentrated in the transformation zone and squamo-columnar junction of the cervix (Morris *et al,* 1983a; Lehner *et al,* 1991; Rebello and Green, 1975; Kutteh *et al,* 1988). One study demonstrated alterations in immunocompetent cell proportions within the endometrium in HIV-infected women (Johnstone *et al,* 1994a) but obtaining endometrium is more invasive than obtaining cervical samples. The transformation zone of the cervix also has the advantage of being easily identified on colposcopic inspection and provides a reference point to ensure that samples are obtained from the same anatomical site in each subject.

Colposcopically-directed cervical biopsy is an established method of making histological diagnosis in women with cervical cytological abnormalities. Colposcopically-directed biopsy of the squamo-columnar junction was felt to be the appropriate sample for this study for several reasons. Colposcopic assessment allows a direct inspection of the cervix and the squamo-columnar junction is easily identified. When metaplastic epithelium is exposed to factors responsible for neoplasia, the transformation zone becomes atypical and this is manifest as aceto-white epithelium, altered vascular patterns and irregular surface contour. These changes are easily recognisable through the colposcope and combined colposcopy and histology are the mainstay of clinical management of cervical intraepithelial abnormalities (reviewed by

Copplestone *et al,* 1993). Thus colposcopic inspection in this study enabled the operator to identify and exclude women with histologically-abnormal areas on their cervixes. Biopsies are taken under direct vision and the same anatomical site can be sampled in all cases. Intact tissue of 3-5mm is obtained so that histological assessment can be performed and the distribution of immunocompetent cells and numbers per unit area can be established. Sufficient tissue is also available for cytokine and viral load quantification. The technique is minimally invasive and causes only minor discomfort for the subject.

Staining techniques to identify immunocompetent cells

Immunohistochemical techniques, using monoclonal antibodies to identify the disposition of immunocompetent cells, have been successful in furthering our knowledge of immune mechanisms in other mucosal surfaces. In particular, the relative distribution of lymphocytes and macrophage subsets is now well established for the mucosa of the gut (Mahida *et al,* 1988) and the lung (Spiteri *et al,* 1991; Power *et al,* 1993). These techniques are in standard use in our laboratories and experience has shown the results from these techniques to be accurate and reproducible on a variety of different tissues.

In Situ Hybridisation

The molecular biology technique of in-situ hybridisation provides a powerful tool to elucidate the expression of messenger RNA (mRNA) at a cellular level. It has been successfully utilised in qualifying cytokine production in immunocytes in various pathological conditions (Navikas *et al,* 1995; Ichinose *et al,* 1995). Staining techniques using cytokine-specific monoclonal antibodies (Sander *et al,* 1991) can be used to establish the correlation between the detection of cytokine mRNA by in-situ hybridisation, and the ultimate expression of cytokine by the cell. These methods have been applied clinically in the analysis of viral infection and more recently, in the analysis of mRNA expression in various disease states. Previous studies on cytokine production in the genital tract have focused on the cytokine content of genital secretions (Belec *et al,* 1995a), with the disadvantage that the source of the cytokines cannot be determined. The application of in-situ hybridisation and cytokine staining methods enabled us to study the cytokine producing ability of immunocompetent cells within genital tract tissues, thus shedding some important light on the functional capacity of these cells.

Polymerase Chain Reaction

Before the technique of polymerase chain reaction became widely available for HIV detection, quantitative culture of peripheral blood mononuclear cells or plasma was used to estimate the infectious titre of HIV in blood (Ho *et al,* 1989). However, fewer

than 50% of patients with CD4 lymphocyte counts greater than 200 x 10^6/ml had positive plasma cultures. Attempts to quantify viral load within the genital tissues were also done by viral culture (Wofsy *et al,* 1986; Voght *et al,* 1986). Such cultures were liable to contamination by candida and other infectious agents in the genital tract. Polymerase chain reaction (PCR), a system of amplifying and quantifying viral nucleic acid has recently been effective in measuring viral load in the blood and in other tissues (Nielsen *et al,* 1996; Liuzzi *et al,* 1996). Some authors have reported success in semi-quantitative detection of HIV by PCR in genital tract secretions (Zorr *et al,* 1994). Detection of HIV RNA by PCR reveals measurable virus in the plasma of virtually all HIV infected patients regardless of clinical stage and this technique has recently been utilised to monitor disease progression and response to therapy. Three commercially available plasma HIV RNA assays, branched DNA (bDNA), RT-PCR and Nucleic Acid Based Amplification (NASBA) are currently available. The bDNA technique amplifies the signal from a captured viral RNA target by sequential oligonucleotide hybridisation steps while RT-PCR and NASBA use enzymatic techniques to amplify target HIV RNA into measurable amounts of nucleic acid product. Despite the differences in methodological approach, plasma RNA measurements obtained with the three assays are strongly correlated (reviewed by Saag *et al,* 1996). The RT-PCR system utilised in this project (Amplicor, Roche diagnostics, Hertfordshire, UK) has the added advantage of having a protocol for extracting viral RNA from solid tissues, allowing it to be used on cervical biopsy specimens as well as serum specimens.

Part 6: Thesis Objectives

From this introduction, it is evident that little is known about the immune system of the female genital tract, despite an increasing recognition of its importance in HIV disease. Studies utilising the techniques described above were therefore designed:

1. To define the study population in terms of clinical, menstrual, sexual and reproductive history.

2. To define the distribution and functional capacity of immunocompetent cells in the normal, healthy genital tract mucosa and to establish the effects of the menstrual cycle on these parameters.

3. To determine how genital tract mucosal immunity is altered in HIV disease, characterising the phenotypic and functional features, including cytokine profiles, of the immunocompetent cells and relating this to systemic immune function.

4. To determine if a relationship exists between the alterations in immune cell disposition and distribution in HIV-positive women and local and systemic HIV load.

CHAPTER 2: SUBJECTS

Demographic details of background population

The Ian Charleson Day Centre

Same Day Testing Clinic

Subject Selection

Physical examination and clinical investigations

Colposcopy

Other measurements

Ethical considerations

Statistical Analysis

CHAPTER 2: SUBJECTS

Demographic details of background population.

The Ian Charleson Day Centre

All HIV-positive women participating in this study were recruited from the Ian Charleson Day Centre (ICDC), the dedicated HIV outpatient unit of the Royal Free Hospital. This unit, which was set up in 1987, has the largest cohort of HIV-infected women attending a single treatment centre in England and Wales. There is a weekly women's outpatient clinic in which HIV-positive women are seen, on average every three months for a medical check and blood tests. Sequential CD4 lymphocyte counts are performed routinely at each 3 month visit. Women are offered prophylaxis against Pneumocystis carinii pneumonia (PCP) if their CD4 lymphocyte count falls below $200/mm^3$ or if they develop symptomatic HIV disease. Cotrimoxazole (Septrin) is most frequently used, with nebulised pentamidine offered to those intolerant of Septrin.

Antiretroviral medication is also offered to women with CD4 counts below $200/mm^3$ or those with clinical symptoms, with zidovudine, either alone or in combination with other nucleoside analogues or proteinase inhibitors being most commonly used. Women are also encouraged to participate in clinical trials of antiretroviral medication where appropriate. Although serial estimation of plasma HIV RNA load is now being introduced into clinical practice as a means of monitoring response to antiretroviral

therapy, it was available only as a research tool at the time of undertaking this project and HIV RNA levels were not routinely measured.

On their first visit, or soon after, all women are offered a gynaecology screening examination, during which details of the patients' reproductive history, gynaecological history, lifestyle and attitudes are collected and a gynaecological examination is performed. Cervical smears are obtained and colposcopy, with biopsy if indicated, is routinely performed. Women are also routinely screened for other sexually transmitted infection. The gynaecological screening examination is repeated every six months and data is collected prospectively and stored on a computerised data-base. An on-site gynaecological service was established in 1990; women were referred to another centre prior to that date. Complete medical and gynaecological data are thus available on all women seen for gynaecology screening since 1990 (Table 2:1). Women were mostly young, with a mean age of 32.7 (sd 6.7). It is evident from table 2:1 that the majority of women acquired HIV infection through heterosexual intercourse. Women were classified as acquiring HIV infection through intravenous drug use if they had ever injected intravenous drugs. However, we recognise that these women could have been infected through heterosexual intercourse.

The commonest AIDS defining diagnoses made during follow-up of these women were: PCP, oesophageal candidiasis, atypical mycobacterium infections, toxoplasmosis

and Kaposi's sarcoma. Contrary to previous data suggesting an association of HIV infection with a high frequency of STDs (Hoegsburg *et al,* 1990), the prevalence of sexually transmitted diseases is relatively low in this group of women, with new STDs detected in 9.5% of women at first gynaecology visit and none at subsequent visits. As expected, there is a high incidence of cervical abnormality, with 47.6% of smears being reported as abnormal at first visit, and similar proportions of abnormal smears at subsequent visits (Olaitan *et al,* 1997).

TABLE 2:1 **Demographic & lifestyle details of background population** (n=185)

Patient Characteristics		n (%)	CD4 counts at initial presentation			% with AIDS at first visit
			Mean (SD)	Median	90% range	
Total		185 (100)	356 (270)	310	10-865	13.8
Demography						
Race	Black	90 (48.6)	268 (201)	230	10-610	19.2
	White	83 (44.8)	421 (291)	410	16-990	9.5
	Other	12 (6.5)	509 (392)	390	110-1176	12.5
*E.C	Hetero	145(78.4)	352 (258)	320	10-830	14.1
	IVDU	30 (16.2)	431 (287)	423	16-1010	5.9
	Other	10 (5.4)	204 (298)	110	3-1176	26.3
Sexual Lifestyle						
Mean Age at coitarche (sd)		17.8 (2.67)				
Median No. of partners (range)		3 (1-100)				

*Exposure Category
Hetero: Heterosexual

Same Day Testing Clinic

In addition to caring for HIV infected women, the ICDC also provides a same-day antibody testing clinic four times a week, which is open to the general public. The HIV-negative women participating in this project were recruited from this clinic. Attenders are often self-referred because of some perceived risk of HIV exposure. The majority of patients tested (97%) are found to be HIV-negative. The women utilising this service are mainly Caucasian and tend to be self-motivated and able to access health care effectively (Olaitan *et al,* 1996). Black African women are far less likely to attend this clinic for voluntary testing and most of the HIV-positive Black African women attending the ICDC underwent HIV testing either because a sexual partner was found to be HIV-positive or because the woman presented with an opportunistic infection (Appendix 3). Of a sample of 65 women attending the clinic in the three months from September to December 1994 (the period when recruitment to the study commenced), 50 (77.0%) were White, two (3.1%) were Black African and 13 (20.0%) were from other ethnic groups. The most common reason for presenting for testing was unprotected sexual intercourse with a male of unknown HIV status. All 65 women tested negative after the appropriate window period.

Subject selection

Consecutive HIV-positive attenders of the ICDC women's outpatient clinic who satisfied the selection criteria were invited to participate in the study. Recruitment

occurred between September 1994 and July 1996. The HIV-negative women were recruited from same day testing clinic over the same time period. Exclusion criteria included: pregnancy, previous hysterectomy, amenorrhoea, cervical colposcopic, cytological or histological abnormalities, inadequate visualisation of the squamo-columnar junction at colposcopy, and current sexually transmitted disease. Women who were deemed too ill to participate (such as women with severe intercurrent infection, lymphomas or other malignancies), or those with bleeding disorders, were also excluded from the study.

Eighty-seven of the HIV-positive regular attenders to the ICDC were eligible for inclusion in the study. Of these, 45 women agreed to participate but five women were subsequently excluded because of significant cervical abnormality that required treatment. Twenty-one HIV-negative women agreed to participate in the study but one woman was found to have significant cervical abnormality and consequently excluded. Therefore a total of 40 HIV-positive women and 20 HIV-negative, healthy volunteers, all with documented negative HIV antibody tests, participated in the study. The HIV-positive subjects constituted approximately half (46%) of the clinic attenders eligible for selection and were representative of the whole clinic cohort in terms of age, race and CD4 lymphocyte count. They were at various stages of disease, as reflected by the serum CD4 count, and the majority acquired their HIV through heterosexual intercourse (Table 2:2).

Demographic data, including social, sexual and menstrual history, was obtained by structured questionnaire (Appendix 1). All subjects were in their reproductive years and had normal menstrual cycles and were well matched in terms of age, sexual and menstrual history (Table 2:3). There were no intercurrent illnesses.

TABLE 2:2 **HIV-disease stage of HIV-positive subjects** (n=40)

Subject Details	**Number**
Ethnic Origin	
White (%)	17 (42.5)
Black African (%)	22 (55.0)
Other (%)	1 (2.5)
Exposure Category	
Heterosexual (%)	36 (90.0)
IVDU (%)	4 (10.0)
Other (%)	0 (0)
Serum CD4 lymphocyte count/ml	
Mean (sd)	344.8 (288.3)
Median (range)	288 (10-880)
***Medication**	
None (%)	18 (45.5)
Septrin (%)	13 (32.5)
Antiretrovirals (%)	13 (32.5)
Other (%)	9 (22.5)

Other: Acyclovir, Rifanah, Fluconazole, Ganciclovir, Prednisolone
*Some women were on more than one drug
(Full medical details on all HIV-positive subjects are available in Appendix 3)

Table 2:3 **Demographic and lifestyle details of HIV-positive and HIV-negative subjects**

Demographic details	HIV-positive n= 40	HIV-negative n=20
Median age in years (range)	33 (23-48)	32 (20-42)
Median age at menarche(range)	14 (11-18)	12 (12-16)
Median age at coitarche(range)	18 (14-22)	17 (14-21)
Mean number of sexual partners (sd)	6.0 (4.9)	10.4 (6.5)
Number of women with children (%)	19 (47.5)	2(10)%
*Previous STD (%)		
None	22 (55.0)	16 (80%)
Warts	5 (12.5)	2 (10%)
Herpes	7 (17.5)	1 (5%)
Other	9 (22.5)	1 (5%)
Contraception (%)		
No sex	9 (22.5)	2 (10.0)
**Condoms only	23(57.5)	6 (30.0)
Contraceptive Pill	7 (17.5)	2 (10.0)
Other	1 (2.5)	10 (50.0)

* Some women had more than one STD

No sex: Not currently sexually active

**All sexually active HIV-positive subjects used condoms, alone or in addition to another contraceptive method.

Physical Examination and Clinical Investigations

All women first underwent a bimanual pelvic examination to exclude gross pelvic abnormality. Then, with the subject in the lithotomy position, a Cuscoe's speculum

was inserted to visualise the cervix and vaginal walls. A cervical smear (Ayre's spatula and brush specimen) was taken from all subjects and the following swabs obtained to exclude genital infection: a urethral swab in Aimes transport medium for gonococcal infection (GC), cervical swabs for GC, Chlamydia (ELISA) and viral culture (Cytomegalovirus, Herpes Simplex virus) and a high vaginal swab in Bushby's medium for Candida albicans, Trichomonas vaginalis and Gardnerella vaginosis.

Colposcopy

After inspection with the naked eye, the cervix was examined with a colposcope (Olympus OC5-3) and stained with 5% acetic acid and aqueous iodine solution to identify the transformation zone and any abnormal epithelium. In all cases a biopsy of the squamo-columnar junction (3-5mm of tissue) taken for immunological and viral studies, using a pair of Tischler-Morgan biopsy forceps (Rocket Medical, Watford, Herts). Additional biopsies, preserved in formalin for histological analysis were taken from cervixes showing colposcopic abnormalities (women in whom histological abnormality was confirmed were excluded from the study). As there is evidence that local immune responses are affected by hormonal changes in the menstrual cycle (Schumacher, 1988), biopsies were taken at two phases of the menstrual cycle, i.e. the pre-menstrual and post-menstrual phase of five subjects from the HIV-negative group who were not using hormonal contraception. Biopsies were obtained in consecutive menstrual cycles to allow for healing at the site of the previous biopsy. Care was taken

to ascertain that the biopsies were taken from opposite cervical lips (upper lip first biopsy, lower lip second biopsy) to avoid the possibility of iatrogenic inflammation generated from the healing biopsy site. All other biopsies were obtained in the pre-menstrual phase. To avoid infection and to facilitate healing, subjects were advised to avoid sexual intercourse and internal sanitary protection for three weeks after the cervical biopsy was taken. Pelvic examination and colposcopy were performed by the author in all cases.

Other measurements

Ten millilitres of peripheral blood was collected from all HIV positive subjects by veno-puncture into Vacutainer tubes with EDTA for automated leucocyte and differential counting. A further 10mls was taken into heparinised tubes for CD4 counts. CD4 counts were performed using a whole blood analysis method and the percentage of CD4 lymphocytes was expressed as absolute CD4 counts on the basis of lymphocyte counts. A monoclonal antibody, RFT4 (CD4), was used as described previously (Bofill *et al,* 1992). Clotted blood, 10mls in plain tubes was also collected for viral studies

Ethical considerations

Each study had received prior approval by the Royal Free Hospital Ethical Practices Committee. The study was explained to each subject and informed consent obtained prior to recruitment to the study. An information leaflet was given to each subject at the time of recruitment. (Appendix 2)

Statistical Analysis

The statistical methods applied in this thesis are those in standard practice for the calculation of the median, mean and standard deviation of the mean. Spearman's rank correlation was used to assess correlation between different parameters. The student's *t* test was used to determine the significance of the difference between means when the observations under study were assumed to be drawn from normally distributed populations, with paired *t* tests applied for matched pairs. The Mann Whitney U test was used when this assumption could not be made, with the Wilcoxon signed rank test applied to paired samples. A probability value (p) of less than or equal to 0.05 was taken as indicating statistical significance. All tests of significance were two-sided.

CHAPTER 3: THE NORMAL MUCOSA

Introduction

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CHAPTER 3: 'THE NORMAL MUCOSA'

The Distribution of Immunocompetent cells in the mucosa of the lower genital tract in HIV-negative females.

Introduction

Basic Histology

The vagina is lined by stratified squamous epithelium, which is composed of five cell layers separated from the stroma by a basement membrane. The cell layers, starting from the basement membrane are: the highly cellular basal layer, the parabasal layer, the intermediate and transitional layers which consist of large polygonal cells and the superficial layer. These cells, which do not secrete mucus, are derived from differentiation from the basal layer. Secretions seep between the cells to moisten the vagina. The superficial and intermediate cells which contain glycogen are constantly exfoliated. The exfoliated cells release their glycogen content which is acted upon by Doderlein's bacillus, a normal inhabitant of the vagina, to produce lactic acid which is responsible for the normal acidity of the vagina and explains the relative resistance to infection (Llewllyn-Jones 1986). Although the blood vessels do not extend beyond the basement membrane, a system of intercellular channels traversing the vaginal epithelium has been described which allows macromolecules, fluids and cells to migrate from the basement membrane to the vaginal lumen. The vaginal epithelium can also absorb drugs, particularly oestrogen

The vaginal portion of the cervix (the ectocervix) is lined by a stratified squamous epithelium which is identical with vaginal epithelium. The endocervical canal is lined by tall columnar mucus-secreting epithelium containing numerous complex, highly branched tubular glands which also secrete mucus (Wheater *et al,* 1979). The area of transition between squamous and columnar epithelium, the squamo-columnar junction, is located within the transformation zone. Histo-morphological changes characteristic for phases in the menstrual cycle can be observed. Hormonal changes associated with different phases of the menstrual cycle are illustrated in figure 3:I. Oestrogen stimulation results in an increase in the number of mucus-containing cells in the enlarged cervical crypts (Gaton *et al,* 1982). The quantity and quality of mucus produced also undergoes cyclical changes, with oestrogen promoting the secretion of copious thin mucus. Following ovulation, under the influence of progesterone, the mucus becomes highly viscid and forms a plug which inhibits the entry of micro-organisms into the cervical canal.

The connective tissue underlying the cervical epithelium has a high content of collagen fibres which interlace with smooth muscle fibres of the myometrium. A moderate number of lymphocytes and plasma cells are present in the sub-epithelial stroma of the cervix but polymorphonuclear lymphocytes are usually seen only during the luteal phase or under inflammatory conditions (Schumacher, 1988; Morris *et al,* 1983b). Langerhans' cells have also been demonstrated in the lower genital tract mucosa, and

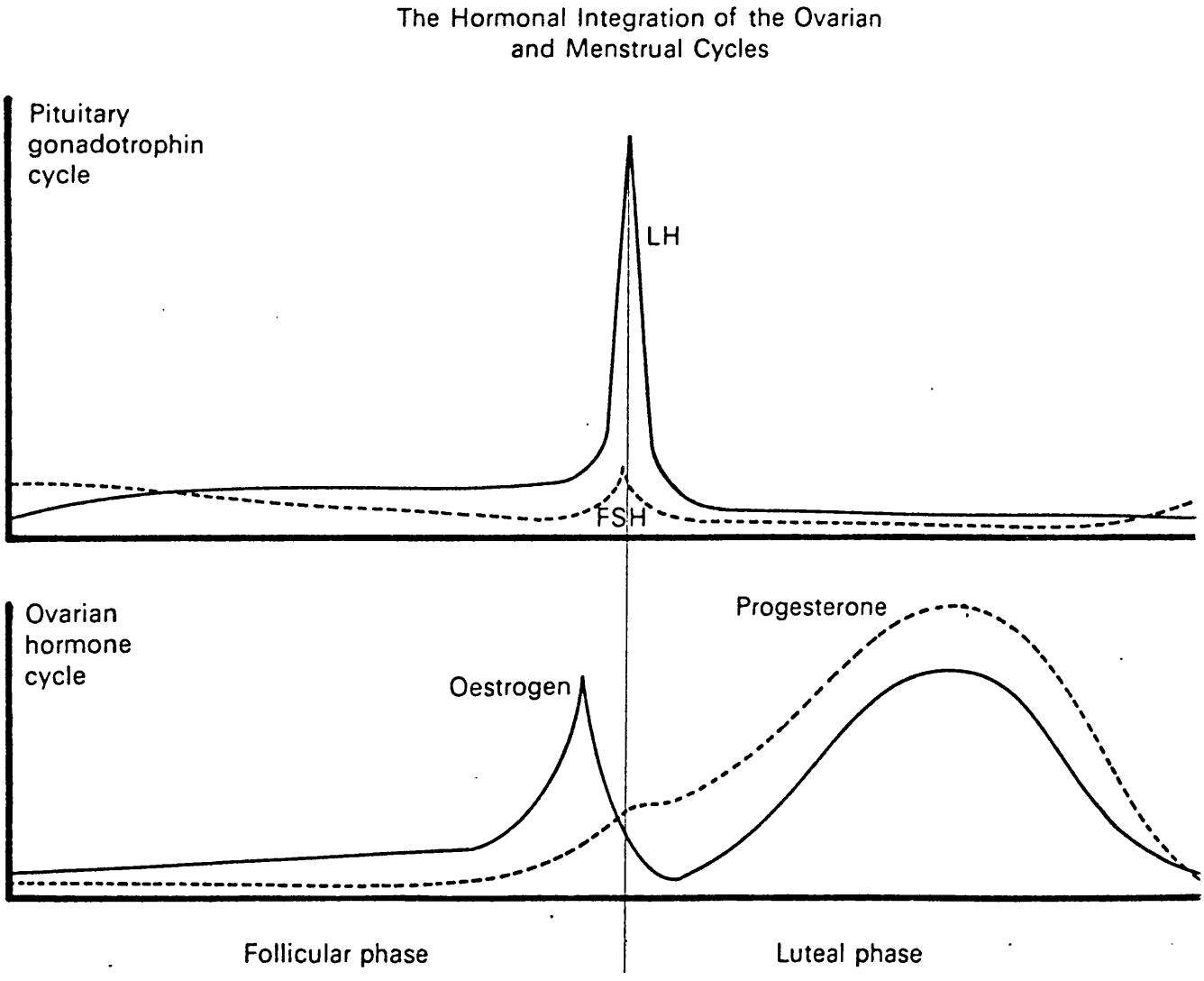
ppear to be a constant feature of the transformation zone and ectocervical epithelium (Morris *et al,* 1983b; Lehner *et al,* 1991). Lymphocytes also appear to be chiefly concentrated in the endocervix (Rebello and Green, 1975; Kutteh *et al,* 1988).

The increase in heterosexually-acquired HIV infection in women has highlighted the paucity of information on the mucosal immunity of the female genital tract and its role in the prevention of infection. It is not known where in the genital tract HIV transmission occurs but the circumstantial evidence suggests that the uterine cervix is an important site. Inoculation of the cervix of a female chimpanzee with simian immunodeficiency virus (SIV)-infected fluid resulted in viraemia (Pomerantz *et al,* 1988). Inoculation of the vagina in the female macaques with SIV showed that the first cellular targets for SIV were in the lamina propria of the cervico-vaginal mucosa (Spira *et al,* 1996). In humans, cross-sectional studies indicate that cervical ectopy and inflammation are associated with increased shedding of HIV into cervico-vaginal secretions (Clemetson *et al,* 1993; Kreiss *et al,* 1994). This, and the evidence presented above that immunocytes are mainly concentrated in the cervical transformation zone led to the decision to focus this study on the squamo-columnar junction of the cervix. Thus, immunohistochemical and double immunofluorescence techniques were used to define the distribution and proportions of immunocompetent cells in the cervix of HIV-negative women.

Figure 3:1 **The Menstrual Cycle**

Relationship to hypothalamic/pituitary axis.

The normal menstrual cycle lasts for 28 days, with menstruation occurring on day 1-5, and ovulation on day 14. Considerable variation can occur and is compatible with normal functioning. After menstruation, rising levels of oestrogen exert a negative feedback on the pituitary, inhibiting follicular stimulating hormone (FSH) release. Towards mid-cycle, still higher levels of oestrogen exert a positive feedback and cause as sudden peak in luteinising hormone (LH) which induces ovulation. FSH levels also rise. If conception fails to occur following ovulation, oestrogen and progesterone levels fall after about 7 days and menstruation occurs.



Materials and methods

Subjects

The subjects for this section of the study consisted of the 20 HIV-negative women described in chapter 2. None of the subjects had current genital infections and all cervical smears were reported as normal. There were two smokers in the group, each smoking 20 cigarettes a day. All subjects had cervical biopsies taken as previously described and, in addition, five of the subjects had additional biopsies taken so that two phases of the menstrual cycle were studied: the post-menstrual and pre-menstrual phases. None of the subjects had had sexual intercourse in the 24 hours prior to biopsy.

Preparation of biopsy specimens

The cervical biopsies were placed in optimal cutting temperature medium (OCT, BDH, Poole) on small cork discs and frozen in isopentane cooled in a bath of liquid nitrogen. Using a cryostat maintained at -25^{0}C, 6 μm sections were cut from one biopsy from each subject and transferred to poly-l-lysine coated slides. These were air dried, fixed in a mixture of chloroform and acetone (1:1), wrapped in cling film and stored at -20^{0}C until use. At least 40 sections were cut from each biopsy specimen. Sections from all biopsy specimens were stained with Haematoxylin and Eosin and Toluidine Blue (0.1%W/V) to show morphology before further processing.

Analysis by Immunohistochemistry

A standard protocol for the indirect immunoperoxidase method was used to identify individual cell surface antigens (Mason *et al,* 1983). Figure 3:2 illustrates the steps involved in this procedure. After 10 minutes incubation with 1% normal rabbit serum (NRS), the biopsy sections were incubated with the appropriate monoclonal antibody at optimal dilution for one hour. NRS swamps non specific binding sites before application of the first layer reagent. The working concentrations and incubation times of monoclonal antibody (usually 1:5) had been established in the laboratory previously by titration studies using human tonsil sections. The specific monoclonal antibodies (MoAb) used are indicated in the table 3:1. After washing with phosphate buffered saline (PBS), the sections were further incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO, Copenhagen) and a 1:25 solution of normal human serum (NHS) for one hour. The sections were then washed in PBS and developed for five minutes in freshly made diaminobenzidine (DAB) and hydrogen peroxide (H_20_2) solution. DPX, the mounting medium is produced by a mixture of Distrene, (a polystyrene) and plasticizer (Dibutyl Pthalate) and Xylene.

All staining was carried out at room temperature in a humidified chamber to prevent drying of the sections or evaporation of the antibody. Simultaneous negative controls, in which the antibody was omitted from the cervix biopsy section were set up on each occasion. Human palatine tonsil was used as a positive tissue control, except when

taining for Langerhans' cells, when normal human skin was used. Isotype controls for all MoAb used were performed on tonsil sections using an irrelevant antibody. The immunoperoxidase method was used to quantify macrophages, T lymphocytes, B lymphocytes, plasma cells and Langerhans' cells within the cervical epithelium.

Table 3:1 Monoclonal Antibodies Used In This Study

Monoclonal antibody	Immune cells	Source
RFTmix (CD2, CD5, CD8)	Mature T cells	RFHSM
CD22 (RFB4)	B Lymphocytes	RFHSM
CD68 (EBM1)	Macrophages	*Dako
CD1 (RFT6)	Langerhans' cells	RFHSM
RFD1	Antigen presenting cells	RFHSM
RFD7	Phagocytic macrophages	RFHSM
RFD1/RFD7	Suppressor macrophages	RFHSM
CD4 (RFT4)	Helper/inducer T cells	RFHSM
CD8 (RFT8)	Suppressor/Effector T cells	RFHSM

RFHSM: Royal Free Hospital School of Medicine

*Dako Ltd, High Wycombe, England, UK

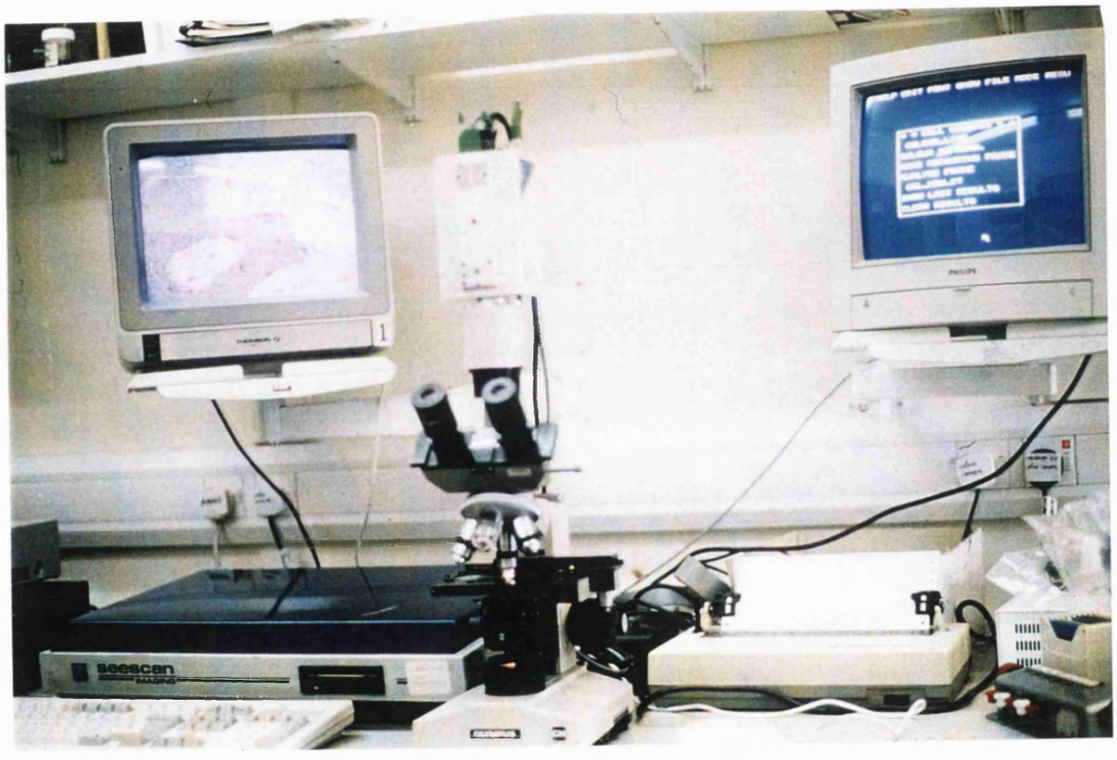
The distribution of positive cells was measured using an image analyser (Seescan, Cambridge). This equipment generates a computerised image of the section which enables the observer to draw a frame around the area of interest in the section (figure 3:3). The drawing mechanism is flexible, allowing one to select areas of interest like epithelium or stroma, while omitting irrelevant areas or artefact. The software package calculates the area of the selected frame ($10^4 \mu m^2$). Cells within the frame exhibiting identifiable reactions on their cell surfaces and cell membranes were scored as positive and point counted on the screen. Background staining was identified by comparison with the negative control sections. The cell count is then automatically related to the area of the frame to generate a count/unit area. Using the x 40 objective, the positive cells within each framed area of field (three per section) were point counted and the mean cell count of the three areas calculated. As the biopsy sections were usually very small, this normally represented the whole section. In all sections from all biopsy specimens, the variability between selected areas of any one specimen was far less than the variability between specimens. All results given therefore quote the range throughout the group, thus reflecting the greatest variability seen. In each measurement the number of cells counted was divided by the area of framed tissue to obtain a figure representing the number of cells per $10^4 \mu m^2$(unit area). Each section was checked by two independent observers (LP, AO).

Figure 3:2 **Analysis by immunohistochemistry**

Schematic diagram illustrating steps involved in the indirect immunoperoxidase method used throughout this thesis.

Specimen Preparation	Sections thawed to room temperature
	Specimens circled with polysiloxane (water repellent)
	Placed in a humidity chamber
	⇓
First Layer	50µl 1:100 NRS applied for 10 minutes
	50µl of MoAb applied after draining excess NRS
	Incubated for 45 minutes
	Rinsed twice in PBS
	⇓
Second Layer	50µl of 2nd layer MoAb (1:100 P161 & 1:25 NHS) applied for 45 minutes
	Rinsed twice in PBS
	50µl of developing solution (DAB tablet dissolved in16.6mls PBS, + 166 µl 1% H_2O_2) for 5 minutes
	Rinsed in tap water
	⇓
Counterstaining	Counterstained with Haematoxylin
	Dehydrated through graded alcohols
	Mounted in DPX

Figure 3:3 Image Analyser (Seescan, Cambridge)



Analysis by immunofluorescence

The simultaneous expression of two different antigens on one single cell was identified by the double immunofluorescence technique, using two different fluorochromes (fluorescein-isothiacynate, FITC and tetramethyl-rhodamine-isothiocyanate, TRITC). Both these fluorochromes are excited by UV radiation but they display a different spectrum of light emission: green for FITC, red for TRITC (Janossy *et al,* 1983). Cervical biopsy sections were allowed to equilibrate to room temperature and incubated in a moist chamber with 50µl of each selected MoAb (table 3:1). After washing with PBS, a 50µl mixture of goat anti-mouse immunoglobulin M TRITC and goat anti-mouse IgG FITC (Southern Biotechnology Associates Birmingham AL) was added for 45 minutes as a second layer (figure 3:4). Goat immunoglobulin second layer reagents were used in view of previous reports of binding of aggregated immunoglobulin to Fc receptors on human cells during such tests. Goat immunoglobulin binds inefficiently to human Fc receptors and thus gives cleaner results.

Immunofluorescent counting was performed on a fluorescence microscope (Zeiss, Oberkochen, Germany) at x 40 magnification with narrow band barrier selective filters for FITC and TRITC. Background fluorescence was determined using positive and negative controls as in the immunoperoxidase method. Cells were either positive for FITC or TRITC or both. As before, three representative fields, or a minimum of 100

cells were counted. The subsets were expressed as a percentage of the whole using the equation illustrated below. Doubly-staining cells are represented in the results as a percentage of total positive staining cells present. This method was used to quantify macrophage and T lymphocyte sub-set proportions within the genital tract mucosa. For example:

% of D1+ cells= (D1+)x 100/(D1+) + (D7+) + (D1+/D7+)

Figure 3:4 **Analysis by Immunofluorescence**

Schematic diagram illustrating the main steps involved in the immunofluorescence method used throughout this thesis.

50µl of MoAb applied to specimen
Incubated in moist chamber for 60 mins

⇓

Rinsed twice in PBS

⇓

50µl of goat class-specific 2nd layer Ig
conjugated with FITC & TRITC respectively

⇓

Rinsed twice in PBS

⇓

Mounted in 10% PBS/90% glycerol

Results

Cervical Smears and Genital Infection Screening

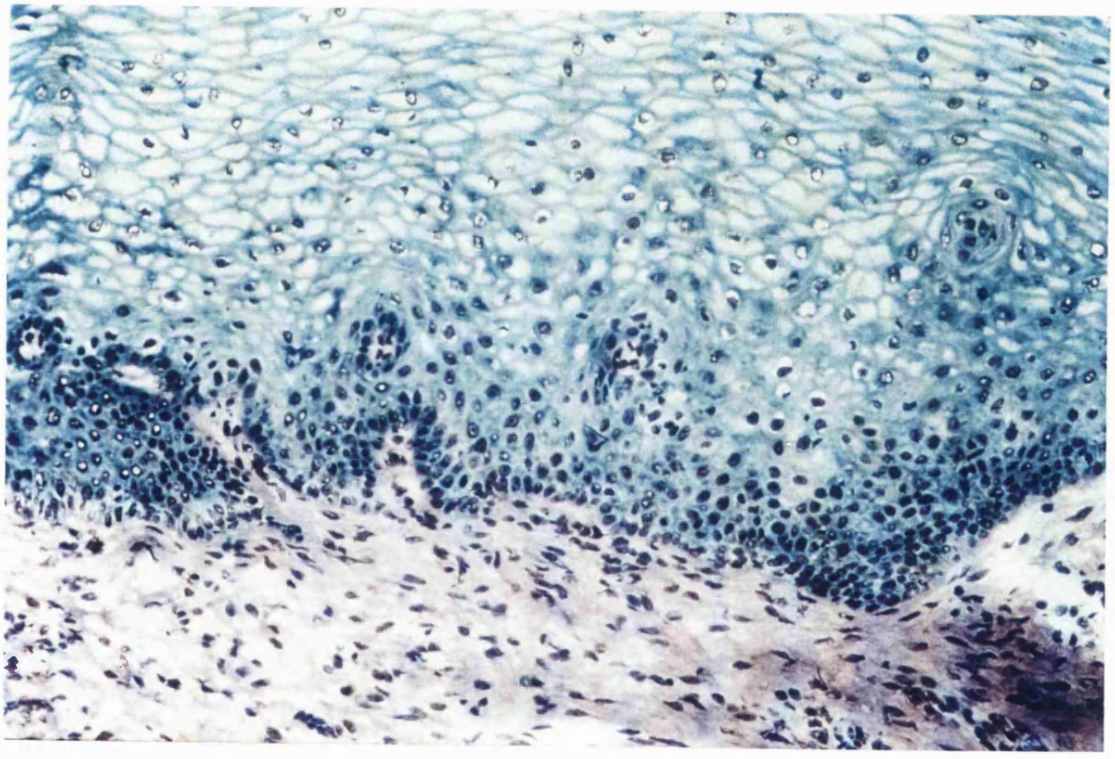
There were no colposcopic abnormalities in this group of patients and all cervical smears were reported as normal. Genital infection screening confirmed the absence of sexually transmitted diseases and other genital infections.

Histology

Haematoxylin and eosin staining confirmed that none of the samples showed any pathological changes, although a variable degree of cellularity and epithelial integrity was observed. Some epithelial disruption was observed in some sections and this was felt to be artefactual, resulting from freezing and cryostat sectioning of small samples. The classical pattern of stroma, basement membrane and mature, differentiated squamous epithelium was seen in each subject (figure 3:5). There was no tissue oedema or thickening of the basement membrane observed. Toluidine blue staining revealed small numbers of mast cells: one to three per section in all samples. Frozen tissue sections are however inappropriate for studying granulocytes which require more elaborate tissue fixation and embedding and use of specific monoclonal antibodies. Thus further definition of the granulocyte population was not undertaken as this was felt not to be directly relevant to the central theme of this thesis: the cell-mediated immune response.

Figure 3:5 Section of Cervix from HIV-negative subject stained with H&E

(Original magnification x400)



Lymphocyte, Langerhans' cell & Macrophage Counts

Lymphocytes and macrophages were found mainly in the sub-epithelial stroma and all analyses described below refer to this area. Langerhans' cells, which showed a body and one to three cytoplasmic processes extending between adjacent squamous epithelial cells, were observed mainly in the epithelium (figure 3:6). The mean cervical macrophage, T and B lymphocyte, plasma cell and Langerhans' cell counts are shown in table 3:2. Table 3:3 shows the relative cell counts at the two phases in the menstrual cycle, illustrating that, although there is a tendency to greater cellularity in the luteal phase, there is no significant difference in immunocompetent cell proportions in the follicular and luteal phases of the menstrual cycle.

Table 3:2 The Distribution of Immunocompetent Cells in HIV-negative subjects

Cells types	**Mean cell counts/$10^4\mu m^2$ (sd)**
Macrophages	8.29 (4.30)
T Lymphocytes	11.59 (9.60)
B Lymphocytes	1.29 (1.49)
Plasma Cells	4.85 (2.50)
Langerhans' Cells	13.37 (5.80)

Table 3:3

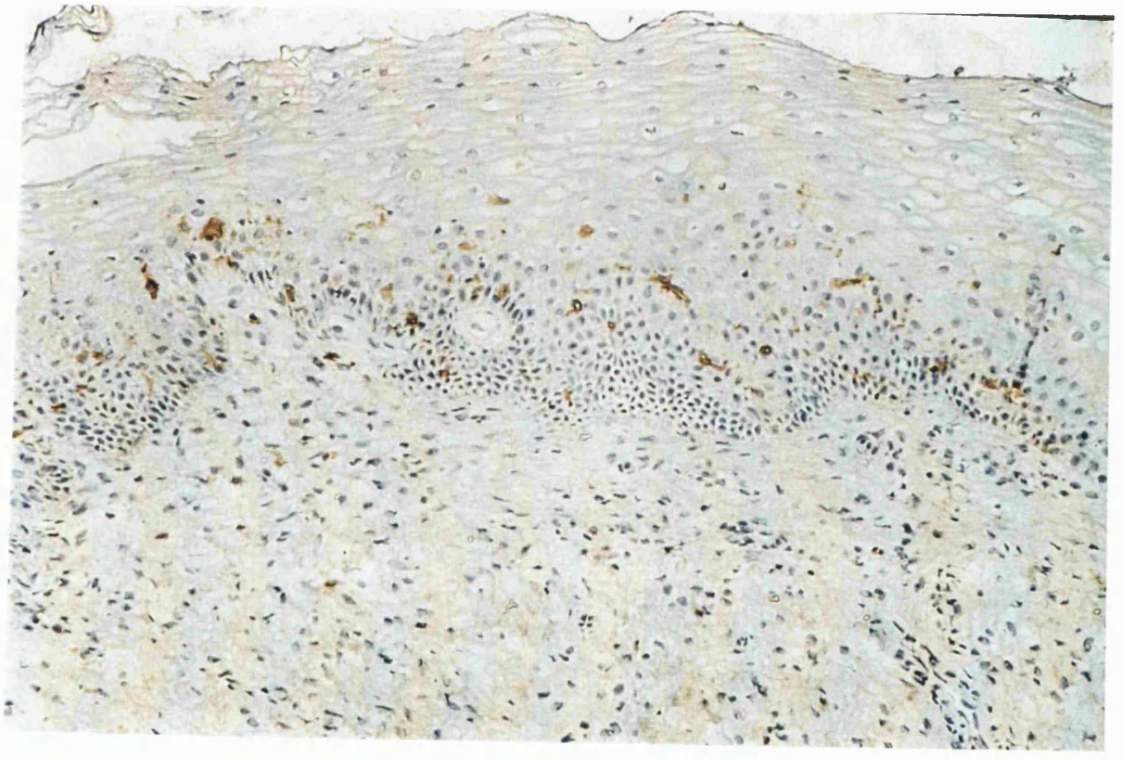
Variation of immunocompetent cell count through menstrual cycle n=5

Median (range)

Immunocompetent Cells/$10^4 mm^2$ (sd)	Follicular Phase	Luteal Phase	p*
Macrophages	10.0 (4.0-16.0)	19.70(11.0-26.5)	0.1
T cells	8.25 (4.5-24.3)	15.5(12.7-21.5)	0.3
B cells	0.5 (0-1.75)	2.25 (0-2.5)	0.3
Plasma cells	4.25 (0.5-6.5)	6.1 (2.5-8.6)	0.4

*Wilcoxon rank sum test

Figure 3:6 Biopsy of cervix from HIV-negative subject demonstrating the distribution of Langerhans' cells. Immunoperoxidase staining with CD1 (Original magnification x 400)



Macrophage & Lymphocyte subset ratios

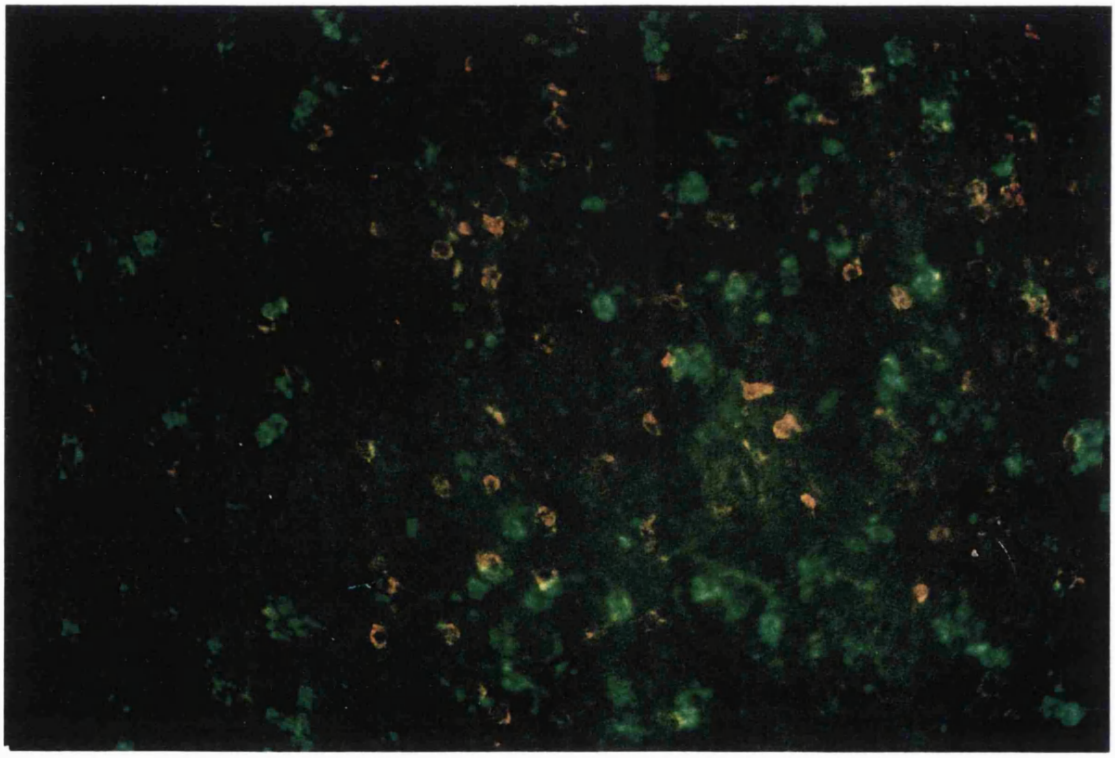
The majority of macrophages were RFD7 positive with smaller proportions being positive for RFD1 and RFD1/D7, respectively (Table 3:4). Figure 3:7 illustrates the appearance of RFD1/D7 cells stained with the double immunofluorescence technique. The T lymphocytes were predominantly CD4+ cells with a minority expressing CD8 antigens (Table 3:4).

Table 3:4 Macrophage and lymphocyte subset proportions in cervix sections from HIV-negative subjects

	Mean % (sd)	**Median % (range)**
Macrophages		
RFD1	8.6 (5.3)	7.3 (3.9-17.5)
RFD7	87.8 (7.8)	85 (75.5-95)
RFD1/D7	4.13 (3.7)	3.9 (0-8.6)
T Lymphocytes		
CD4	73.2 (7.0)	74.3 (63.0-83.0)
CD8	26.7 (7.0)	25.7 (17.0-37.0)

Figure 3:7 Section of cervix from HIV-negative subject: Immunofluorescence staining to demonstrate RFD1/RFD7 macrophage subsets. (Magnification x 400)

D1 - TRITC (red), D7- FITC (green), D1/D7 - doubly-stained, appear orange.



Discussion

This is one of the first comprehensive studies into the distribution of immunocompetent cells in the lower genital tract of normal women. Most previous work has been focused on the immune response to cervical cancer (Fukuda *et al,* 1993; Bell *et al,* 1995) and wart virus infection (Morris *et al,* 1983a). The presence of macrophages, Langerhans' cells, and lymphocytes in the cervical mucosa has been previously noted but there have been few attempts to assess their relative proportions in the normal state. Previously published data are scanty and often contradictory.

This work has confirmed that immunocytes are present in the cervical mucosa at all stages of the menstrual cycle, which is consistent with the observation of Morris *et al* (1983b) that chronic inflammatory cells are a common finding in the transformation zone stroma. The T cells are mainly of the T-helper (CD4+) sub-type, which is in contrast with the work of Pomerantz *et al* (1988) where they found a 1:1 ratio of CD4 to CD8 cells in the cervix of HIV-negative women. Morris *et al* (1983b) found that the majority of T cells in the normal tissue they studied were CD8+, with CD4 cells seen uncommonly. However both these group of authors only looked at two 'normal' subjects each in their study, and, in the case of Pomerantz *et al* (1988), both were intravenous drug users. There is, however, as demonstrated by these results, substantial inter-subject variation in the distribution and proportions of immunocompetent cells, and accurate representations cannot be expected if only two subjects are studied.

We found that B lymphocytes and plasma cells were scanty in the sub-mucosa which is consistent with previous publications (Morris *et al,* 1983b; Schumacher, 1988). In contrast to this, Crowley-Norwick *et al* (1995) found that B lymphocytes were the predominant cell type in normal cervical tissue, and were at higher concentrations than in the peripheral blood. However, they used a digestion technique and the possibility of contamination of cervical tissue from circulating blood lymphocytes cannot be excluded. Rebello and Green (1975) in a study of 58 uteri obtained at hysterectomy found that plasma cells were aggregated maximally at the squamo-columnar junction and beneath areas of squamous metaplasia but they did not attempt to compare the numbers with relative proportions of other immune cells.

Changes in serum sex hormone concentrations have been shown to affect immunoglobulin concentrations in cervico-vaginal secretions, with immunoglobulin concentrations lowest at the time of ovulation, less than 10% of those in the post-menstrual phase (Forrest *et al,* 1991). It has been suggested that oestrogen reduces immunoglobulin concentrations in genital tract secretions by a direct suppression of the mucosal plasma cell immunoglobulin production (Schumacher 1988). Murdoch *et al* (1982) found that IgA-containing plasma cells were apparently decreased under the influence of oestrogen and increased under the influence of progesterone. We found increased plasma cell concentrations in the luteal (progestogenic) phase of the menstrual cycle, but the difference was not statistically significant. The study may have

lacked the power to detect a small difference as numbers studied were relatively small. However, consistent with our observations, Rebello and Green (1975) found no difference in plasma cell concentrations in 58 cervix sections between the different phases of the menstrual cycle. Despite the relatively small proportions of plasma cells detected, there is evidence that the induction of specific local immunity is acquired after exposure to various antigens, in experimental and natural settings. In herpes simplex type 2 (HSV-2) infection of the cervix, the local anti-HSV-2 secretory IgA antibody is associated with significantly shorter duration of viral shedding (Merriman *et al,* 1984). Specific antibodies in genital tract secretions have also been demonstrated against candida (Bohler *et al,* 1994) and other genital tract pathogens.

We found that there were substantial number of macrophages within the cervical stroma, in contrast to Pomerantz *et al* (1988) and Morris *et al* (1983b). The question of macrophage heterogeneity has assumed increasing importance in recent years with greater appreciation of the multiplicity of roles of these cells in the immune response. Macrophage subset proportions seem to vary at different mucosal surfaces which implies that there is a customised adaptation to meet the varied requirements at each mucosal surface. Unlike in the gut (Liu *et al,* 1993b) and lung (Lipman *et al,* 1995), antigen-presenting cells (RFD1+) appear to predominate in the normal cervix, with smaller proportions of phagocytic (RFD7+) and suppressor (RFD7+) macrophages.

As expected, we found Langerhans' cells within the epithelium. It has been reasonably well established that these cells play a pivotal role in the process of exogenous antigen recognition. Morris *et al* (1983b) suggest that the central role of Langerhans' cells is to take up and process exogenous antigen and to present it to stromal lymphocytes and macrophages in an immunologically appropriate way. A close association was observed between Langerhans' cells and cytotoxic T cells leading to the hypothesis that activated T cells may migrate to the epithelium to eradicate antigens in response to signals from Langerhans' cells. Thus, the integrity of Langerhans' cells may be essential for the normal functioning of T cells in the genital tract mucosa . Maclean *et al* (1984) suggested that Langerhans' cells may play a role in the healing of cervical mucosa after local ablative therapy, implying that Langerhans' cells may have the additional function of maintaining the health of cervical tissue.

Conclusion

This study confirms previous findings that immunocompetent cells are present in the normal cervical mucosa. In addition, it has been possible, for the first time, to accurately quantify the proportions of these cells and their subsets. It has also been possible to observe the effect of the menstrual cycle on these cell populations. These observations, which confirm the existence of a local immune system within the female lower gental tract, provide a baseline to study the function of immunocompetent cells

within the normal genital tract mucosa and to determine if there are alterations associated with HIV infection.

Chapter 4: THE GENITAL TRACT MUCOSA OF HIV-POSITIVE WOMEN

Introduction

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CHAPTER 4: THE GENITAL TRACT MUCOSA OF HIV-POSITIVE WOMEN

Alterations in immunocompetent cell proportions associated with HIV infection

Introduction

It is evident from the foregoing chapter that the cells responsible for humoral and cell-mediated immune responses are represented in the genital tract mucosa. It would be expected therefore that there is the capacity to mount an immune response at a local level to invading bacteria and viruses. Thus, exposure to HIV should lead to the generation of a cell mediated immune reaction and resolution of infection as described in part 5 of the introduction. However, heterosexual exposure to HIV in many women leads to a chronic viral illness and a profound impairment of the systemic immune response culminating in AIDS, thus implying a failure of the local immune system to resist infection with the virus. In addition, women with HIV infection are subject to cervical intraepithelial neoplasia and recurrent viral and fungal infections of the genital tract even when peripheral CD4 lymphocyte counts are in the normal range, implying a disturbance in local genital tract immunity. The aim of this study is to determine if HIV infection is associated with changes in the distribution of immunocompetent cells in the genital tract which may explain the failure of the local immune system to eliminate the virus and prevent systemic infection after heterosexual exposure to HIV. Alterations in immunocompetent cell distribution may also explain the susceptibility to genital infections observed in HIV-infected women.

Materials and Methods

Subjects

The forty HIV-positive women described in chapter 2 were included in this section of the study. As previously indicated (chapter 2), HIV-positive women were included in this study only if they were in their reproductive years and with normal menstrual cycles. Colposcopy, biopsy and screening for STDs were performed as described (chapter 2) and women were excluded if they had positive cytology, abnormal or inadequate colposcopy or abnormal histology. Women with current STDs were also excluded. Peripheral blood was collected for immune studies on the same day the biopsy was taken.

Laboratory techniques

Immunoperoxidase and fluorescence techniques, as described in chapter 3, were used to study the immunocompetent cell distribution in the cervical biopsy sections. In addition to the antibodies used in chapter 3, double immunofluorescence was performed on the cervix biopsy sections using anti-CD8 antibody in combination with each of the antibodies listed in table 4:1. Systemic CD4 counts were performed using a whole blood analysis method, and the percentage of CD4 lymphocytes was expressed as absolute CD4 counts on the basis of lymphocyte counts, as previously described. Comparisons were made between immunocompetent cell proportions in the cervix and peripheral blood of HIV-positive women. Comparisons were also made

between the immunocompetent cell proportions in the cervix of HIV-positive women and the immunocompetent cells in the cervical biopsy sections of the 20 HIV-negative women described in the preceding chapter.

Table 4:1 Monoclonal Antibodies Used In This Chapter

Monoclonal antibody	Immune cells	Source
CD28	Activated T cells	Serotec*
CD25	Activated T cells (IL-2 receptor)	RFHSM
Bcl-2	Gene product inhibiting cell apoptosis	Dako‡
CD57	Natural killer cells	RFHSM
CD45ro (UCHL1)	Primed T lymphocytes	Dr P. Beverly#
Perforin	Cytolytic Granules	Prof. B. Dupont**
TIA-1	Cytolytic granule-associated protein	Coulter♠

* Serotec, Kidlington, England

‡ Dako Ltd , High Wycombe, UK

University College Medical School, London

** Sloane Kettering Cancer Centre, New York, USA

♠Coulter Electronics, Florida, USA

Results

Characteristics of study population

The demographic details of the study population are summarised in chapter 2 (Table 2:2 and 2:3). The median age of the subjects was 33 years (23-48) and the median peripheral CD4 lymphocyte count was 288/mm^3 (10-880). There were no STDs or cervical abnormalities in this group.

Histology

Haematoxylin and eosin staining confirmed that none of the samples showed any histological abnormalities. The classical pattern of stroma, basement membrane and mature, differentiated squamous epithelium was seen in all sections. Toluidine Blue staining showed one to three mast cells per section in all samples.

Immunohistology

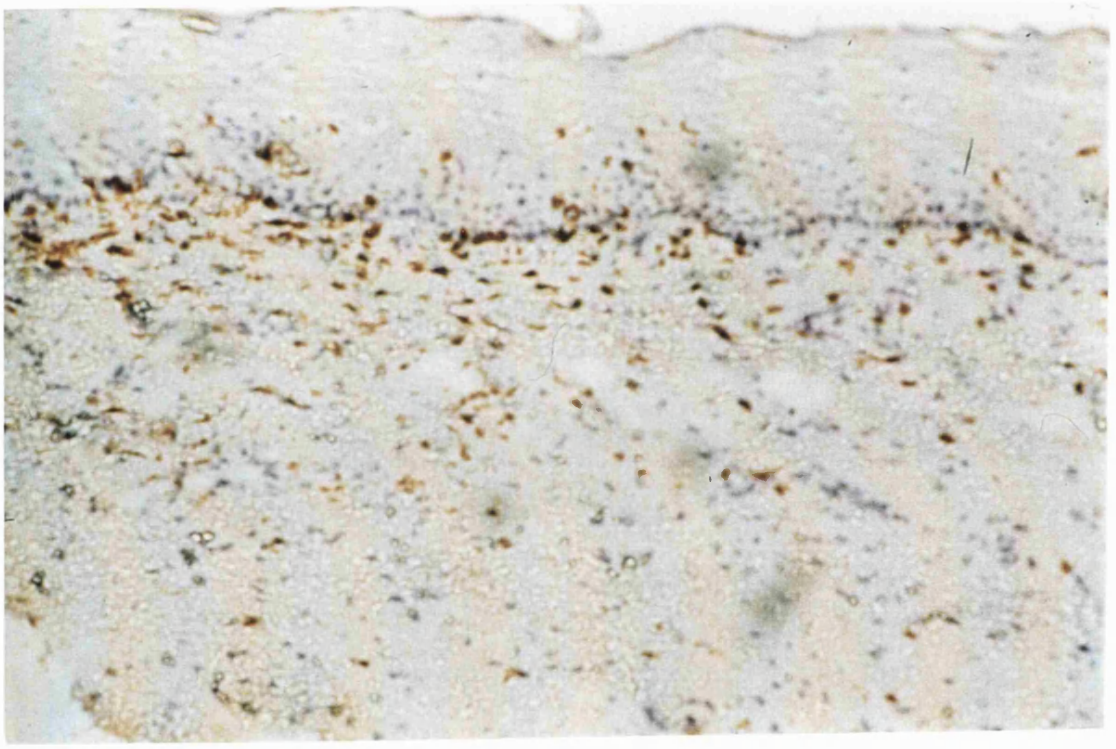
Table 4:2 shows the mean lymphocyte, macrophage and Langerhans' cell counts in HIV-positive women. There were numerous T lymphocytes and macrophages but very few Langerhans' cells, B cells and plasma cells per unit area in the cervical biopsy sections. Figure 4:1 shows T lymphocytes stained with the immunoperoxidase technique in a section of cervix from an HIV-positive subject.

Table 4:2 Mean Immunocompetent Cell Counts: HIV-positive subjects ($10^4 \mu m^2$)

Cell Type	Mean count (sd)
T Cells	21.2 (8.7)
Macrophages	18.3 (12.6)
B Cells	1.2 (0.9)
Plasma Cells	3.6 (0.8)
Langerhans' cells	3.8 (2.2)

Figure 4:1 Section of cervix from HIV-positive subject stained with immunoperoxidase (RFTmix) to show distribution of T lymphocytes

(Original magnification x 400)



Macrophage and lymphocyte subset ratios

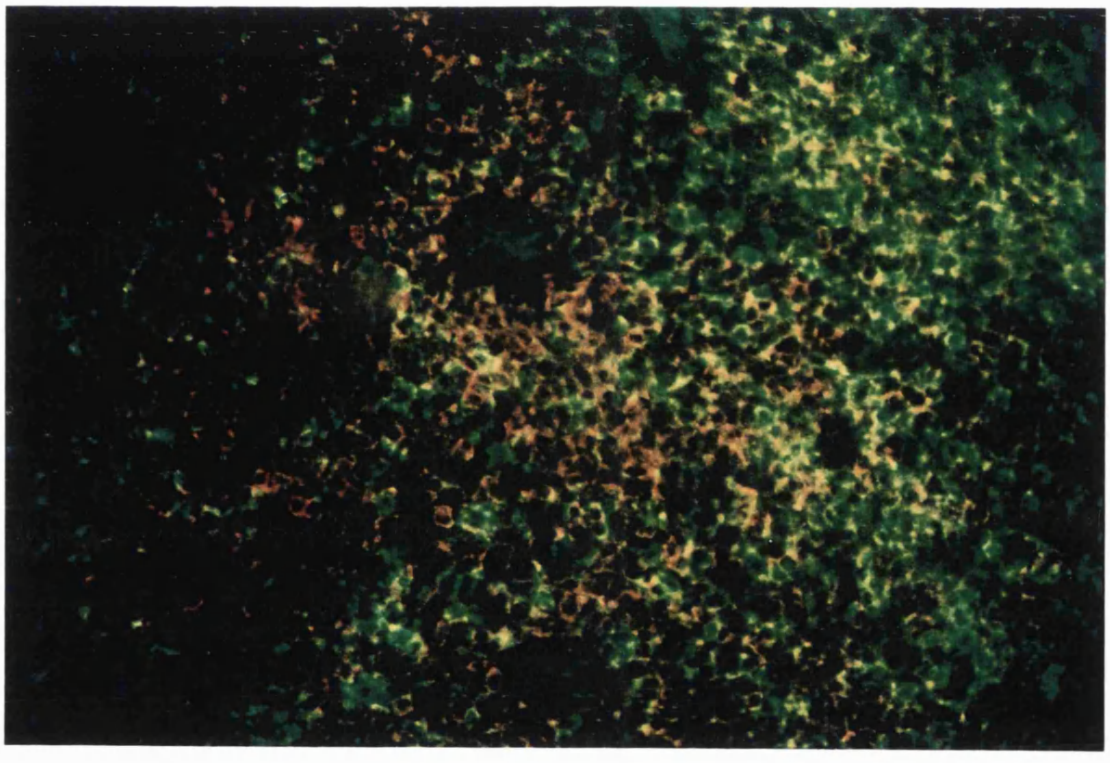
Table 4:3 shows the mean RFD1/D7 macrophage subset and the CD4/CD8 T lymphocyte subset proportions as revealed by double immunofluorescence staining in cervical biopsy sections from HIV-positive women. It can be seen that the majority of macrophages from these sections are RFD7+ and the majority of T lymphocytes are CD8-positive. These proportions were consistent in all cervical biopsy sections studied from the HIV-positive subjects. Figure 4:2 shows a section of cervix from an HIV-positive subject stained with the double immunofluorescence technique to show CD4/CD8 lymphocyte subsets.

Table 4:3 Macrophage and lymphocyte subset proportions in cervix sections from HIV-positive subjects

	Mean % (sd)	**Median % (range)**
Macrophages		
RFD1	7.9 (6.6)	4.2 (1.2-18)
RFD7	86.2 (6.7)	84.6 (78.0-95.8)
RFD1/D7	4.4 (0.9)	1.6 (0-12.6)
T Lymphocytes		
CD4	26.4 (9.3)	28.5 (6.7-39.7)
CD8	74.4 (9.6)	74.2 (64.0-93.0)

Figure 4:2 Section of cervix from HIV-positive subject: Immunofluorescence staining to demonstrate CD4/CD8 T lymphocyte subset distribution. (x 400)

CD4 - red (TRITC), CD8 - green (FITC)



Comparison with HIV-negative cervical biopsy sections

Haematoxylin and Eosin staining showed that there was no difference in histological appearance between HIV-positive cervical biopsy sections and the HIV-negative cervical biopsy sections described in the previous chapter. Mast cell numbers and distribution, as revealed with Toluidine blue staining, were similar in cervical biopsy sections from both groups. The mean Langerhans' cell count was significantly reduced and the T lymphocyte count significantly increased in the HIV-positive group compared with the HIV-negative group (Table 4:4). No difference was observed in the macrophage, B lymphocyte and plasma cell proportions between the two study groups.

Table 4:4 Immunocompetent Cell Counts: HIV-positive & HIV-negative Subjects

Cell Type	HIV-positive Median (Range)	HIV-negative Median (Range)	p*
T Cells	23.0 (6.0-36.8)	7.7 (3.5-30.6)	0.002
Macrophages	14.8 (4.0-35.5)	12.4 (4.7-39.0)	1.0
B Cells	1.5 (0-2.0)	2.3 (0-2.7)	0.36
Plasma Cells	3.4 (3.0-4.6)	6.7 (2.0-7.3)	0.10
Langerhans' Cells	3.3 (1.5-8.5)	13.5 (5.6-21.0)	0.0045

* Mann Whitney U test

There was no difference in the RFD1/D7 macrophage subset ratios in HIV-positive and HIV-negative cervix sections (figure 4:3). However, there was a reduction in the CD4 subset proportions and an increase in the CD8 proportions in the HIV positive compared to HIV-negative sections, resulting in an inversion of the CD4/CD8 ratios (figure 4:4). The increase in CD8 lymphocyte proportions represented an absolute increase in CD8 count, since T cell numbers were significantly increased in the HIV-positive group. It was therefore decided to examine the phenotype of CD8 cells in HIV-positive and HIV-negative subjects, using double immunofluorescence with the monoclonal antibodies illustrated in table 4:1. Direct comparisons were made between the CD8 phenotype in HIV-positive and HIV negative cervical biopsy sections.

Figure 4:3

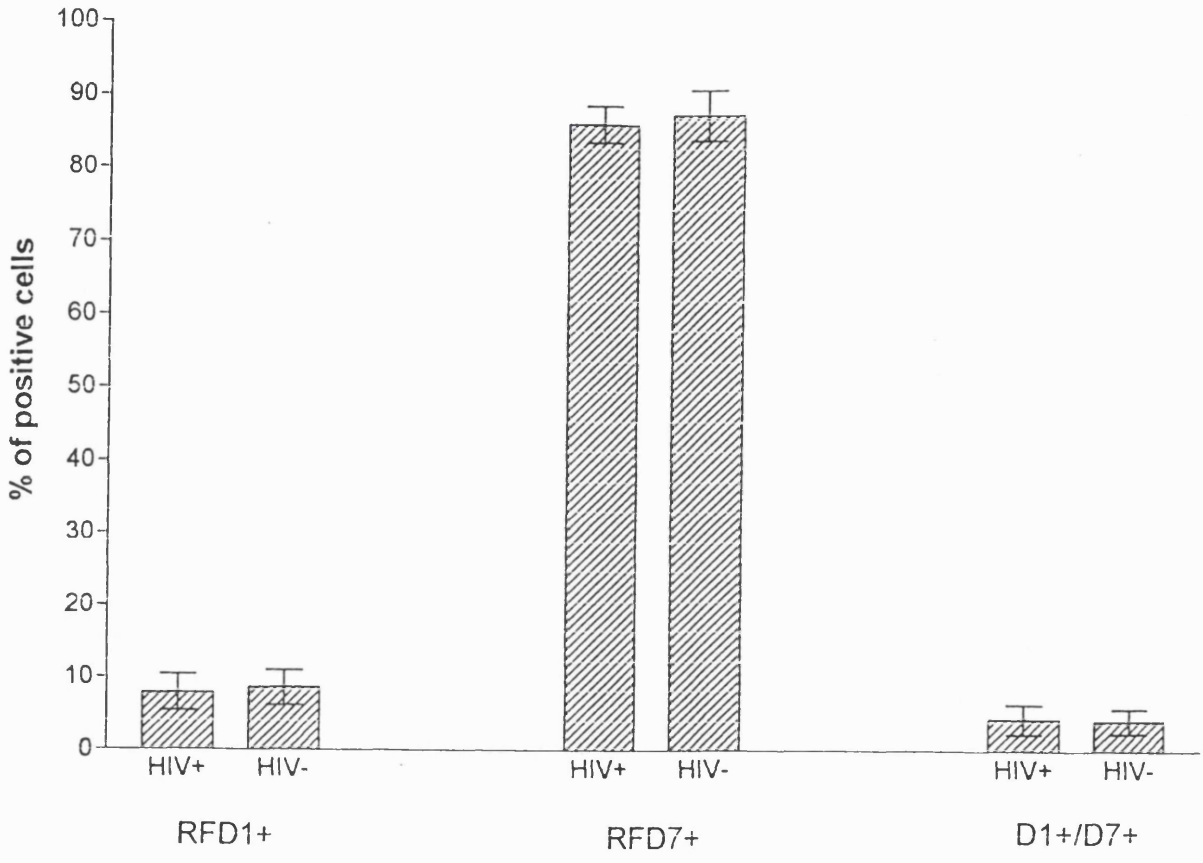


Diagram showing the distribution of RFD1/RFD7 macrophage subsets in HIV-positive and HIV negative subjects. (Counted by double immunofluorescence)

Mean and standard error shown

Figure 4:4

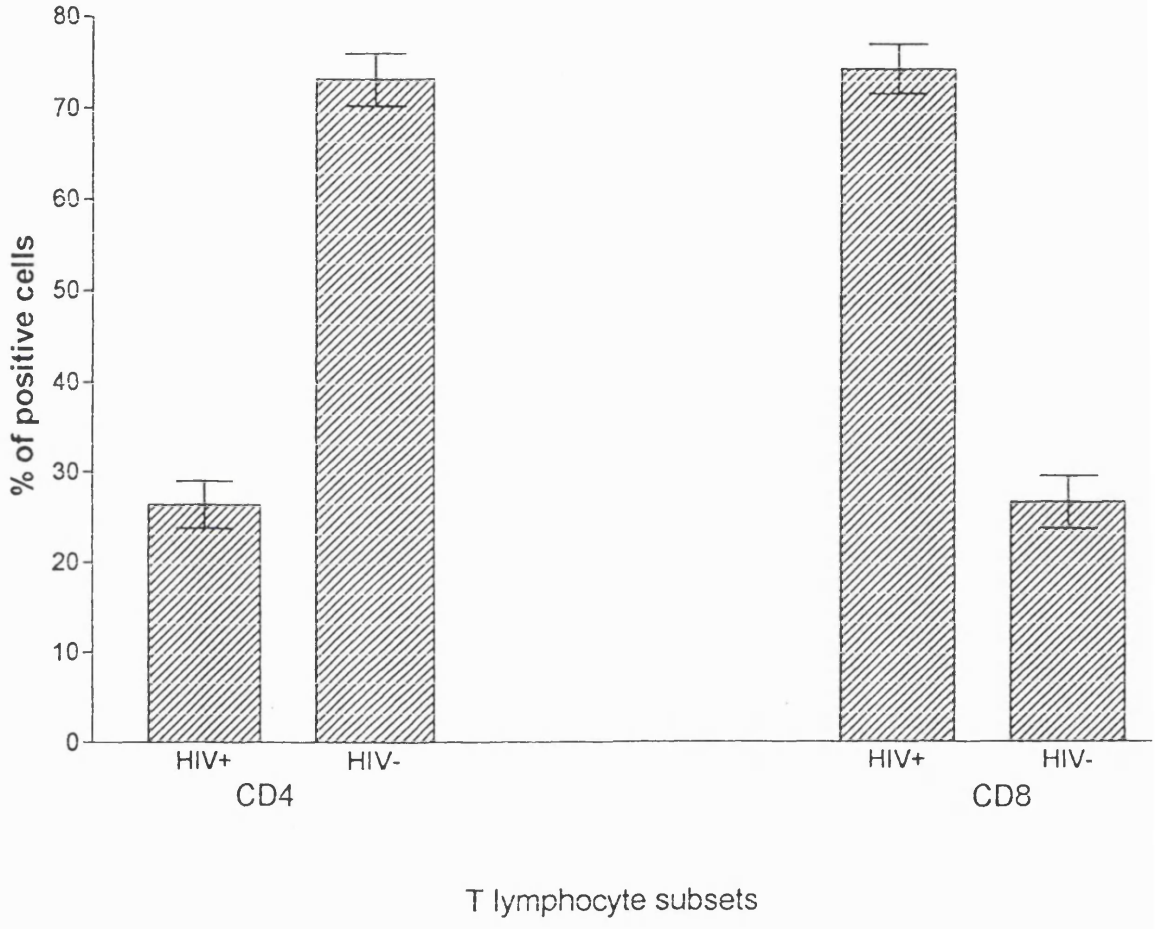


Diagram showing the distribution of CD4/CD8 T lymphocyte subsets in HIV-positive and HIV negative subjects. (Counted by double immunofluorescence)

Mean and standard error shown

Phenotypic characterisation of CD8+ cells

Table 4:5 shows the distribution of CD8+ phenotypes in cervix biopsies from the HIV-positive and HIV-negative groups. It can be seen that the CD8+ cells in biopsies from both the HIV-positive and HIV-negative groups were negative for perforin, CD25 and CD28. A significantly lower proportion of CD8 cells from the HIV-positive group than the HIV-negative group expressed TIA-1. No CD57+ cells were observed in biopsies from either group. However, CD8+ cells in both groups expressed Bcl-2 and the mean proportion of the CD8+ cells expressing Bcl-2 in the HIV-positive group was not significantly different from the HIV-negative group. However, when the Bcl-2 expression was related to the peripheral blood CD4 lymphocyte counts, it was shown that in HIV-positive women with CD4 counts of less than 400/l cervical CD8+ lymphocytes showed significantly less Bcl-2 expression (median=32.5 (17.0-41.5)) than cervical CD8+ lymphocytes in the HIV-negative subjects (p=0.01, Man Whitney U). There were significantly more CD45ro+ CD8+ T lymphocytes in cervix biopsies from the HIV positive group compared with the HIV- negative group.

Table 4:5 Distribution of CD8+ phenotypes in cervical biopsies from HIV-positive & HIV-negative subjects

Cell Types	HIV-positive % Median (range)	HIV-negative % Median (range)	p*
CD8+/CD25+	nil	nil	-
CD8+/CD28+	nil	nil	-
CD8+/Perforin	nil	nil	-
CD8+/TIA-1+	43.9 (28.0-51.0)	85 (70.9-95.0)	0.02
CD8+ CD57+	nil	nil	-
CD8+/Bcl-2	36.0 (17.0-55.0)	47.3 (35.5-47.5)	0.13
CD8+/CD45ro+	80.0 (77.0-94.0)	40.0 (30.0-41.0)	0.02

* Mann Whitney U test

Correlates with peripheral blood count

HIV-positive women with normal peripheral CD4 lymphocyte counts showed a high proportion of CD8+/Bcl-2+ T lymphocytes in the cervix. However, the proportion of Bcl-2+ CD8+ lymphocytes in the cervix fell with declining peripheral CD4+ counts (Figure 4:5). A positive correlation (r=0.8, p=0.002, Spearman rank) was detected between peripheral blood CD4 lymphocyte counts and the proportion of Bcl-2+ CD8 lymphocytes in the cervix. We also compared the cervical CD4/CD8 ratios in the

HIV-positive women to the CD4/CD8 ratios in their peripheral blood. It can be seen that the CD4/CD8ratios in the cervix are less than the CD4/CD8 ratios in the peripheral blood in subjects with peripheral CD4 counts greater than $330x10^6/l$ (table 4:6) but there was no statistically significant correlation. The proportions of memory cells in the HIV-positive patients did not correlate with disease stage.

Table 4:6 CD4/CD8 ratios in the cervix compared with blood CD4/CD8 ratios

Blood CD4 Count	Blood CD4/CD8 ratio	Cervix CD4/ CD8 ratio
880	0.75	0.25
820	1.30	0.50
770	1.45	0.38
760	1.13	0.29
760	0.62	0.43
590	0.59	0.30
590	0.49	0.27
550	0.30	0.14
550	1.17	0.59
550	0.60	0.56
410	0.57	0.31
400	0.34	0.17
330	0.30	0.15
330	0.25	0.56
288	0.40	0.31
230	0.41	0.29
210	0.09	0.39
190	0.10	0.22
120	0.11	0.41
100	0.14	0.47
40	0.03	0.01
30	0.12	0.29
30	0.115	0.10
20	0.06	0.23
20	0.09	0.07
10	0.01	0.2

Figure 4:5

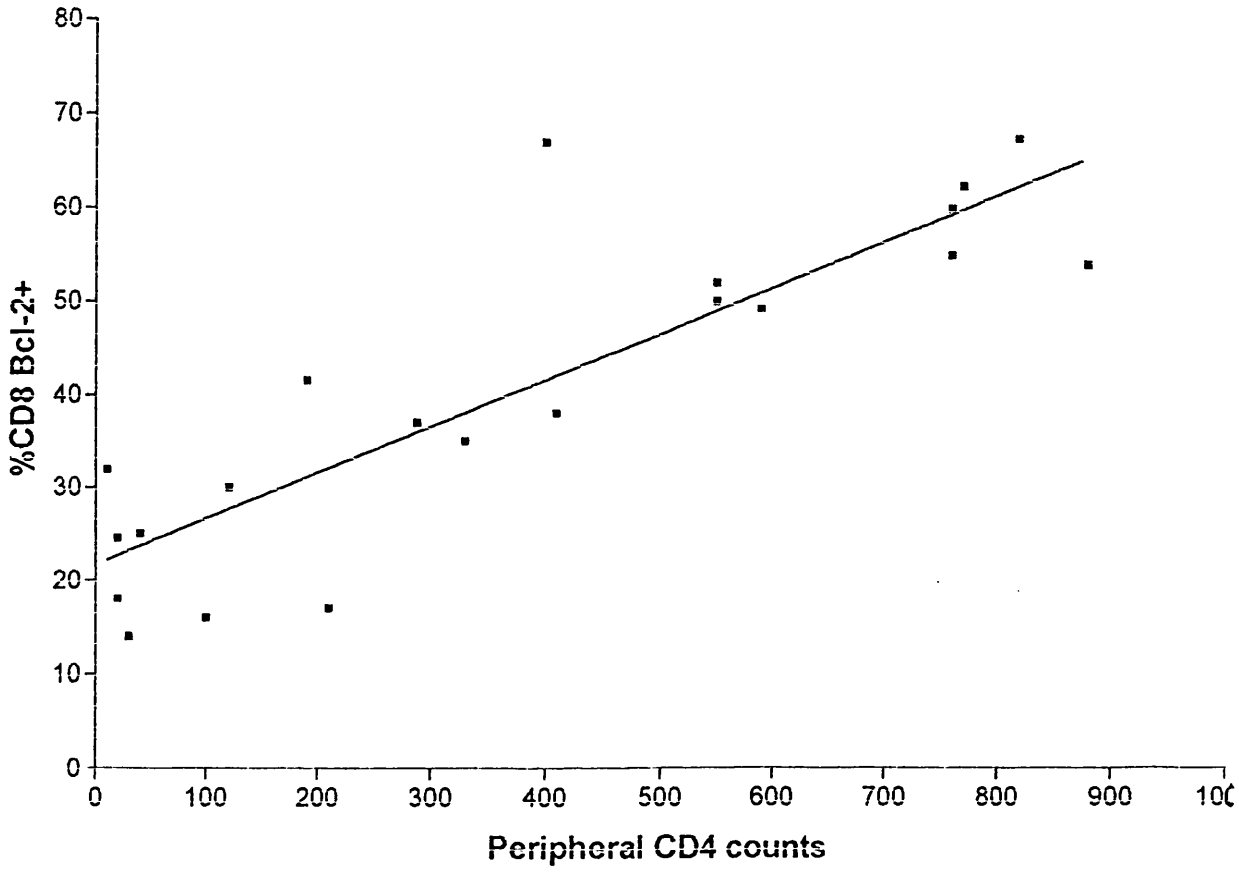


Diagram to show correlation between CD8+/Bcl-2+% of CD8+ T lymphocytes in the cervix (double immunofluresecence) and peripheral CD4 lymphocyte count (x 10^6/ml)

Discussion

The cervical mucosa of HIV-positive women was found to be normal on histological examination, and not different from the cervical mucosa of HIV-negative women, implying that there is no overt pathology. This observation is consistent with findings at other mucosal surfaces (Lipman *et al*, 1995 Lim *et al* 1993 a & b)

HIV infection is associated with an inversion of the CD4/CD8 lymphocyte ratios in the genital tract mucosa, a finding that has been demonstrated at other mucosal surfaces (Lim *et al,* 1993a). It was shown in this study that the CD4/CD8 inversion in the cervix is largely due to an increase in the CD8 cell proportions. As previously discussed, CD8 cell expansion is a normal reaction to virus infection (Doherty, 1993). The fact that the majority of these cells expressed T lymphocytes CD45ro supports the theory that these cells are virally stimulated. However, these cells appear to be functionally impaired. They do not, as would normally be expected, show signs of activation by displaying the IL-2 receptor, CD25. This 'naive' to memory cell shift has been described in peripheral blood and other tissues (Bofill *et al,* 1995; Prince *et al,* 1991) and several theories have been postulated to explain these findings. Janossy *et al* (1992) in a comprehensive review article suggested that 'memory' T cells (CD45r0+) would normally show at least a weak expression of IL-2α receptor (CD25) and MHC class II molecules while resting 'naive' cells are negative. Therefore, an accumulation of 'memory' cells should lead to an increased rather than a decreased display of IL-2α

chains. As a constant level of background stimulation is required to maintain memory, this process may deteriorate when the infectious virus mutates and may remain susceptible to severe dysregulating influences. If then, for example, there is inadequate IL-2 synthesis (because of the reduction in CD4 cell proportions), the most efficient cohort of IL-2 receptor-positive T 'memory' cells (CD45ro+) dwindle and are replaced by other functionally impaired subsets with less stringent IL-2 requirements.

The observation that these CD8+ cells appear to be functionally impaired, as evidenced by the absence of perforin and low TIA-1 expression supports Janossy *et al*'s (1992) theory. As perforin is the first step in the cytotoxic process (Clark, 1988), later mediated by TIA-1 and other granule-located enzymes, this suggests that these CD8+ cells were not functionally cytotoxic at the time the biopsy was taken. Bofill *et al* (1995) found similar changes in lymph nodes from HIV-infected patients, with large numbers of CD8+CD45ro+ T cells, which contained TIA-1 but showed no detectable levels of perforin, infiltrating the paracortex and germinal centres. As TIA-1 is found in both mature and immature granules, Bofill *et al* (1995) suggest that the CD8+ cells from their lymph node sections are cytotoxic precursors. However, a far more likely explanation, especially when considering the fact that these cells are already primed, is that these CD8+ cells are terminally differentiated end-stage cells that have lost their perforin expression and therefore their cytotoxic activity.

Under normal circumstances, the acute CD8+ cell expansion in response to viral infection is followed by resolution during which the activated CD4 and CD8 T cell numbers return to normal levels. Persistence of CD8+ cells in the peripheral blood is a feature of HIV-infection. The observations in this chapter indicate that this also occurs in cervical tissue. The increased levels of CD8+/Bcl-2+ cells found in cervical biopsy sections of HIV-positive women with higher peripheral CD4 counts may indicate that these cells are over-stimulated, and attempting to respond to viral infection. Decrease in peripheral CD4+ cell levels is associated with a reduction in Bcl-2 expression in these cervical CD8+ cells. This may reflect a reduction in cytokine-mediated stimulation of CD8+ cells, leading to a loss of Bcl-2, and hence, programmed cell death. The addition of interleukin IL-2 to Bcl-2-negative cells can result in the restoration of normal Bcl-2 levels (Tian *et al,* 1991). Relevant to this point is the reported deficient production of IL-2 in HIV-1 infected patients. This is probably a result of the loss of CD4 cells as they have been shown to be the most efficient IL-2 producing cells (Akbar *et al,* 1991). The finding in this study of high proportions of CD8+/Bcl-2+ cells in early HIV disease, and decreasing CD8+/Bcl-2+ with advancing disease is therefore consistent with the theory of Bofill *et al* (1995) that the persistence of CD8+ population in HIV-infection is a result of inappropriate over-stimulation of these cells. No correlation was detected between peripheral CD4 lymphocyte count and the proportion of CD8+/CD45ro+ cells in the cervix. It is recognised however that a

failure to show a correlation may be due to the fact that numbers studied were too small, particularly if the relationship is non-linear.

Specific antibody against HIV has been detected in the cervico-vaginal secretions of women at risk of HIV infection through infected male partners (Archibald *et al,* 1987; Belec *et al,* 1989) but the relative absence of plasma cells and B cells in HIV-positive cervical biopsies would imply that much of the antibody response is not mounted at the mucosal surfaces, but elsewhere, probably in local lymph nodes. A study in SIV-infected monkeys showed an absence of IgA-containing plasma cells in the cervical and vaginal basal lamina. Furthermore, vaginal washes from infected animals contained only IgG, while non-infected monkeys had both IgA and IgG in their vaginal washes (Miller *et al,* 1992). This may indicate, as has been previously suggested (Witkin, 1993), that IgG in the vaginal fluid is mainly a transudate from serum while IgA is locally produced. Lu *et al* (1993), using more sensitive techniques, found IgA in cervico-vaginal secretions but showed that IgG levels were up to 100-fold that of IgA. They demonstrated a correlation between cervico-vaginal and serum concentrations of specific IgG, and concluded that the antibody could have transudated from serum to the cervico-vaginal mucosa. Taken together, these data would imply that genital tract plasma cells are not the main source of antibodies in cervico-vaginal secretions, and this may have implications for the design of vaccinations.

The loss of Langerhans cells in the cervical epithelium of HIV-positive women has been previously reported (Barton *et al,* 1990), and it has been suggested that the loss of immunosurveillance may play a role in the aetiology of CIN. Langerhans' cell reduction has also been demonstrated in cervical papilloma virus infection (Morris *et al,* 1983 *a*), which again is associated with CIN. Various hypothesis have been put forward to explain this loss of dendritic cells during viral infections: HIV can directly infect Langerhans cells (Lehner *et al,* 1991) and may exhibit a direct cytopathic effect. Dendritic cells from peripheral blood have also been shown to be a target for HIV infection (Patterson *et al,* 1995). Alternatively, antigen-primed Langerhans cells may migrate from the cervical epithelium to regional lymphoid tissue where they stimulate an immune response. However, as antigen-primed Langerhans' cells become D1+ (Lenz *et al,* 1993), and as we detected no increase in D1+ cells in HIV-positive biopsies, the latter hypothesis is not borne out by our results. Whatever the mechanism, the loss of Langerhans' cells may result in an impairment of the antigen-presenting arm of the immune system.

Experiments with dendritic cells in the peripheral blood have shown that HIV-infected dendritic cells caused low levels of stimulation of allogenic lymphocytes in mixed lymphocyte reactions (Macatonia *et al,* 1990). Thus Langerhans' cell infection and depletion in HIV disease may be one of the factors underlying the defective T cell response observed in this chapter, and may, in part explain the susceptibility to

recurrent opportunistic genital tract infections in HIV-positive women. The 'healing role' of Langerhans' cells suggested by Maclean *et al* (1984) may be lost or impaired when Langerhans' cell numbers are depleted and this may, in part, contribute to the susceptibility of HIV-positive women to CIN.

The inversion of the CD4/CD8 ratios in the cervix appear to precede systemic immunosuppression. The normal range quoted for CD4/CD8 ratios in the peripheral blood is 0.7 - 3.5 (Royal Free Immunology Laboratories), and although peripheral T lymphocyte subset counts were not measured in the HIV-negative subjects, it may be assumed that they will fall within the normal range. The mean CD4/CD8 ratio in the cervical samples of these women was 2.75, which is within the normal range quoted for peripheral blood. However, in the HIV-positive women, there is an inversion of the CD4/CD8 ratios of the cervix even in women with normal or near normal peripheral CD4/CD8 ratios (table 4:6). This observation that there may be a localised disturbance in immune function in the genital tract before systemic immune function is impaired may in part explain the observation of Imam *et al* (1990) of recurrent vaginal candida infection in HIV-positive women with normal peripheral CD4 lymphocyte counts, oral candida in women with declining CD4 counts and oesophageal candida in women with AIDS.

SIV-specific antiviral cytotoxic T lymphocyte responses have been documented in the genital tract of the rhesus macaques (Lohman *et al,* 1995), but never before in the human genital tract. While the absence of other detectable viral infections or sexually transmitted disease would imply that the impaired cytotoxic responses observed in this chapter are HIV-specific, there is no direct proof. Furthermore, if these are viral specific changes, one would expect them to be confined to women with heterosexually acquired HIV infection and yet similar changes were observed in subjects infected by intravenous drug use. However, as indicated in chapter 2, all the women presumed to have acquired infection through intravenous drug use were sexually active and their risk may actually have been heterosexual exposure. Alterations in immunocompetent cell proportions have been observed at other mucosal surfaces in HIV-infected subjects (Lim *et al*, 1993a & b; Lipman *et al*, 1995) and it may be that the changes we observed are part of a general pan-mucosal response to HIV infection. This would perhaps argue against the CD8+ lymphocyte expansion at this site being a specific anti-HIV cytotoxic lymphocyte response. However, contrary to results from other studies of mucosal surfaces in HIV (Lipman *et al,* 1995; Lim *et al,* 1993b), we found no difference in the proportions of macrophage subsets between the HIV-positive and HIV-negative groups. Further studies using triple immunofluorescence techniques to look for the presence of HIV antigens in these activated T lymphocytes are required to clarify this issue.

Conclusion

This study has demonstrated that HIV infection is associated with alterations in the distribution of immunocompetent cells in the female genital tract and that these changes precede systemic immunosuppression. Although the proportions of CD8+ lymphocytes are increased compared with HIV-negative women, these CD8+ cells appear to be of impaired cytotoxic potential. These changes, which may be HIV-specific, indicate that there is a disturbance in local immune function which may explain the susceptibility of HIV-positive women to recurrent genital tract infections. A study of cytokine environment and of HIV load in the genital tract may help to further elucidate the fuctional significance of these changes and these issues are addressed in the subsequent chapters.

Chapter 5: The Cytokine Profile of the Cervical Mucosa

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CHAPTER 5: The Cytokine Profile of the Cervical Mucosa

A study of the cytokine profile of cells from HIV-positive and HIV-negative cervical biopsies using in-situ hybridisation and cytokine staining techniques

Introduction

Cytokines, soluble products of immunocompetent cells are important regulators and effectors of the immune response. In particular, they are known to be responsible for initiating and regulating T cell responses. When viruses first gain entry into the body they are taken up by macrophages and other dendritic cells, leading to the release of the cytokine interleukin-1 (IL-1). IL-1 acts by specific receptors to activate T lymphocytes which in turn produce specific cytokines which serve to inactivate virus and co-ordinate the immune response. Two distinct patterns of cytokine were originally defined among a panel of mouse T cell clones (Mossman *et al,* 1986). T helper-1 (Th-1) cells produce IL-2 and IFN-γ while Th-2 cells express IL-4, IL-5 and IL-10. Evidence from strong immune responses in both mice and humans suggest that Th-1 and Th-2 cell patterns occur and are important in-vivo (Sander *et al,* 1995; Ichinose *et al,* 1996). Th-1 responses are usually immunostimulatory while Th-2 responses are inhibitory. The response pattern induced by a particular pathogen is usually predictable and appropriate (Mossman and Moore, 1991) but there are severe consequences if an incorrect pattern is induced. HIV establishes a close interaction with the immune system that can result in increased or decreased production of various cytokines (Lai *et*

al, 1991). It has been postulated that the HIV-associated change in peripheral cytokine production may contribute to the pathogenesis of AIDS by upregulating HIV replication and inducing immune dysfunction (Poli and Fauci, 1992). Elevated levels of serum cytokines, cytokine production, and soluble cytokine receptors have been reported in patients with HIV infection and AIDS (Martinez-Maza,1992). In particular, there is growing evidence of a switch from Th-1 to Th-2 T-helper lymphocyte cytokine production pattern which may favour progression to AIDS (Clerici *et al* 1994, Del Prete *et al* 1995).

Cytokines may have a very short half-life in plasma (Steiniger *et al,* 1988) or may be produced and act locally in tissues without ever entering the circulation (Pantaleo *et al,* 1993). Thus the plasma concentration of cytokines may not reflect local production indicating that a study of cytokine production by genital tract immunocytes should be conducted at a local level. Belec *et al* (1995a) demonstrated that pro-inflammatory cytokine levels were raised in cervico-vaginal secretions of HIV-infected women. In this study, there was no correlation between plasma concentrations and genital tract production of IL-1β. The inherent problem with studies of this sort is that the presence of cytokines in secretions may not be reflective of local production and may, in part, comprise transudate from plasma.

The observation recorded in the previous chapter that the genital tract T-lymphocytes may be functionally impaired underlines the need to study the cytokine environment of

these cells, particularly as the cytokine-synthetic ability of cells is reflective of their immunological function and conversely, their functional capacity may be affected by the cytokine environment. The aim of this study therefore was to study the cytokine repertoire of genital tract immunocytes by applying methods of in situ hybridisation and biotin streptavidin alkaline phosphatase staining.

In Situ Hybridisation

The discovery that the genetic message is written in a four letter code, and the ability to read that message by sequencing the DNA has given a technique of great power to biologists. The process which underlies all methods based on molecular hybridisation is the formation of the DNA double helix from two complementary strands. In the late 1960s, Gall and Pardue (1969) and John *et al* (1969) independently discovered a way of localising the position of specific sequences in the nucleus or chromosomes by carrying out hybridisation reactions on cells fixed to microscope slides - the method of in situ hybridisation. Two developments have contributed to the accessibility of this technique: firstly, the availability of synthetic oligonucleotide probes and secondly, the use of non-radioactive detection methods (Guitteny *et al,* 1988; Hankin *et al,* 1989; Larrson *et al,* 1990; Pringle *et al,* 1990). These facts have also facilitated the transfer of the in situ hybridisation technique into the clinical laboratory (Warford, 1988)

The process of in situ hybridisation permits the precise location of target nucleic acid while preserving cell and tissue morphology. This has been applied clinically in the analysis of viral infection and, more recently, in the analysis of mRNA expression in various disease states. Thus the capacity of an immunocyte to produce cytokines can be studied using in situ hybridisation to detect the specific messenger RNA (mRNA) for the cytokine of interest.

To ascertain that the presence of specific mRNA correlated with the production of the particular cytokine in the genital tract mucosa, the biotin streptavidin alkaline phosphatase technique, using specific anti-human cytokine antibodies, was used to detect the presence of the cytokines.

Materials and Methods

In situ Hybridisation

Biopsy specimens from the HIV-positive and HIV-negative subjects described in chapter 3 were used in this investigation. The cytokines studied in this chapter are listed in table 5:1. Six micrometre cryostat sections were mounted on poly-l-lysine-coated slides as previously described. To avoid contamination with exogenous RNAse, all sections were handled with gloves and sterile instruments were used throughout. Laboratory glassware and metal spatulas were sterilised by baking at 180°C for nine hours. All small instruments and the necks of open vessels were covered with foil. Disposable sterile plastic instruments were used where possible, otherwise plastics were rendered RNAse-free by rinsing with chloroform. All solutions were freshly made up at the start of the procedure. The diethyl pyrocarbonate (DEPC)-treated water was prepared as follows. 1 ml of DEPC was added to 999ml of de-ionised water in a fume cupboard. The bottle was shaken vigorously so that the inside of both the bottle

and cap were exposed to DEPC. The bottle was then left to stand at room temperature in a fume cupboard overnight. The DEPC-treated water was then autoclaved at 121°C/15psi to destroy any residual DEPC.

The in-situ hybridisation technique outlined in figure 5:1 (page113-114) consists of three main steps: tissue fixation, hybridisation and detection. Tissue fixation ensures the preservation of tissue morphology and the retention of the target mRNA in its original location by cross-linking the cell matrix. 4% paraformaldehyde was used as this is recommended in particular for cells or cryostat sections.

Pre-treatment of the slides improves access of the specific oligonucleotide probe to the cellular RNA. Acetylation of the specimen prevents non-specific binding of the probe. In order to demonstrate that the target nucleic acid was RNA, controls were prepared in which biopsy sections were pre-treated with RNAse before application of the specific oligonucleotide probe. All the mRNAs within the section would then be degraded and any resultant labelling would be non-specific. Additionally, positive controls in which sections were incubated with poly-dT oligonucleotide probes which detects all animal mRNAs were prepared. Simultaneous negative controls in which the specific oligonucleotide probe was omitted were also set up.

The slides are washed after hybridisation to remove non-specifically bound RNA prior to detection. Digoxigenin-labelled (DIG) synthetic single-stranded DNA oligonucleotide probes (R&D Systems, Abingdon, UK) to identify mRNA for the cytokines listed in table 5:1 and detection was carried out with anti-Digoxigenin antibody (Boehringer, East Sussex, UK). The slides were developed with 5-

bromo-4-choloro-3-indolyl phosphate (BCIP/NBT) (Sigma, Dorset, UK). After developing, the slides were washed in de-ionised water to remove the coverslip. They were wiped carefully and mounted in PBS glycerol. mRNA expression was quantified on each section by measurement of optical density using a monochrome video camera attached to a microscope with a x40 objective. The relative density of the reaction product was recorded using a computerised image analyser (Seescan Cambridge, UK). Frames were drawn around positively staining cells as described in chapter 3, again selecting three areas per slide. The software automatically calculates the area within the frame and the optic density per unit area is generated by the software. The optical density of all positively staining cells within each frame was measured and the average optical density per biopsy section calculated. Optical density measured per unit area was directly proportional to the intensity of the staining. The average optical density for each cytokine was compared between HIV-positive and HIV-negative sections.

Table 5:1: Cytokines studied in this chapter

Cytokine	Cells mainly responsible for production	Function in vivo
Human interleukin 2 (hIL-2)	CD4+ Cells	Released in response to initial stimulation by antigen-presenting cells. Activates cytotoxic T cells, ? blocks programmed cell death
Human interleukin 4 (hIL-4)	Cytotoxic T lymphocytes, CD4+ cells	Promote B cell responses, induces activated B cells to switch from IgM to IgG and IgE
Human interferon-γ (hIFN-γ)	Cytotoxic T cells, CD4+ lymphocytes	Inhibits viral replication
Human interleukin 5 (hIL-5)	CD4+ Cells	Terminal differentiation of eosinophills
Human interleukin 10 (hIL-10)	CD4+ cells	Suppresses IL-2, and to a lesser extent IF-γ production by CD4+ cells. Blocks IL-2-mediated T cell proliferation

Biotin streptavidin Alkaline Phosphatase Staining Technique

Expression of cytokines by mononuclear cells was assessed by staining with specific anti-cytokine antibody using the biotin streptavidin alkaline phosphatase technique. Alkaline phosphatase hydrolyses napthol phosphate esters to phenolic compounds and phosphates. The phenols couple to chromogens (fast red or fast blue) to produce insoluble coloured azodyes. Endogenous alkaline phosphatase is inhibited by levamisole. Fast red salt (Sigma) was used in these tests as it gives a more distinctive stain than fast blue. Unlike other staining techniques described in this thesis, this staining procedure requires freshly cut frozen tissue and special fixation techniques. The sections were fixed in a mixture of acetone/methanol (50/50v/v) at -20°C for 10 minutes, washed with 0.05% tris-HCL buffered saline, pH 7.6 (TBS) and stained as illustrated in figure 5:2 (page115-116). A panel of specific anti-human mouse monoclonal antibodies were used to identify IL-2, IL-4 and IFN-γ (table 5:2). The working dilution of the MoAb had been previously determined in the laboratory by titration studies using human palatine tonsil sections. Negative controls, where the primary antibodies were replaced with TBS, were set up to check for non-specific binding of the biotin layer. One section from each specimen was incubated with the substrate alone to exclude the detection of endogenous alkaline phosphatase activity in epithelial cells. As the fast red product is soluble in alcohol and other organic solvents, an aqueous mounting medium was used. Assessment was performed using the image analyser as described in chapter 2. Background staining was assessed by comparison

with the negative control. A semi-quantitative calculation of the proportion of positively staining cells was made, with staining described weak if there were less than five positively staining cells per unit area, moderate if there were five to ten cells, and strong if there were more than ten. All sections were assessed by two independent observers. The distribution of positively-staining cells was compared visually with the pattern obtained with in situ hybridisation to determine if mRNA detection correlated with expression of IL-2, IL-4 and IF-γ.

Table 5:2 Monoclonal antibodies used in this study

Antibody	Source	Cell Specificity (antigen)	Dilution
IL-2	♦Serotec	Recombinant and human IL-2	1:200
IL-4	♠Endogen	Natural and recombinant human IL-4	1:50
IFN-γ	♣Genzyme	Human IFN-γ. No cross reaction with IFNα or β	1:20

♦Serotec, Kidlington, Oxford UK
♠Endogen Inc, Cambridge, MA, USA
♣Genzyme diagnostics, Kent, UK

Figure 5:1 Steps involved in the in situ hybridisation technique used in this chapter

All procedures carried out at room temperature except otherwise indicated.

Key
SSC: 0.15M NaCl. 0.015M trisodium citrate, pH7.0

TNMT: 0.1M Tris-HCL (pH 7.5), 0.1M NaCl, 2M $MgCl_2$, 0.05% Triton X-100

NBT: 0.1M Tris-HCL (pH 9.6), 0.1M NaCl, 0.5% 1M $MgCl_2$

(See overleaf)

Step 1 Tissue preparation & fixation	Fix in 4% paraformaldehyde, room temperature
Step 2 Pre-treatment & hybridisation	Wash slide in PBSM 10 minutes (40ml 10xPBS + 2ml 1M $MgCl_2$ + 358ml DEPC-H20)
	Immerse in PBSM+0.25%Triton-X100 +0.25%Nondet P40, Wash in PBSM, 5 minutes x2
	Dip slides in 20% acetic acid at 4°C for 15 seconds
	Wash in PBSM 5 minutes x 2
	Incubate in 20% glycerol for 1 hour
	Rinse briefly with SSC x 2
	*Incubate with RNase (200mg/ml) 2 hours at 37°C
	Rinse briefly with SSC x 2
	Place slides in moist chamber and add 30ml of probe at appropriate dilution in pre-hybridisation solution. Incubate for 20 minutes at 70°C and at 37°C overnight
Step 3 Treatment after hybridisation and detection	Wash coverslips off in 4xSSC, 15 minutes x4
	Wash in 2xSSC, 20 minutes, 60°C
	Wash in 0.2xSSC, 20 minutes, 42°C
	Wash in 2xSSC, 10 minutes
	Wash in TNMT, 10 minutes
	Block slides in 3% BSA (bovine serum albumin) 1 hour
	Wash in TNMT, 10 minutes x 2
	Incubate with anti-DIG-antibody, 1/600
	Wash in NBT 5 minutes x2. Add developer, cover with coverslip, incubate overnight

Figure 5:2 Steps involved in the biotin streptavidin alkaline phosphatase staining technique used in this chapter

To avoid drying out of reagents, this procedure was carried out in a moist chamber

Key

PBS: Phosphate buffered saline

BSA: Bovine serum albumin

TBS: Tris buffered saline

(See overleaf)

Step 1 Tissue Preparation	Cut 6µm tissue sections. Air dry for 1 hour, ring with polysiloxane
	Fix in methanol:acetone (1:1) -20°C for 10 minutes
	Rinse in PBS, 30 seconds x 2
Step 2 Staining	Apply 150ml of mouse anti-human-antibody at appropriate dilution in PBS+0.05% BSA and incubate overnight at 4°C.
	Wash in TBS and dry slides carefully
	Apply second layer of biotinylated anti-mouse IgG (150µl) 1:100 PBS-BSA for 1 hour at room temperature.
	Wash in TBS, 2 minutes
	Apply 100 µl streptavidin-alkaline phosphatase 1:100 PBS-BSA and incubate 1 hour.
	Wash in TBS, 2 minutes
	Apply substrate* for 20-30 minutes.
	Wash in tap water for 2 minutes
	Counterstain with Mayer's haematoxylin for 3minutes Wash in running water, 2 minutes
	Rinse in distilled water, 2 minutes x2
	Mount in PBS Glycerol

* Substrate:
0.01g Napthol ASBI phosphate
20ml Tris-HCL (ph 8.2)
400ml Dimethyl formide (DMF)
0.02g fast red
10 drops levamasole.
Mix and filter

Results

Cytokine mRNA expression

The median optical density measurements for each of the cytokines under study are shown in table 5:3. It can be seen that there was significantly less IL-2 mRNA, and more IL-4, IL-5 and IL-10 mRNA detected in the HIV-positive compared with the HIV-negative biopsy sections. There was no significant difference IFN-γ between the two study groups.

Table 5:3 Optical Density of Cytokine mRNA in Cervical Biopsies

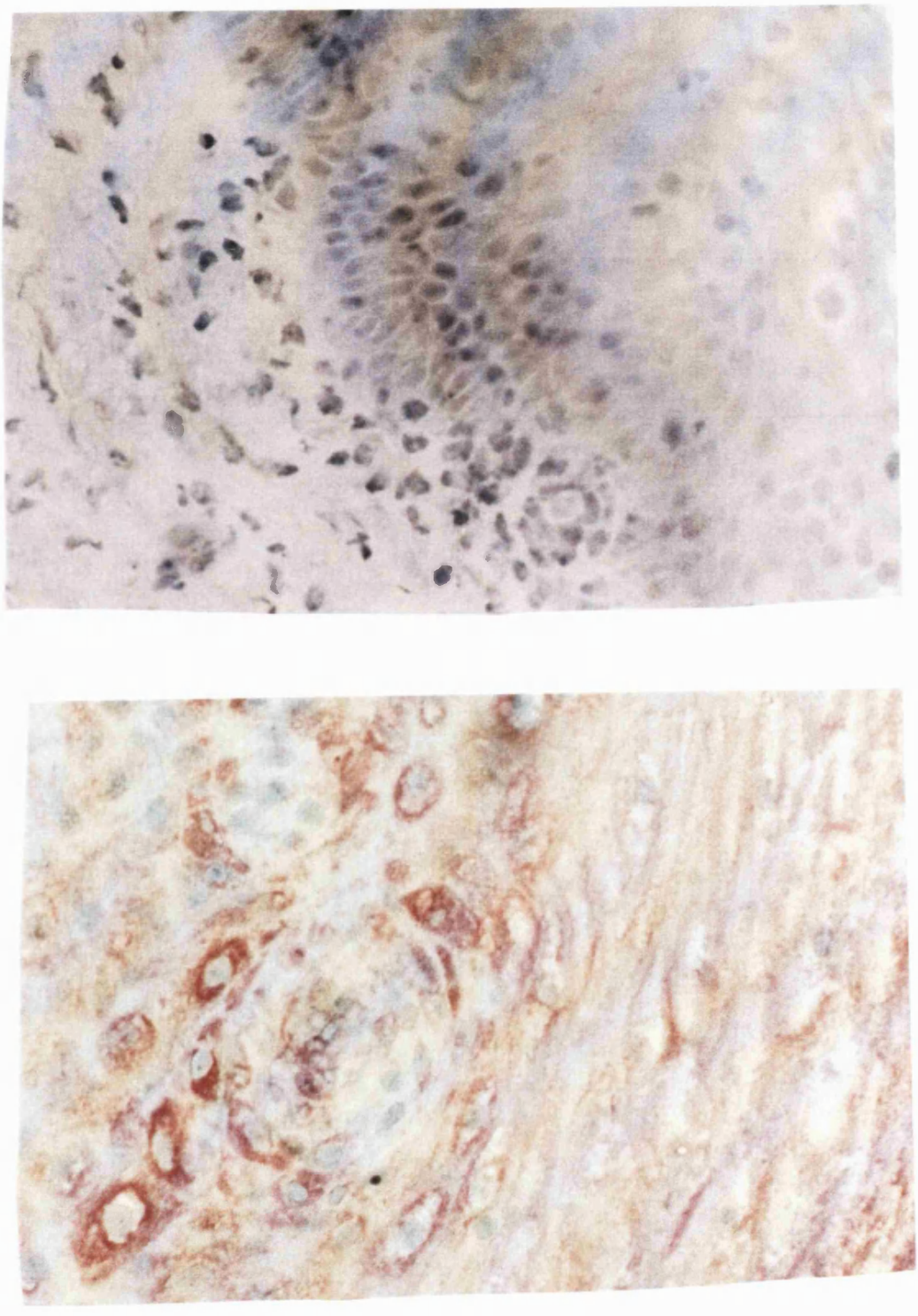
Cytokine	HIV-positive Median (range)	HIV-negative Median (range)	p*
hIL-2	0.12 (0.09-0.17)	0.20 (0.12-0.30)	0.04
hIL-4	0.28 (0.19-0.54)	0.09 (0.07-0.16)	0.0006
hIFN-γ	0.18 (0.14-0.36)	0.26 (0.15-0.32)	0.50
hIL-5	0.34 (0.28-0.63)	0 (0-0.4)	0.045
hIL-10	0.34 (0.25-0.40)	0 (0-0.39)	0.02

*Mann Whitney U test

Correlation of cytokine mRNA with cytokine expression

Cytokine staining confirmed that the pattern of cytokine expression mirrored the mRNA detection, with HIV-positive sections showing weak staining for IL-2, moderate for IFN-γ, and strong for IL-4. HIV-negative sections showed moderate staining for IL-4 and IFN-γ, and weak staining for the other cytokines. The positively-staining cells were mainly confined to the sub-epithelial stroma. The morphological appearance and the distribution of these positively staining cells was consistent with the appearance and distribution of lymphocytes as revealed by immunoperoxidase staining in the preceding chapter (figure 5:3).

Figure 5:3 Sections of cervix from HIV-positive subject to demonstrate the distribution of IL-4 using in-situ hybridisation (top photograph) and biotin streptavidin (lower photograph) techniques. (Magnification x 400)



Correlates with peripheral blood

No relationship was detected between peripheral CD4 lymphocyte count and cervical cytokine mRNA optical density in HIV-positive women. In addition, there was no relationship detected between cervical CD4/CD8 ratios and cervical mRNA optical density (Table 5:4).

Table 5:4 Relationship between cervical cytokine profile, CD4/CD8 ratios in cervix and peripheral CD4 counts of HIV-positive subjects

Peripheral CD4x10^6/ml	Cervix CD4/CD8	IL-2	IL-4	IFN-γ	IL-5	IL-10
880	0.25	0.11	0.48	0.14	0.33	0.25
760	0.29	0.17	0.19	0.15	0.35	0.40
550	0.59	0.09	0.54	0.20	0.33	0.35
400	0.17	0.17	0.28	0.34	0.554	0.33
330	0.15	0.12	0.21	0.14	0.28	0.35
210	0.39	0.09	0.28	0.31	0.63	0.34
120	0.41	0.12	0.23	0.20	0.32	0.36
30	0.29	0.09	0.2	0.36	0.32	0.30
20	0.23	0.15	0.30	0.20	0.35	0.33
10	0.2	0.14	0.35	0.14	0.37	0.35

Discussion

This study demonstrates that there is a predominance of Th-2 cytokines in the cervix of HIV-positive women. This observation reflects the Th-1 to Th-2 shift said in the literature to be associated with HIV disease. This shift to a Th-2 pattern of cytokine production is said to predispose to HIV disease progression.

Th-1 and Th-2 responses occur naturally and may be an immunologically appropriate response to invasion by certain pathogens. For instance, there is experimental evidence to show sequential production of Th-1 and Th-2 cytokines in response to live bacillus Calmette-Guérin (Sander *et al,* 1995). The initial response was dominated by macrophage cytokines (monokines), followed by a marked production of Th-1 cytokines (IL-2, IF-γ) at days four to five. Later in the reaction (days 10-12) a Th-2 response, with IL-4, IL-5 and IL-10 production, was observed. There is substantial evidence of cross-regulation between the Th-1 and Th-2 T-helper cell sub-sets (Mossman & Moore, 1991). For example, IL-10 inhibits synthesis of cytokines by Th-1 cells which can result in decreased proliferation. IF-γ which is itself inhibited by IL-10, inhibits the growth of Th-2 cells and IL-4 preferentially stimulates the growth of Th-2 cells. Sander *et al* (1995) therefore concluded that the production of Th-2 cytokines late in the response to BCG may reflect a dynamic in vivo balance, preventing over-expression of inflammatory, potentially tissue-damaging Th-1 cytokines.

The resting T cell (CD4+CD45ro-) that has not recently been stimulated may produce only IL-2 on first contact with antigen cells. As the cells become 'primed' (i.e. CD45ro+), they begin to produce IL-4 and other Th-2 cytokines, with less IL-2 production. Thus, the T cells producing more Th-2 cytokines may represent later, more differentiated phenotypes. Under normal circumstances, if the viral infection were overcome by the immune system, the inhibition of IL-2 producing cells by IL-10 would lead to Bcl-2 down-regulation, apoptosis, programmed cell death and resolution.

The observation of a predominance of Th-2 cytokines may therefore not necessarily reflect an HIV-induced pathological shift in cytokine production, but may be an exaggeration of a normal process. The chronicity of HIV, with the constant background antigenic stimulation, leads to an accumulation of virally stimulated cells which produce type 2 cytokines. This theory is consistent with observation in the previous chapter that there is an accumulation of primed lymphocytes in the cervix of HIV-positive women. Paradoxically, the accumulation of type 2 cytokines may predispose to the chronicity of the viral infection. IL-10 inhibits Th-1 cytokine synthesis, and as murine cytotoxic T lymphocytes synthethise the Th-1 pattern of lymphocytes (Fong and Mossman, 1990), and the same may occur in humans, IL-10 may have a suppressive effect on their ability to kill the virus. Other conditions, such as IgA nephropathy have been described where an excess of Th-2 cytokine production is associated with a disease process (Field *et al,* 1993, Ichinose *et al,* 1996).

The cytokine pattern observed may also explain some of the alterations in immune cell proportions observed in the previous chapter. The loss of Bcl-2 observed in women with more advanced disease may be related to the reduction in IL-2 synthesis. No correlation was observed between IL-2 levels and HIV disease stage as defined by peripheral CD4 lymphocyte counts but there may be other confounding and regulating factors (such as the use of antiretroviral therapy) which were not directly studied. Th-2 cytokine patterns are said to favour B-cell responses. In particular, IL-5 enhances IgE production and accumulation of eosinophills. Hypergammaglobulinaemia is a feature of HIV disease and as previously discussed, antibody production in genital tract secretions has been described. We however found no increase in B cells or plasma cells in the cervix of HIV-positive women, and there may be other virally-mediated or macrophage-derived regulatory factors involved in this process.

Finally, the susceptibility of HIV-positive women to cervical neoplasia may, in part, be explained by this cytokine pattern. Type-1 cytokines are immunostimulatory and are thus capable of limiting tumour growth while type-2 cytokines are immunoinhibitory and are capable of stimulating tumour growth (Clerici *et al,* 1997). Thus the predominance of type-2 cytokines in the cervical tissues of HIV-positive women may permit neoplastic change, either alone or in conjunction with other co-factors such as HPV.

Conclusion

The observation of an altered cytokine profile in the cervix of HIV-infected women is consistent with the finding in the previous chapter of functionally impaired, virally stimulated cytotoxic T lymphocytes within the cervical mucosa. The observed alterations in cytokine profile and cytotoxic T cell phenotype may explain why HIV-infected women are susceptible to recurrent viral and fungal infection of the genital tract and CIN. The predominance of Th-2 cytokines in the cervix of HIV-infected women may occur because of an accumulation of terminally differentiated cytotoxic T lymphocytes, presumably in response to the chronicity of the viral infection. If this hypothesis is correct, it would be expected that there would be expected that there would be high levels of HIV in the cervical tissues. The relationship of local viral load to local and systemic immunity is explored in the next chapter.

Chapter 6: HIV Load in Female Cervical Tissues

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Materials and Methods

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CHAPTER 6: HIV LOAD IN FEMALE CERVICAL TISSUES

Relationship to local and peripheral immune changes and to peripheral viral load

Introduction

Recent published data have shed some important light on our understanding of the virological and immunological dynamics of HIV infection (Havir and Richman, 1996). Even in early disease, there is a high level of viral replication in lymphoid tissue, with half the viral population in the plasma turned over within hours (Pantaleo *et al,* 1993; Ho *et al,* 1995). The rate of viral replication stabilises after primary infection at a particular level or set point for each individual and remains relatively stable in asymptomatic patients over months and possibly years (Mellors *et al,* 1995). Plasma RNA measurements are an indirect reflection of viral replication in fixed lymphoid tissue. HIV RNA levels appear to be more predictive of HIV disease progression to AIDS than CD4 counts, particularly in patients with counts above 350 x 10^6/ml (Mellors *et al,* 1996).

There is increasing evidence that there may be a correlation between plasma HIV-1 RNA levels and mother-to-child transmission of HIV. Some workers have postulated that maternal plasma HIV-1 RNA levels are a major determinant of mother to child transmission and that maternal RNA levels may predict the risk of transmission (Fang

et al, 1995). However, experience at this centre (Reid W and Johnson MA, unpublished data) and published reports (Weisser *et al,* 1994) have shown that materno-fetal transfer can occur in women with early HIV disease as defined by low viral load or other surrogate markers of disease progression. This implies that maternal viral load is not the only determinant of transmission, and other factors such as maternal immune response, properties of the mother's virus and obstetric indicators, such as mode and timing of delivery, may play a role.

There is growing evidence to suggest that vertical transmission of HIV occurs principally during parturition (European Collaborative Study, 1992; Goedert *et al,* 1991) and that delivery by caesarean section may protect against transmission (European Collaborative study, 1996). The risk of vertical transmission increases when the foetal membranes are ruptured for more than four hours before the delivery (Landesman *et al,* 1996). These facts imply that contact with maternal genital tract secretions may predispose to HIV-1 infection in the neonate. Of relevance to this is the observation that HIV-1 is present in female genital tract secretions, probably at higher concentrations in pregnant women than in non-pregnant women (Henin *et al,* 1993) and that genital tract HIV load may not necessarily correlate to peripheral viral load (Wofsy *et al,* 1986; Nielsen *et al,* 1996). These observations also have implications for horizontal transmission. A study of HIV-1 viral load in men also

showed a lack of correlation between viral load in semen and plasma (Liuzzi *et al,* 1996), supporting the theory that different viral compartments exist and implying that HIV-infected men may have a high risk of HIV transmission through sexual intercourse even when peripheral HIV load is low.

The aim of this study therefore was to quantify the HIV-RNA load within the cervical biopsy sections and to relate this to the local immune changes described in the preceding chapters and to peripheral viral load, to further develop our comprehension of the local immune mechanisms involved in the vertical and horizontal transmission of HIV.

Materials and Methods

The forty HIV-positive women described in chapter two were included in this study. The demographic details are summarised in table 2:2 and table 2:3. Colpopscopically-directed cervical biopsies for viral load quantification were obtained and frozen as previously described (chapter 2). Peripheral blood was collected by venopuncture at the time of taking the biopsies: 10mls of clotted blood into a plain tube and 10mls of defibrinated blood into an EDTA-containing tube.

Processing of peripheral blood

Peripheral Blood Mononuclear Cells

Mononuclear cells were isolated from the defribrinated blood samples as follows: The blood was diluted in an equal volume of sterile PBS and 20mls of the diluted blood was used to overlay 10 mls of lymphoprep in a universal container. The preparation was then centrifuged at 650G for 15 minutes. The band of mononuclear cells which formed in the middle of the preparation was carefully harvested with a sterile pastette and transferred into a fresh universal container. The cells were resuspended in PBS and centrifuged for five minutes at 1500rpm. After discarding the supernatant, the pellet was dispersed by gently agitating the tube. The cells were washed twice by resuspending in PBS and centrifuging for five minutes at 1200rpm. The resultant cells were then suspended in five millilitres of PBS and a cell count performed with a modified Neubauer haemocytometer. Cellular exclusion of trypan blue was used to assess viability. The cells were then frozen at -70°C until required

Serum Collection

The serum was separated from clot by centrifuging the samples at 2000G for 10 minutes and carefully removing the supernatant from the cell pellet. The specimens were then frozen until required.

Polymerase chain reaction

HIV RNA quantification was performed using the Amplicor HIV monitor test (Roche Diagnostic Systems, Hertfordshire, UK) which has a detection threshold of 200 RNA copies/ml. Twelve samples of serum, PBMC, cervix or control were run in any one batch, as recommended by the manufacturers. A negative control which contained no HIV RNA, and a positive control which contained a known amount of HIV RNA was included in each batch. The main features of the test are sample preparation, amplification and detection.

Sample preparation

The RNA was extracted from 200µl of sample (serum or PBMC) by incubating with 600µl of a lysis reagent containing guanidine thiocynate and 'Quantitation Standard RNA' for 10 minutes at room temperature. Cervical biopsies were weighed and homogenised in 600µl of lysis reagent and then processed in the same manner as liquid specimens. The RNA was precipitated by adding 800µl of isopropanol and centrifuging at 1600x g for 15 minutes. The pellet was resuspended in one ml of 70% ethanol and centrifuged at 1600xg for five minutes. The precipitated RNA was then resuspended in 400µl of a buffer containing carrier RNA. The 'Quantitation Standard (QS) RNA' is a synthetic RNA molecule with primer sites identical to the HIV target and a unique probe sequence specific to the QS RNA molecule. A known amount of

QS is introduced into each sample with the lysis reagent to permit quantitation of HIV RNA from a comparison of resulting optical densities following amplification and detection

Amplification

Amplification occurred in a thermal cycler. A 142 base pair sequence in the *gag* gene of HIV is amplified by reverse transcription and polymerase chain reaction in a single reaction. The reaction employs the thermostable recombinant enzyme DNA polymerase, which has both reverse transcriptase and DNA polymerase activity in the presence of manganese. The primers are biotinylated at the 5' ends to yield biotinylated amplification products.

Manganese was added to the DNA polymerase and 50µl of sample added to wells containing 50µl of the enzyme mixture. The pink dye in the manganese acts as a colour indicator to ensure that each DNA polymerase is added to each sample. The tray was placed in the thermal cycler which was set to the following program:

Hold	2 minutes at 50°C
Hold	30 minutes at 60°C
4 cycles	10 seconds at 95°C, 10 seconds at 55°C, 10 seconds at 72°C
26 cycles	10 seconds at 90°C, 10 seconds at 60°C, 10 seconds at 72°C
Hold	15 minutes at 72°C

Detection

The biotinylated HIV and QS amplified products are detected in separate wells of a microwell plate coated with HIV-specific and QS-specific oligonucleotide probes, respectively. To measure HIV and QS amplification products over a large dynamic range, 5-fold serial dilutions of the amplification products were performed in the HIV-specific and QS-specific wells of the microwell plate. The bound, biotinylated amplification products were quantified by incubating with an Avidin-horseradish peroxidase conjugate for 15 minutes at 37°C. After washing the plate with a semi-automated plate washer, a colorimetric reaction for the horseradish peroxidase was performed by incubating the plate with a photosensitive substrate for 10 minutes in the dark. The reaction was stopped and the optical density of the wells measured at 450nm.

The HIV RNA copy number per ml was then calculated from the known input copy number of the QS RNA, the optical densities of the HIV-wells and QS-wells that fell within a defined range, and the dilution factors associated with the specific wells. The HIV copy number per gram for the cervical biopsies was calculated by dividing the result with the biopsy weight. The HIV copy number per peripheral blood mononuclear cell was calculated by dividing the RNA copy number per ml by the number of PBMCs per ml of sample.

Results

HIV load results are available from 20 cervical biopsies (table 6:1). Twenty samples were destroyed during storage The results were invalid in three cases as the optical density results in the quantitation standard wells did not fall within the specified range (0.3-2). The assay was considered to have failed in these cases. HIV load results from serum are available from 15 subjects; the other samples were destroyed during storage or handling. Assay failure occurred in two cases. Paired serum and cervix results are available in 14 cases. PBMC viral load results were available in only two women (1629 and 2586 copies in samples each containing $3x10^5$ cells/ml) and this parameter was therefore excluded from all further analyses.

Table 6:1 shows the RNA copies per unit area in serum and cervix, the subjects' peripheral CD4 lymphocyte count, mode of HIV acquisition and antiretroviral therapy. It can be seen that there is a tendency for serum viral load to increase with decreasing peripheral CD4 count, but there was no statistical correlation. In all except one case where the cervical HIV load was below detection threshold, the number of RNA copies per gram of cervix was greater than the RNA copies per ml of serum, and this difference was more marked in women with more advanced HIV disease, as indicated by the blood CD4 count. The difference between mean RNA copies per gram of cervix (median 3564119 95% confidence intervals 192978-21849316) and per ml of serum (median 69724, 95% confidence interval 9042-270371) is highly significant ($p=0.008$, Wilcoxon signed rank). However, there was considerable variability in viral load even between women with similar blood CD4 lymphocyte counts and no statistical relationship was established between peripheral CD4 count and viral load in either cervix or serum. Formal statistical analysis to determine the influence of factors such as the duration of HIV infection, use of antiretroviral medication and the duration of use which may have an unequal effect on the cervical and serum viral load, was precluded by the small numbers. The number of women thought to have acquired HIV through intravenous drug use is too small to enable a comparison to be made with viral load in women with heterosexually-acquired HIV infection.

Correlation with immune changes in the cervix

No statistical correlation was observed between the CD4/CD8 ratios in the cervix and the cervical HIV load although there appeared to be a trend of higher viral loads being associated with higher cervical CD4/CD8 ratios (table 6.1).

Table6:1 Serum CD4 lymphocyte counts, HIV RNA copies in the serum and cervix and cervical CD4/CD8 ratios related to use of antiretroviral medication and HIV exposure category

Blood CD4 x10^6/ml	Serum HIV RNA copies/ml	Cervix CD4/CD8 ratio	Cervix HIV RNA copies/g	Antiretrovirals (Duration)	Exposure category
20	9753	0.1	455715	AZT (5 months)	HS
20	132424	0.07	385955.	AZT,ddC (9months)	HS
30	538856	0.29	7404878	AZT (2 years)	HS
100	no spec.	0.47	11963066	Saquinovir (1year)	HS
120	no spec.	0.41	37058823	Saquinovir (1year)	HS
190	272514	0.25	20673563	Nil	HS
210	2734	0.39	13142	AZT (5 years)	HS
288	no spec	0.44	<threshold	AZT,ddI (1 year)	IVU
330	1990	0.56	failed assay	Nil	HS
330	16198	0.15	23273	Nil	IVU
400	no spec	0.17	95216	AZT,ddI (3 years)	HS
410	137088	0.31	<threshold	Nil	IVU
550	failed assay	0.22	<threshold	Nil	IVU
550	failed assay	0.56	17724705	Nil	HS
590	<threshold	0.49	43698633	Nil	HS
760	1886	0.29	18500	Nil	HS
760	31241	0.43	1014099	Nil	HS
770	30450	0.32	5837500	Nil	HS
820	278	0.5	failed assay	Nil	HS
880	3456	0.25	failed assay	Nil	HS

HS: Heterosexual

Discussion

Previous studies have demonstrated the presence of HIV in cervico-vaginal secretions (Wofsy *et al*, 1986; Clemetson *et al,* 1993; Henin *et al,* 1993). In one study (Wofsy *et al,* 1986), virus could be cultivated from both the cell fraction and supernatant of genital secretions, indicating that HIV was present as cell-associated and cell-free virus. Shedding of HIV was unrelated to clinical stage of disease, p24 antigenaemia or zidovudine therapy (Henin *et al,* 1993) but was positively correlated with cervical inflammation (Kreiss *et al,* 1994) cervical ectopy, oral contraceptive use (Clemetson *et al,* 1993) and pregnancy (Henin *et al,* 1993). The fact that virus was isolated from secretions from non-menstruating women would indicate that the presence of HIV in the genital tract secretions is not merely due to contamination with blood. Taken together, these facts imply that there is HIV within cervical tissues.

This study is one of the few (Van de Perre *et al,* 1988; Pomerantz *et al,* 1988) that have confirmed the presence of HIV within the cervical tissues. The mechanism by which HIV in genital tract tissues gains access to cervico-vaginal secretions is unknown. Cervical cells are constantly shed into the genital tract and it may be that HIV-infected cells are sloughed directly into the secretions in the same way, possibly in parallel with active production of free virions. Of relevance to the latter theory is

the fact that the presence of transcriptionally active HIV-1 infected cells within cervical biopsy specimens has been demonstrated (Nuovo *et al,* 1993).

The reason why there is higher viral load within the cervix than in the serum is not entirely clear, but may be related to the increased number of T-lymphocytes observed within the cervical mucosa (chapter 4). If, as suggested in the previous chapters, the genital tract is the primary route of HIV infection in this patient group, uptake by functionally-impaired CD8+ T-lymphocytes and the favourable cytokine environment may lead to persistence of virus within the tissues. Systemic infection may occur when the mucosal T cells migrate. The inherent assumption in this theory is that all subjects were infected heterosexually, which may not be the case. Attempts have been made to correlate the presence of virus in the genital tract to mode of HIV acquisition, and it has been suggested that HIV is more likely to be isolated from the genital tract of women with sexually acquired HIV than those acquiring HIV through intravenous drug use (reviewed by Mostad and Kreiss, 1996). The number of women acquiring HIV through intravenous drug use in this study was too small for any valid comparisons to be made with the group with heterosexually acquired infection, particularly as there may be other potentially confounding factors, such as duration of HIV infection, use of antiretroviral medication and recent sexual activity.

Alternatively, the high viral load observed within the cervical tissues may be secondary to changes to immunocompetent cell proportions which, as previously suggested (chapter 4), may be an HIV effect, common to all mucosal surfaces. Thus, the virus-laden lymphocytes may be accumulate in the genital mucosa as a secondary event. Studies of viral load at other mucosal surfaces may shed some important light on this issue. HIV-RNA has been detected by reverse transcription PCR in intestinal biopsies of patients with early and late HIV disease (McGowan *et al*, 1996). Although no correlation was detected between HIV disease stage, as defined by peripheral CD4 count, and HIV detection in intestinal biopsies, the relationship to peripheral viral load was not studied. The authors concluded that the intestinal mucosa may act as a reservoir for HIV infection and active viral replication may occur throughout the natural history of HIV infection. The route of HIV infection was not stated in this paper and it may be that the subjects were all sexually infected homosexual men. Genital tract mucosa may similarly act as a reservoir for HIV infection in heterosexually infected HIV-positive women.

The finding in this study of consistently higher viral loads in cervical tissues than in serum may in part explain why some HIV-infected pregnant women may transmit virus to the neonates even when peripheral CD4 lymphocyte counts are high, and viral load low. This observation also has important implications for horizontal transmission

as it indicates that HIV-positive women are potentially infectious at all stages of disease. Interestingly, a study in men comparing viral load in semen and blood showed that semen contained lower viral load than blood (Luizzi *et al,* 1996).

This study was not designed to demonstrate which cells within the cervix contained virus. However, previous studies have shown that virus is present mainly within lymphocytes (Van de Perre *et al,* 1988) and activated macrophages (Pomerantz *et al,* 1988). HIV has not been demonstrated within epithelial cells, which is not surprising as they are CD4 negative. Pomerantz *et al* (1988) showed that the HIV-infected cells were located mainly within the endocervical aspect of the transformation zone. Thus cervical ectopy and any inflammatory conditions, by exposing a greater surface area of endocervical epithelium to the exterior, would be expected to lead to increased viral shedding, as observed by Kreiss *et al* (1994). The hormonal changes in pregnancy lead to increased cervical vascularity, exudate and ectopy, all of which would contribute to enhanced viral shedding. The large variability we observed in viral load from cervical biopsy specimens may be partly reflective of the quantity of endocervical tissue present within each specimen. Langerhans' cells in the skin have been shown to be infected with HIV in some patients (Tshachler *et al,* 1987) but no published studies have shown that Langerhans' cells from cervical tissue are infected (Mostad and Kreiss, 1996). This is surprising as some reports have indicated that

Langerhans' cells are most readily infected with HIV (Macatonia *et al,* 1991). It was demonstrated in chapter 3 that Langerhans' cell proportions are decreased in HIV-infected women. One of the theories postulated to explain this finding is that the virus exhibits a direct cytopathic effect on these cells, which may explain why the failure to detect HIV-infected Langerhans' cells.

These findings have important implications for the design of strategies to reduce rates of vertical and horizontal transmission. The observation that viral load in the cervix is consistently higher than in the serum, and the probable lack of correlation with antiretroviral therapy indicate that oral antiretroviral therapy may be of limited value in reducing transmission rates. Oral zidovudine has been shown to reduce vertical transmission rates by approximately 70% in previously zidovudine-naive women with CD4 lymphocyte counts of less than 200 x 10^6/ml (Connor *et al,* 1994). However, even within the narrow confines of the study inclusion criteria, transmission rates of nearly 10% occurred in the therapy arm. Locally administered virucidal compounds may have a greater effect at reducing viral load within cervical tissues, thereby reducing transmission rates. The peripartum use of local virucidals has been reported in other settings. Chlorhexidine douches have been used effectively to reduce neonatal group B streptococcal infection (Burman *et al,* 1992). Candidate compounds have been

proposed for local administration to reduce HIV transmission and some are currently under study (reviewed by Minkoff and Mofenson, 1994).

Conclusion

The observation that viral load is higher in the cervix than in the serum supports the hypothesis that impaired local immunity in the genital tract may lead to a failure to resist HIV. In particular, the presence of functionally impaired cytotoxic T lymphocytes and the altered cytokine profile within the cervix may lead to persistence of virus within genital tract tissues. These observations have important implications for the vertical and horizontal transmission of HIV, as women may have high viral loads in the genital tract and thus be potentially infectious even when peripheral HIV load is low. Larger longitudinal studies are required to examine the relationship between viral load in cervical tissues, the genital tract shedding of virus and the effects of potential modifying factors such as the presence of HIV antibody in cervical secretions, sexual intercourse and mode of HIV infection.

CHAPTER 7: DISCUSSION

Summary

The Distribution of immunocompetent cells in the cervical mucosa

Horizontal transmission

Cervical intraepithelial neoplasia

Genital tract infection

Vertical transmission

Suggested further studies

The role of CD8 cells in the cervix of HIV-positive women

Relationship to CIN

Factors influencing HIV load in cervical tissues

Conclusion

CHAPTER 7: DISCUSSION

Summary of Aims

Despite an increasing recognition of the importance of the immune system of the female genital tract and its role in the prevention of infection, there has been limited research undertaken in this area. The emergence of HIV infection in women and the discovery that vertical transmission to the fetus occurs mainly during the intrapartum period (European Collaborative study, 1992; Goedert *et al,* 1991) has once again underlined the importance of female genital tract immunity and the attention of some researchers has been focused on this subject. However, despite this renewed interest, very little is known about the immunity of the normal female genital tract in health and in disease. Epidemiological studies have been successful in identifying some of the factors which predispose to the vertical and horizontal transmission of HIV and genital tract abnormalities associated with HIV. However, there is now a recognition of the need to understand the immunological mechanisms at the mucosal surface. This is the first study that has set out to comprehensively define the immune system of the genital tract in healthy females and to describe how this is altered in HIV disease.

The primary aims of this study have been successfully accomplished. The distribution and cytokine profile of immunocompetent cells have been defined in samples of cervical squamo-columnar junction from a well-defined population of healthy women. The changes associated with HIV infection have been documented and related to peripheral immunity and local and peripheral HIV load. The increase in CD8+ lymphocyte numbers and the resultant inversion of CD4/CD8 ratios has been described at other mucosal surfaces (Lim *et al,* 1993; Johnstone *et al,* 1994) but no attempt has previously been made to relate this observation to function in this manner. It was shown in this study that the CD8+ cells in the genital mucosa show evidence of functional impairment which may be associated with the altered cytokine environment; changes which may, in part, account for the observation that HIV load was greater in cervical tissues than in the serum.

This study was feasible largely because the Ian Charleson day Centre is in the unique position of having the largest and most heterogeneous group of HIV-positive female outpatients in England, providing a 'captive audience' from which volunteers could be recruited. The same day testing clinic also allowed access to a large population of proven HIV-negative women. Both the HIV-positive and the HIV-negative groups proved to be reliable and co-operative. One of the advantages of studying HIV-

positive women from a single treatment centre is that therapeutic approaches are uniform, thus reducing the effects of a potentially confounding variable.

The secondary aim of the project was to postulate theories, on which further research can be based, on the possible role of genital tract immune mechanisms in the vertical and horizontal transmission of HIV and in the aetiology of HIV-associated conditions such as CIN and recurrent viral and fungal infections. These have been met, but only to a limited extent, which is perhaps not surprising when one considers that the interaction between HIV and the immune system is complex at all levels. To this end, our observations that there are impaired cytotoxic T cells and an increased HIV load in the cervix have established the basis for some robust theories which, as well as shedding some important light on these issues, should stimulate and direct further research into the subject.

The Distribution of Immunocompetent Cells in The Genital Tract Mucosa

The female genital tract has a unique mucosal surface which is distinct from other mucosal surfaces in a number of ways. During sexual intercourse, it is exposed to allogenic biological material on a regular basis. Seminal fluid, as well as containing

sperm, contains a variable number of potentially immunogenic cells from the male (Witkin *et al,* 1988). Secondly, the genital tract is subject to hormonal control and alterations occur in its architecture at various phases in the females' lifetime. For example, the pre-pubertal and post-menopausal genital tract epithelium are different from the genital tract epithelium in the reproductive years (table 7:1).

Table 7:1 Changes in the Genital Tract Epithelium Related to Age

	Oestrogen	Epithelium	Glycogen	pH	Flora
Newborn	+	Thick 20-30 cells	+	Acid 4-5	Sterile ⇓ Doderlein's bacilli Secretion abundant
Month-old child	-	Thin 10 cells	-	Alkaline >7	Sparse, coccal& varied flora. Secretions scant
Puberty	Appears	Thickening 10-20 cells	- → +	Alkaline ⇓ Acid	Sparse, coccal ⇓ Rich, bacillary
Mature	++	Thick 30+ cells	+	Acid 4-5	Doderlein's bacilli, Secretion abundant
Post-Menopause	+ → -	Thin < 10 cells	-	Neutral or Alkaline 6 ->7	Varied, Dependent on level of circulating oestrogen Secretions scant

In addition, cyclical changes, associated with the menstrual cycle, occur during the reproductive years, with alterations in the depth and predominant cell type at the mucosal surface. For instance, cell smears taken from the vagina during the proliferative phase of the menstrual cycle which is under oestrogenic control show superficial and large intermediate cells predominating with few leucocytes present, while intermediate cells and leucocytes predominate during the secretory phase which is under progestogenic control (Llewlyn-Jones, 1986). Pregnancy also leads to changes in the architecture and immunity of all the mucosal surfaces of the female genital tract, which is evident in the fact that the fetus, which contains 50% paternally derived chromosomes, is not rejected by the mother. Given such a unique environment, is the distribution of immune cells in the female genital tract the same as other mucosal surfaces?

As mucosal surfaces are functionally adapted to their various roles, it would be expected that certain histological and immunological differences will exist between mucosal surfaces at different sites. For example, the trachea is lined by tall, pseudostratified ciliated epithelium containing numerous goblet cells. The presence of cilia at this mucosal surface is reflective of its role in trapping undesirable material from the inhaled air and ensuring the continuous movement of glandular

secretions towards the pharynx (Wheater *et al,* 1979b). This is in contrast to the endocervical epithelium which is lined by simple, tall columnar, mucus-secreting epithelium. The mucosa of the small intestine on the other hand, is characterised by villi which are lined with simple columnar epithelium containing numerous glands which secrete digestive enzymes. A distinct feature of this mucosal surface is the presence of organised lymphoid tissue of which Peyer's patches, the palatine tonsil and the appendix are examples. Studies have also shown the presence of organised lymphoid aggregates in the uterine endometrium (Yeaman *et al,* 1997). No such lymphoid aggregations have been demonstrated in the cervix in non-pathological conditions, but it was shown in this study that immunocytes are dispersed through out the cervical mucosa at all phases of the menstrual cycle in healthy women.

These immunocompetent cells may be expected to generate an inflammatory reaction to seminal fluid during intercourse and in fact there have been studies reporting a large increase in the concentrations of immunocompetent cells in the cervix which migrate into the vaginal lumen after coitus in response to ejaculated sperm (Thompson *et al*,1991). Thus, immunity to spermatozoa can occur and may be a cause of infertility (Schumacher, 1988). Conception is possible only because under normal circumstances, no specific immune reaction is generated against spermatozoa.

However, the mechanisms underlying the interaction between spermatozoa and female genital tract immunity are poorly understood. A study in which the effect of cervical mucus on immune mediators in cervico-vaginal lavage fluid demonstrated that mid-cycle cervical mucus could alter immune reactivity within the reproductive tract by modifying the availability or function of immunomodulatory substances such as cytokines and immunoglobulins (Ginsburg *et al,* 1997). Previous studies have demonstrated that immunocompetent cell proportions and the level of immunoglobulins in cervico-vaginal secretions are lowest during the peri-ovulatory period (Schumacher, 1988). In support of this theory, Murdoch *et al* (1982) found a decreased density of IgA-containing plasma cells in the cervical squamo-columnar junction of the cervix under the influence of oestrogen and an increased density under the influence of progesterone. However, despite obtaining samples that were carefully timed with the phases of the menstrual cycle, we were unable to demonstrate a cyclical variation in immunocompetent cell proportions in our initial pilot study. Rebello *et al* (1975), in a larger study of 58 uteri showed that, consistent with our findings, the proportions of plasma cells in the squamo-columnar of the cervix were not significantly changed through all phases of the menstrual cycle. These contradictions may be accounted for by the methodological differences which exist in the different studies, making direct comparisons invalid. However, unlike

some of the reported studies which are cross-sectional (Rebello *et al,* 1975), our pilot study was conducted longitudinally so that paired samples from the follicular and luteal phases existed for each subject studied. The failure to conduct longitudinal studies may significantly decrease the validity of some earlier studies, particularly when taking into consideration the large inter-subject variation in immunocompetent cell distribution at any phase in the menstrual cycle. Longitudinal studies with larger subject numbers may be required to determine if small but significant changes in immunocompetent cell proportions occur under cyclical hormonal control.

This study has demonstrated that the CD4/CD8 ratios within normal cervical mucosa are similar to that in the peripheral blood. Large numbers of macrophages, with smaller numbers of B cells, plasma cells and mast cells have also been shown to be present in the cervical mucosa under normal circumstances, and the presence of Langerhans' cells in the epithelium has been confirmed. This comprehensive illustration of the distribution of immunocompetent cells within healthy cervical mucosa provides us with an important baseline from which alterations associated with HIV infection, the probable role of the immune system in the horizontal and vertical transmission of HIV and the aetiology of HIV-related genital tract conditions

can be studied. These observations are consistent with those of a recent publication (White *et*

al, 1997), which confirmed the presence of lymphocytes, macrophages and dendritic cells in the cervical and vaginal mucosa of normal women.

Horizontal Transmission

This study has demonstrated the presence of increased numbers of CD8+ lymphocytes within the cervix of HIV-infected women. The fact that the majority of these cells are 'primed' (memory cells, CD45ro+) indicates that they are virally stimulated, but the absence of perforin and low TIA-1 expression (cytolytic granules) suggest that these cells were not functionally cytotoxic at the time the biopsies were taken. Does this increase in primed CD8+ lymphocytes represent an HIV-specific cytotoxic lymphocyte response to genitally acquired HIV infection?

The idea that the increased numbers of CD8+ cells observed within the cervical mucosa represent an impaired HIV-specific cytotoxic lymphocyte reaction is compelling. The theory is in keeping with what is known about the immune response to viral agents and may explain why virus acquired from this site persists in the

tissues and leads ultimately to systemic infection. Under normal circumstances, acute CD8+ expansion in response to viral infection is followed by resolution after the infection is overcome (Akbar *et al,* 1993). However, HIV is known to establish a close relationship with the host immune system, and to have a direct effect on the cytokine environment. Results from this study show an increase in IL-4, IL-5 and IL-10 (Th-2 cytokines) and decrease in IL-2 (Th-1 cytokine) in cervical tissue. This 'Th-1 to Th-2 shift', which may be HIV-mediated, is said to favour HIV replication in T lymphocytes (Poli & Fauci, 1992). Thus, the observed cytotoxic T cell impairment may be due to the interaction of HIV with the immune system, leading to inappropriate signals from cytokines, or an inappropriate response to these cytokines. Of relevance to this is the fact that dysregulation of cytokine gene expression has been demonstrated at other mucosal surfaces in HIV-infected individuals. McGowan *et al* (1994) found increased expression of proinflammatory cytokine mRNA in the intestinal mucosa of patients with HIV infection.

The immune cell and cytokine profile changes documented in the cervix may lead to a failure of the cytotoxic T-cells to overcome local HIV infection and consequently, a lack of resolution of cytotoxic T cell expansion. The high HIV RNA levels detected in cervical tissue may reflect the inability of cytotoxic T cells to kill virus.

Consistent with this theory is the fact that specific HIV anti-cytotoxic lymphocyte activity has been described in the genital tract mucosa of rhesus macaques infected with HIV by intravaginal inoculation (Lohman *et al,* 1995). The T-cell changes observed in this study are probably not limited to the cervix but occur through out the genital tract mucosa. CD4/CD8 inversion has been reported in the endometrium (Johnstone *et al,* 1994). The vagina is lined by squamous epithelium which is identical to the lining of the ectocervix and it may be expected that immunocompetent cells are similarly distributed in the vaginal mucosa and that similar HIV-associated alterations in immune cell composition may occur. Thus the susceptibility or resistance of an individual to HIV infection may depend on the ability to overcome HIV infection at a local level. Systemic infection may occur if local resistance fails and virally infected T cells may migrate to local lymph nodes.

This theory of acquisition of systemic HIV infection through the genital tract has its limitations as it fails to take account of certain observations, both from this study and from other published reports. For example, there are studies demonstrating impaired cytotoxic T-cell function, possibly secondary to an altered cytokine profile at other mucosal surfaces in HIV infection. In one published report, T cells recovered from broncho-alveolar lavage (BAL) specimens of HIV infected patients at various stages

of disease were shown to exhibit impaired cytotoxic function. Addition of IL-2 enhanced spontaneous killing and elicited a lymphocyte-activated killing phenomenon (Agostini *et al,* 1990). McGowan *et al* (1994) found a significant increase in pro-inflammatory cytokines in HIV-infected compared with control small intestinal samples. Bofill *et al* (1995) found increased proportions of primed T cells, with no detectable levels of perforin, infiltrating the paracortex and germinal centres of lymph node in HIV-infected women. These facts suggest that the changes within the cervical mucosa may not be in direct response to genitally-acquired HIV infection but may represent a generalised impairment of the immune system mediated by HIV at all mucosal surfaces, regardless of route of infection.

The fact that similar changes in genital tract immunity were observed in subjects in this study presumed to be infected with HIV through intravenous drug use would support the suggestion that these are generalised changes, unrelated to the mode of HIV acquisition. However, as previously discussed (chapter 2), it is difficult to establish that the women ascribed to the intravenous drug use infection route group were not infected through heterosexual intercourse. It is important to remember also that alterations in immunocompetent cell distribution and high levels of HIV RNA were observed in some women with early, asymptomatic HIV disease, before there

was any evidence of peripheral immune impairment. The observation that alterations in immune cell distribution in the cervix preceded systemic immunosuppression lends weight to the theory that the changes in cervical immunocompetent cell proportions occur as a direct response to genitally-acquired HIV infection. The question of whether the changes in immunocompetent cell distribution and cytokine profile in the cervical mucosa of HIV-infected women are in direct response to the genital tract route of infection is of considerable clinical importance as it has implications for the design of strategies to reduce the rate of vertical transmission and for the administration of vaccines through the mucosal route. Further studies are required to investigate this issue.

Cervical Intraepithelial Neoplasia

The increased risk of CIN in HIV-positive women compared to HIV-negative women has been quoted as between four and ten-fold (Schafer *et al,* 1991; Marte *et al,* 1992; Wright *et al,* 1994). These findings have led to the assumption that HIV infection is an independent risk factor for CIN. However, it has been difficult to establish a causal relationship because the multifactorial origin of CIN (table 7:2) means that behaviour that puts women at risk of HIV may also increase their risk of pre-malignant and malignant disease of the cervix.

Table 7:2 Predisposing factors for CIN

Predisposing factor	**Risk**
Age at 1st intercourse	50% excess risk if before 20th birthday (Rotkin 1973)
Number of sexual partners	11fold risk if > 6 (Harris *et al* 1980)
Lack of barrier contraception	Increased risk
Smoking	12x risk if smoked for > 12 years (Anderson *et al* 1992)

Also, several of the studies reporting an increased risk of CIN in HIV-infected women have been uncontrolled, meaning that potential confounding factors were not addressed. However, in one study in which HIV-positive women were carefully matched with seronegative controls, thereby controlling for potential confounding factors, a significantly higher proportion of HIV-positive women than their seronegative controls had abnormal smears (Johnstone *et al,* 1994b). This implies that there is a relationship between HIV and CIN beyond common predisposing behaviour.

The increased risk of CIN appears to be related to the degree of immunosuppression (Smith *et al,* 1993; Maiman *et al,* 1993), which is perhaps not surprising as iatrogenic immunosuppresion in organ donor recipients is also associated with increased risk of CIN (Porrecco *et al,* 1975), supporting the theory that the host immune response influences the development of cervical neoplasia. Gemigiani *et al* (1995) found that HIV-negative women with invasive cervical cancer had lower peripheral CD4 counts than women without cervical neoplasia. At a local level, women with persistent as opposed to spontaneously regressing CIN have been shown to have decreased numbers of Langerhans' cells, and helper-inducer T lymphocytes in the cervix (Fukuda *et al,* 1993). Cigarette smoking (Barton *et al,* 1988; Poppe *et al,* 1995) and wart virus infection (Morris *et al,* 1983), both recognised risk factors for CIN (Wiggle & Grace, 1980; Levine *et al,* 1993), are also associated with Langerhans' cell depletion. Poppe *et al* (1996) showed that Langerhans' cell proportions in the cervix are decreased pregnant women and tobacco smokers. Pregnancy is known to be immunosuppressive and pregnant women may suffer recurrent vaginal candida infection or proliferation of genital warts while smoking may predispose to CIN.

These findings suggest that the altered immune mechanisms in the genital tract associated with HIV may be important in the aetiology of HIV-associated CIN. A 'healing role' for Langerhans' cells in the cervical mucosa was suggested by Maclean (1984) following the observation that Langerhans' cells are present in the mucosa following local destructive treatment for CIN. Barton *et al* (1990) described a reduction in Langerhans' cell proportions in the cervical mucosa of HIV-infected women but, despite the potential importance of local immunity in the development of CIN, little other research has been undertaken into the subject.

The altered immune cell profile and phenotypic T cell abnormalities demonstrated among the HIV-positive subjects in this study may underlie the susceptibility of HIV-infected women to CIN. A reduction in Langerhans' cell numbers was observed. It has been suggested that Langerhans' cell depletion may facilitate oncogenesis either independently or in association with other factors such as the human papilloma virus (Morris *et al*, 1993a). The prevalence of HPV 16 and 18, which are considered to be of high oncogenic potential, is increased in cervix of HIV-positive women with, or without CIN (Agarossi *et al,* 1992; Van Doornum *et al,* 1993). However, even HPV of low oncogenic potential (such as types 6 &11) have been shown to be associated with a decrease in cervical Langerhans' cells

(Morelli *et al,* 1993). Woodworth *et al* (1993) demonstrated that HPV infection and CIN were associated with abnormal cytokine secretion. Clerici *et al* (1997) showed a reduction in IL-2 production and an increase in IL-4 and IL-10 production by PBMCs from women with HPV-associated high grade CIN, particularly in a sub-group with extensive genital HPV. The studies for this MD thesis were not designed to investigate HPV in the cervix but the question must arise: does HIV-induced alteration in cervical immune cell distribution alone predispose to CIN or does it increase the oncogenic effect of HPV? A study of HPV 16 in minor grade CIN showed that the presence of HPV 16 DNA was a poor long-term predictor of CIN disease progression, regardless of copy number (Downey *et al*, 1994). These findings suggest that other factors other than HPV may be important in the aetiology of CIN and cervical cancer. Carson *et al* (1986) proposed a link between HIV-induced immunosuppression, HPV and CIN by suggesting that immunosuppression from any cause permits recurrent or persistent HPV infection which allows neoplastic transformation.

Further research relating the documented immunocompetent cell and cytokine changes to the presence or absence of HPV in the cervix of HIV-infected women may further enhance our understanding of the aetiology of CIN in this group.

Genital Tract Infection

The cellular immune response is usually the first line of defense against viral and fungal pathogens. The abnormalities in the phenotype of cytotoxic T cells observed even in early HIV disease may explain why HIV infected women are susceptible to recurrent viral and fungal infections even before peripheral immune function is compromised, and may underlie the hierarchical pattern of mucosal candidal infection of vaginal candida in women with normal peripheral CD4 counts, oral candida in those with declining counts and oesophageal involvement in those with AIDS, described by Imam *et al* (1990). The fact that recurrent genital tract infections precede systemic immunosuppression is consistent with our observation that abnormalities in local immune function occur before there is any evidence of disturbance in peripheral immune status.

The decrease in IL-2 and increase in IL-4, IL-5 and IL-10 observed in HIV-positive women compared to the HIV-negative subjects may underlie the phenotypic CD8 abnormalities. Agostini *et al* (1990) found that the addition of IL-2 restores cytotoxic activity to functionally impaired T cells in BAL from HIV-infected subjects. This implies that IL-2 deficiency, which is possibly HIV-mediated, may be at least in part,

responsible for the cytotoxic T-cell abnormality. This observation may pave the way for a unique therapeutic approach to the prophylaxis of recurrent genital herpes, warts and candida. These conditions, although not life-threatening, may be difficult to treat and lead to considerable impairment of quality of life in HIV-positive women.

Vertical Transmission

Traditionally, much of the interest in HIV-positive women has been focused on the issue of vertical transmission but, despite extensive study into this subject (Brossard *et al,* 1995; European Collaborative study 1991, 1992, 1996), it is still not understood how materno-fetal transmission occurs. Studies of viral load have shown that high viral loads in maternal plasma are associated with an increased probability of vertical transmission, but there is no critical threshold below which materno-fetal transmission does not occur (Weisser *et al,* 1994). This, and other evidence to suggest that contact with maternal genital secretions predisposes to fetal infection, would imply that the quantity of HIV in genital tract tissues and secretions may be more important than peripheral viral load in determining the likelihood of vertical transmission. Caesarean section is thought to protect against vertical transmission of HIV (European collaborative study, 1996), despite the fact that the neonate may come into contact with large quantities of virus-containing maternal blood during the procedure. The risk of transmission increases when the fetal membranes are ruptured for more than four

hours before the delivery (Landesman *et al,* 1996), suggesting that fetal contact with maternal genital tract tissues or secretions is a more important determinant of vertical transmission than contact with maternal blood during Caesarean section.

The observation in this study that HIV load is higher within cervical tissue than in serum may, in part, explain the findings listed above. No direct relationship was detected between serum and cervical HIV load but the small sample size in this study meant that factors which could potentially influence viral load such as the use of antiretroviral medication and mode of HIV acquisition could not be formerly analysed. The cross-sectional design of the study also meant that temporal variations in viral load could not be studied. Clearly more study is required into this subject. An understanding of the effects of antiretroviral medication on cervical viral load may potentially influence the design of strategies to reduce the vertical transmission rate.

It is not known how HIV in genital tract tissues enters genital tract secretions but previous studies have demonstrated the presence of cell-free and cell associated virus in cervico-vaginal secretions (Wofsy *et al,* 1986). It is known that epithelial cells are constantly shed into the lumen of the genital tract and it is on this principle that the collection of samples for cervical cytology is based. In addition to epithelial cells,

genital tract secretions also contain variable proportions of inflammatory cells. It is probable therefore that some HIV is shed into the genital tract lumen contained within inflammatory cells. The free virus may be actively produced as Nuovo *et al* (1993) have demonstrated the presence of transcriptionally active cells within cervical biopsies. Thus, the relationship between viral load in genital tract tissues and the amount of virus in genital tract secretions may not be linear. It is important that this relationship is understood as a baseline to design studies to determine whether HIV levels in genital secretions or HIV levels in genital tract tissues are more predictive of vertical transmission.

Suggested Further Studies

It is impossible to encompass all aspects of the genital tract mucosal immune system in HIV disease within one study. However, what has been achieved through this project is a clearer picture of the distribution of immune cells and cytokines in the cervix in healthy women, the alterations associated with HIV disease and a baseline understanding of the relationship between viral load in the cervix and the serum. However, this study has probably generated more questions than it has answered and the observations resulting from the study may be viewed as a basis to stimulate and direct further research into the subject.

The role of cytotoxic lymphocytes and other immune cells in the cervical mucosa of HIV-positive women

The assumption that the quantitative and phenotypic alterations in CD8+ lymphocytes observed within the cervix of HIV-positive women represent an impaired specific cytotoxic reaction to genitally acquired HIV infection can be proven if these changes can be demonstrated, together with the presence of infectious HIV, in the genital tract of HIV-infected women at the time of seroconversion. However, in practical terms, most people are not aware of their HIV seroconversion date and the few who are aware usually recognise this retrospectively. A study of the immunological events in the genital tract at the time of HIV seroconversion would therefore be difficult, if not impossible, to design. However, double immunofluorescent staining for HIV antigens in cytotoxic T lymphocytes in cervical biopsies may confirm the assumption that the functionally inept T lymphocytes are unable to destroy virus.

Considerable information may be gained by comparing the immunological events in the cervix of heterosexually-infected HIV-positive women with women infected through other routes. The inherent difficulty in designing a study to compare HIV effects in the genital tract of women with heterosexually acquired infection with

those who acquired infection through intravenous drug use lies in the difficulty of excluding the possibility of heterosexual exposure in the latter group.

A study of discordant couples where HIV negative females are known to have unprotected sexual intercourse HIV positive men may help to further elucidate the mucosal immune response to HIV. There at least ten such women, who remain uninfected despite multiple exposures to HIV, known to clinicians at the Ian Charleson Day Centre. A study of the cervical immune response in such women following exposure to HIV in the seminal fluid may determine if an effective cervical cytotoxic T lymphocyte reaction is the key to resistance to HIV.

There have been some reports of antibody to HIV in genital secretions of HIV seronegative sexual contacts of HIV-positive men (Archibald *et al,* 1987; Belec *et al,* 1989), suggesting that a humoral response may be mounted at a local level, but the cellular immune response in these cases has not been investigated. A comprehensive investigation into humoral and cellular responses under these circumstances may help elucidate the mechanisms of horizontal transmission.

The cross-sectional design of this study meant that the immunological changes in the genital tract mucosa were studied at a single point in time for each of the subjects involved in the study. A longitudinal study, ideally with larger subject numbers, investigating the changes in genital tract immunocompetent cell distribution, cytokine profile and HIV load as HIV disease progresses, and relating these parameters to peripheral immune function may yield valuable information to enhance our understanding of the genital tract immune response.

Relationship of local immune dysfunction to CIN

The assumption has been made, based on circumstantial evidence, that the observed alterations in the immune system of the cervical mucosa predisposes HIV-infected women to CIN. A prospective, longitudinal follow-up study is required to investigate the relationship between local immune dysfunction and CIN in women with HIV disease, documenting CD4 lymphocyte slopes, peripheral viral load, smoking habits and therapeutic interventions, and relating this to the frequency and severity of CIN and its response to treatment. The role of HPV and its relationship to local immune changes in the cervix also needs to be explored, particularly in the light of studies showing an association between high grade CIN and the presence of HPV16 DNA. (Bavin *et al*, 1993).

Studies at other mucosal surfaces suggest there may be a specific defect in the antigen-presenting arm of the immune system in HIV infection. Although we observed no change in the distribution of macrophage subsets within the cervix, this study was not designed to investigate macrophage function which may have been impaired despite normal subset proportions. The observed depletion in Langerhans' cell numbers and probable subsequent impairment in antigen presentation may underlie the phenotypic abnormalities in CD8-lymphocytes, and there may be even more subtle abnormalities in macrophage function that are, as yet, undetected. An understanding of macrophage and antigen-presenting cell function in the cervix in HIV disease is important as it has been suggested that defects in antigen presentation may predispose to oncogenesis.

Recurrent CIN can substantially impair the quality of life in HIV-infected women and a clearer understanding of the aetiology and natural history of this condition may enable more effective management of the condition.

Factors influencing viral load in genital tract tissues

HIV load in the cervix has been shown to be consistently higher than in the serum but no statistical relationship was detected. A large prospective follow-up study, investigating viral load in genital tract tissues and in peripheral blood, at all stages of HIV disease, is required to better comprehend the relationship between genital tract and peripheral viral load. Also as most published studies have investigated viral load in plasma, direct comparisons cannot be made with this study as viral load was measured in serum, where viral load may be expected to be up to 15% lower (personal communication, Roche). It would be more practical for future studies to be undertaken on plasma.

It is not possible to say from this study precisely where in the cervical tissues the virus was localised. Double immunofluorescent studies, staining for viral antigens and immune and epithelial cells should enable the observer to localise the virus to specific cells. Changes in viral load can also be correlated with immunocompetent cell changes within the cervix.

Pregnancy is immunosuppressive and studies have shown reductions in CD4 and total lymphocyte counts which start in early pregnancy (Johnstone *et al* 1994a). Although it is now recognised that pregnancy does not accelerate the course of HIV disease, it is possible that the immunosuppressive effect of pregnancy may lead to an alteration in the relationships between peripheral and genital tract immune function and viral load. It is important therefore to assess these relationships in pregnant women, but as it is impractical to obtain cervical biopsies from pregnant women, the relationship between viral load in genital tissues and genital tract secretions should be established and genital tract secretions which are easily obtainable in all women, can be used to assess alterations in viral load through pregnancy These findings can be related to fetal outcome. The effects of local and systemic antiretroviral therapies on genital tract viral load should also be assessed in order to devise effective strategies to minimise the rate of horizontal and vertical transmission.

Conclusion

The growing prevalence of HIV infection in women and the risk of transmission to the offspring have led to a recognition of the need to understand the mechanisms of the horizontal and vertical transmission of HIV and HIV-associated conditions specific to women. Epidemiological studies have been effective in increasing our

understanding of factors which predispose to HIV transmission but the mechanisms involved are still poorly understood.

The hypothesis on which this project is based is that an understanding of the immune system of the female genital tract is the key to elucidating the effects of HIV on the female genital tract and the mechanisms of HIV transmission in females. This has been supported by the observations made.

The observation that there are increased numbers of functionally inept cytotoxic T cells in the cervical mucosa, together with an altered cytokine profile, may explain the apparent inability of the local immune system to overcome HIV and explain the high levels of HIV RNA documented in cervical tissues. The reduction in Langerhans' cell numbers, which may reflect an impairment of antigen presentation, and the presence of dysfunctional cytotoxic T cells in the cervical mucosa may explain the susceptibility of HIV-infected women to recurrent fungal and viral infections of the lower genital tract and to CIN. The observation that local immune changes precede systemic immune depletion may explain why recurrent genital infection and CIN are so often the initial presenting complaints of HIV-infected women

The high HIV loads documented in cervical tissue may explain why vaginal delivery is thought to increase the risk of vertical transmission of HIV while Caesarean section may have a protective effect. More research is required with longitudinal follow-up on larger patient groups to confirm and further expand these theories.

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Appendix 1: Structured Questionnaire Used In Study

MUCOSAL IMMUNITY QUESTIONNAIRE Date:

Patient Details

Name: DOB:

Hospital No: Ethnic group:

Address: Telephone:

HIV Details

HIV diagnosis date:

CDC classification: AIDS diagnosis date:

Presumed route of infection: *ADD:

Antiretroviral drugs PCP prophylaxis:

Medical History

Chronic Disease:

Recent illness:

Current illness:

Other Medication:

*Aids Diagnosing Disease

Obstetric History

Viable pregnancies:

STOPS/Miscarriage:

Gynaecology History

LMP:

Contraception:

Menarche:

Cycles

Last cervical smear: (date & report)

Sexual History:

Age at 1st SI:

STDs:

Total No. Partners:

Current Partner: Yes/No
details

Partner's HIV status &

Last sexual intercourse:

With condom:

Personal Habits:

Smokes:

Alcohol:

Drug abuse:

Last drug use:

EXAMINATION

Bimanual:

Colposcopy:

RESULTS:

Date	Ist exam	2nd exam	3rd exam
Cervical smear			
Cervical swab			
HVS			
Virology			
Total Lymphocyte Count			
Serum CD4			
Serum CD8			
ß2 microglobulin			
Day of cycle			

Appendix 2 Information Leaflets Used in This Study

Female Genital Tract Immunity In HIV Infection (HIV positive women)

INTRODUCTION

Sexual intercourse is the commonest mode of HIV transmission world wide. During sex, HIV infected sperm is deposited into the vagina where, by as yet unknown mechanisms, the virus may gain access into the body. The female genital tract has certain natural defence mechanisms (immune system) to prevent entry of infective organisms but very little is known about how it works. We feel that a better understanding of female genital tract immunity will enable us to explain the heterosexual transmission of HIV, and ultimately, to prevention of infection through this route. This project may also help explain why some babies born to women with HIV acquire the infection from the mother while the majority are not infected. The results of this study will probably be available too late to have any direct effect on your management but, by participating in the study, you may benefit by having regular screens for infection and receiving treatment where appropriate.

WHAT THE STUDY INVOLVES

The procedure is similar to the gynaecological screen which you have every six months. Your cervix will be examined with the colposcope and swabs for infection taken from the cervix and vagina. A small amount of fluid (saline) is instilled into the vagina and sucked out with a syringe to collect cells which will later be studied. A cervical biopsy will be taken (you may have had this done before, if you have had an abnormal smear). You may feel a mild pinching sensation during the biopsy and you may notice slight vaginal bleeding for one or two days afterwards. To prevent infection, it is important that you avoid sexual intercourse and wear towels instead of tampons until the bleeding stops completely.

PRACTICALITIES

If wish to discuss any aspects of this study further, or have any questions, please do not hesitate to ask me. Your care will not be in any way affected whether or not you decide to participate in this study, or if you decide to drop out at any stage. If, at any stage during the study, you have any queries or concerns, you can contact me on the telephone number below between 9am and 5pm, Monday to Friday. If you require assistance outside this hours, you may contact your GP who, (with your permission) will be informed of your participation in the study. If you would rather not inform your GP, and you feel that your query requires urgent attention, you can attend Accident and Emergency at the Royal Free where a member of the HIV or gynaecology on call team can be contacted.

Thankyou for your cooperation.

Adeola Olaitan

Research Registrar, Gynaecology/HIV Medicine

Female Genital Tract Immunity In HIV Infection (HIV negative women)

INTRODUCTION

Sexual intercourse is the commonest mode of HIV transmission world wide. During sex, HIV infected sperm is deposited into the vagina where, by as yet unknown mechanisms, the virus may gain access into the body. The female genital tract has certain natural defence mechanisms (immune system) to prevent entry of infective organisms but very little is known about how it works. We feel that a better understanding of female genital tract immunity will enable us to explain the heterosexual transmission of HIV, and ultimately, to prevention of infection through this route. This project may also help explain why some babies born to women with HIV acquire the infection from the mother while the majority are not infected. Although you are not infected with HIV, by participating in the study, you may benefit by having regular screens for infection and receiving treatment where appropriate.

WHAT THE STUDY INVOLVES

You will undergo a full gynaecology screen which involves a pelvic examination and swabs from the cervix and vagina for sexually transmitted diseases. A vaginal washing, which involves introducing a small amount of fluid into the vagina and sucking this out with a syringe is obtained. A cervical smear is also taken and, if this is normal, you do not require one for another three years. At the same time, I shall examine the cervix with a colposcope, an instrument which magnifies the cervix so that any abnormal areas can be clearly seen and biopsied. I will take biopsies of the cervix, even if there are no abnormal areas, as it is an important part of this project to study the appearance of the normal cervix. The colposcopy examination is painless but you may feel a mild, pinching sensation as I take the biopsy and you may notice some slight vaginal bleeding for one or two days afterwards. To avoid infection, it is important that you wear towels instead of tampons and that you avoid sexual intercourse until the bleeding stops completely.I may ask you to return on two more occassions to have the biopsy repeated. I will need to repeat this procedure over two more menstrual cycles.

PRACTICALITIES

If you wish to discuss any aspects of this study further, or have any questions, please do not hesitate to ask me. Your care will not be in any way affected whether or not you decide to participate in this study, or if you decide to drop out at any stage. If, at any stage during the study, you have any queries or concerns, you can contact me on the telephone number below between 9am and 5pm, Monday to Friday. If you require assistance outside this hours, you may contact your GP who, (with your permission) will be informed of your participation in the study.

Thankyou for your cooperation.

Adeola Olaitan

Research Registrar, Gynaecology/HIV Medicine

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Appendix 3: Medical Details of HIV-positive Women

Patient I.D.	MA
Age	31
Ethnic Group	Black African
HIV-positive date	1994
Reason for testing	Chronic diarrhoea
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	30/mm^3 (AIDS)
Medication	Septrin, Zidovudine

Patient I.D.	JB
Age	33
Ethnic Group	Black African
HIV-positive date	1990
Reason for testing	Tuberculous meningitis
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	210/mm^3 (AIDS)
Medication	Septrin, Zidovudine, Rifinah

Patient I.D.	AB
Age	34
Ethnic Group	Black African
HIV-positive date	1996
Reason for testing	?Seroconversion illness
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	760/mm^3(Asymptomatic)
Medication	Nil

Patient I.D.	MC
Age	33
Ethnic Group	Caucasian
HIV-positive date	1992
Reason for testing	Ex-partner HIV-positive
Transmission category	Intravenous drug use
Serum CD4 at entry to study	410/mm^3 (Symptomatic disease)
Medication	Nil

Patient I.D.	DC
Age	35
Ethnic Group	Caucasian
HIV-positive date	1994
Reason for testing	HIV-positive partner
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	880/mm^3 (Asymptomatic)
Medication	Nil

Patient I.D.	SD
Age	23
Ethnic Group	Black African
HIV-positive date	1994
Reason for testing	Persistent pubic folliculitis
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	760/mm^3 (Asymptomatic disease)
Medication	Nil

Patient I.D.	TD
Age	31
Ethnic Group	Caucasian
HIV-positive date	1993
Reason for testing	HIV-positive partner
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	30/mm^3 (AIDS)
Medication	Septrin, Zidovudine

Patient I.D.	MdP
Age	43
Ethnic Group	Black African
HIV-positive date	1994
Reason for testing	Investigation of thrombocytopaenia
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	$10/mm^3$ (AIDS)
Medication	Septrin, Prednisolone

Patient I.D.	PE
Age	31
Ethnic Group	Caucasian
HIV-positive date	1992
Reason for testing	Husband died of AIDS
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	$1101/mm^3$ (Asymptomatic disease)
Medication	Nil

Patient I.D.	TG
Age	28
Ethnic Group	Black African
HIV-positive date	1990
Reason for testing	Investigation of psychotic episode
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	$230/mm^3$ (AIDS)
Medication	Septrin, Zidovudine

Patient I.D.	SG
Age	29
Ethnic Group	Caucasian
HIV-positive date	1987
Reason for testing	HIV-positive partner
Transmission category	Intravenous drug use
Serum CD4 at entry to study	550/mm^3 (Asymptomatic disease)
Medication	Nil

Patient I.D.	CI
Age	32
Ethnic Group	Caucasian
HIV-positive date	1993
Reason for testing	HIV-positive partner
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	590/mm^3 (Asymptomatic disease)
Medication	Nil

Patient I.D.	RF
Age	25
Ethnic Group	Caucasian
HIV-positive date	1992
Reason for testing	Self-referred
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	820/mm^3 (Asymptomatic disease)
Medication	Nil

Patient I.D.	LJ
Age	33
Ethnic Group	Caucasian
HIV-positive date	1996
Reason for testing	Blood donor, mandatory screen
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	770/mm^3 (Asymptomatic disease)
Medication	Nil

Patient I.D.	RK
Age	28
Ethnic Group	Black African
HIV-positive date	1992
Reason for testing	HIV-positive husband
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	330/mm^3 (Symptomatic disease)
Medication	Septrin

Patient I.D.	SK
Age	23
Ethnic Group	Black African
HIV-positive date	1993
Reason for testing	Antenatal screening (abroad)
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	760/mm^3 (Asymptomatic disease)
Medication	Nil

Patient I.D.	NL
Age	26
Ethnic Group	Black African
HIV-positive date	1993
Reason for testing	Raped in high prevalence country
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	60/mm^3 (AIDS)
Medication	Refused

Patient I.D.	VL
Age	42
Ethnic Group	Black African
HIV-positive date	1996
Reason for testing	Miliary tuberculosis
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	20/mm^3 (AIDS)
Medication	Zidovudine, Didanosine, Septrin, Fluoexitine, Rifinah

Patient I.D.	EM
Age	33
Ethnic Group	Black African
HIV-positive date	1994
Reason for testing	Recurrent genital herpes infection
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	120/mm^3 (Symptomatic disease)
Medication	Acyclovir

Patient I.D.	MM
Age	29
Ethnic Group	Black African
HIV-positive date	1993
Reason for testing	Pneumocystis pneumonia
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	40/mm^3 (AIDS)
Medication	Septrin, Zidovudine, Prednisolone

Patient I.D.	SM
Age	29
Ethnic Group	Black African
HIV-positive date	1995
Reason for testing	Pneumocystis pneumonia
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	$20/mm^3$ (AIDS)
Medication	Septrin, Zidovudine, Zalcitabine, Fluoexetine

Patient I.D.	LM
Age	46
Ethnic Group	Black African
HIV-positive date	1991
Reason for testing	Self-referral
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	$400/mm^3$ (Symptomatic disease)
Medication	Septrin, Acyclovir, Didanosine, Zidovudine

Patient I.D.	JN
Age	27
Ethnic Group	Black African
HIV-positive date	1992
Reason for testing	Self-referral
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	$160/mm^3$ (Symptomaticdisease)
Medication	Acyclovir

Patient I.D.	GN
Age	32
Ethnic Group	Black African
HIV-positive date	1991
Reason for testing	HIV-positive partner
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	$20/mm^3$ (AIDS)
Medication	Septrin, Fluconazole

Patient I.D.	BN
Age	35
Ethnic Group	Black African
HIV-positive date	1995
Reason for testing	Oral candidal infection
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	$190/mm^3$ (Symptomatic disease)
Medication	Septrin

Patient I.D.	JN
Age	27
Ethnic Group	Black African
HIV-positive date	1994
Reason for testing	HIV-positive partner
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	$50/mm^3$ (AIDS)
Medication	Septrin

Patient I.D.	NO
Age	33
Ethnic Group	Caucasian
HIV-positive date	1990
Reason for testing	HIV-positive husband
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	$550/mm^3$ (Asymptomatic disease)
Medication	Nil

Patient I.D.	LO
Age	32
Ethnic Group	Black African
HIV-positive date	1994
Reason for testing	Ill health, haemoptysis
Transmission category	Heterosexual
Serum CD4 at entry to study	0/mm^3 (AIDS)
Medication	Septrin, Rifinah

Patient I.D.	CO
Age	27
Ethnic Group	Caucasian
HIV-positive date	1992
Reason for testing	HIV-positive partner
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	550/mm^3 (Asymptomatic disease)
Medication	Nil

Patient I.D.	COt
Age	30
Ethnic Group	Black African
HIV-positive date	1994
Reason for testing	Pulmonary Tuberculosis
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	30/mm^3 (AIDS)
Medication	Septrin

Patient I.D.	EO
Age	27
Ethnic Group	Caucasian
HIV-positive date	1995
Reason for testing	Hepatitis C-positive
Transmission category	Intravenous drug use
Serum CD4 at entry to study	600/mm^3 (Asymptomatic infection)
Medication	Nil

Patient I.D.	HO
Age	26
Ethnic Group	Black African
HIV-positive date	1995
Reason for testing	HIV-positive son
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	120/mm^3 (Symptomatic disease)
Medication	Septrin

Patient I.D.	KP
Age	33
Ethnic Group	Caucasian
HIV-positive date	1990
Reason for testing	Husband died of PCP
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	70/mm^3 (AIDS)
Medication	Septrin, Zidovudine, Zalcitabine

Patient I.D.	VR
Age	41
Ethnic Group	Asian
HIV-positive date	1993
Reason for testing	Recurrent shingles
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	100/mm^3 ((AIDS)
Medication	Septrin, Zidovudine, Saquinovir

Patient I.D.	CS
Age	31
Ethnic Group	Caucasian
HIV-positive date	1990
Reason for testing	Salmonella septicaemia
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	150 mm^3 (AIDS)
Medication	Pentamidine

Patient I.D.	ST
Age	29
Ethnic Group	Black African
HIV-positive date	1989
Reason for testing	HIV-positive partner
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	550 mm^3 (Symptomatic infection)
Medication	Nil

Patient I.D.	NtP
Age	48
Ethnic Group	Caucasian
HIV-positive date	1989
Reason for testing	Persistent lymphadenopathy
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	10 mm^3 (AIDS)
Medication	Septrin

Patient I.D.	GV
Age	42
Ethnic Group	Caucasian
HIV-positive date	1984
Reason for testing	Self referred
Transmission category	Intravenous drug use
Serum CD4 at entry to study	330 mm^3 (Symptomatic disease)
Medication	Nil

Patient I.D.	SV
Age	29
Ethnic group	Caucasian
HIV-positive date	1987
Reason for testing	Self referred
Transmission category	Intravenous drug use
Serum CD4 at entry to study	288 mm^3 (Symptomatic disease)
Medication	Zidovudine, Didanosine

Patient I.D.	BvP
Age	37
Ethnic Group	Caucasian
HIV-positive date	1991
Reason for testing	Antenatal screening
Transmission category	Intravenous drug use
Serum CD4 at entry to study	600 mm^3 (Asymptomatic disease)
Medication	Nil

Patient I.D.	FW
Age	32
Ethnic Group	Black African
HIV-positive date	1984
Reason for testing	Pre- in vitro fertilisation treatment
Transmission category	Heterosexual intercourse
Serum CD4 at entry to study	590 mm^3 (Asymptomatic disease)
Medication	Nil