RESISTANCE TO HIV-1 INFECTION AMONG A GROUP OF CONTINUOUSLY EXPOSED WOMEN

BY

KEITH R. FOWKE

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RESISTANCE TO HIV-1 INFECTION AMONG A GROUP OF CONTINUOUSLY EXPOSED WOMEN

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT:

Despite intense sexual exposure to the human immunodeficiency virus type 1 (HIV-1) a small group of women within the Nairobi Prostitute Cohort Study have remained persistently HIV-1 seronegative for at least 3 years. The goal of this project was to determine if these women are resistant to HIV-1 infection and if so, by what mechanism(s). HIV-1 gene amplification confirmed that the majority (91%) of those women were truly uninfected. Survival analysis modelling of the time to seroconversion of all initially seronegative prostitutes suggested that statistically these women should be infected. Multivariate analysis suggested that HIV-1 exposure factors (condom use and the number of sex partners) and acquisition co-factors (sexually transmitted diseases) could not account for the inverse relationship between exposure to HIV-1 and risk of infection. Considering that statistically these women should have been infected but were not, and that differences in HIV-1 exposure or acquisition co-factors could not account for this lack of infection, it was concluded that these women were resistant to HIV-1 infection. What was the mechanism of resistance? Analysis of the cDNA for the cellular receptor for HIV-1, CD4, and *in vitro* HIV-1 infection of peripheral blood mononuclear cells suggested that the resistance to infection was not at the level of the susceptible cell. HLA class I analysis showed that two alleles in particular, A69 and B18, and the general rarity of the HLA haplotype were significantly associated with remaining seronegative. Cellular immune responses to HIV-1 (cytokine production in response to HIV-1 antigens and HIV-1 specific cytotoxic response) were present in resistant women but not low risk seronegative controls. A small group of women within the Nairobi Prostitute Cohort may to be resistant to HIV-1 infection. Evidence from this study suggest resistance is not at the level of the susceptibile cell but may involve cellular immune responses.

DEDICATION:

I would like to thank my wife Colette Fowke, who more than any other, has given me the strength and confidence to believe in myself. Without her support, this thesis would not have been possible.

I would like to also thank my parents Stan and Edna Fowke for their constant encouragement and love.

Colette, Mom and Dad, I dedicate this thesis to you.

ACKNOWLEDGEMENTS:

Since no researcher is an island unto him/her self, I would like to thank a number of people without whose help this thesis would not have been possible.

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Finally, a special thank you to the women of the Nairobi prostitute cohort whose spirit of co-operation is fundamental to the hope we place in research.

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A. Overview:

It has been estimated that by the year 2000 the human immunodeficiency virus type 1 (HIV-1), the etiological agent of the acquired immune deficiency syndrome (AIDS), will have infected 40-110 million people worldwide (Greene WC, 1993). With current therapies, most of those infected will develop AIDS within ten years and die within two additional years (Rutherford GW, et al. 1990; Hessol NA, et al. 1994). Deaths due to HIV-1 will have dramatic economic and social effects on endemic regions, most of which are developing countries. Since the most economically productive portion of the population (ages 15-49) has the highest prevalence of HIV-1 infection (DeCock KM, et al. 1990), it has been estimated that in some areas of Africa, 15-20% of the labour force will die of AIDS (Anderson RM, et al. 1988). The social impact of the AIDS epidemic in Africa will be unparalleled including an estimated 10 million orphans (Anderson RM, et al. 1988). Despite wide spread public health education and control programs, the rate of viral spread worldwide has continued to increase, especially in Southeast Asia (Blattner WA, 1991; Merson MH, 1993). The continuing spread of HIV-1 in poorer countries emphasizes the need for effective prevention and treatment strategies. Scientists are currently debating the antigenic nature of a vaccine and which portion of the immune system it should activate. A natural model of immunity to HIV-1 infection would provide direction for the development of an effective vaccine. In Nairobi, Kenya we have followed a group of prostitutes since 1985 (Plummer FA, et al. 1991; Simonsen JN, et al. 1990) and have observed that, despite intense levels of sexual

exposure to HIV-1, some have remained persistently seronegative for up to nine years. This thesis addresses the following questions: are these women resistant to HIV-1 infection and if so what is the mechanism of that resistance?

B. The Discovery of AIDS and the Etiological Agent:

In 1981 the Centers for Disease Control (CDC) in the United States reported a cluster of five cases of *Pneumocystis carinii* pneumonia (PCP) in healthy men (CDC, 1981). By the end of 1982 over 800 cases of PCP or other conditions associated with immunodeficiency had been reported (Kaposi's sarcoma, mucosal candidiasis, disseminated cytomegalovirus infection and chronic perianal herpes simplex virus ulcers). Initial cases were predominantly among homosexual men or intravenous drug users (Gottlieb MS, et al. 1981; Masur H, et al. 1981; Siegal FP, et al. 1981) , however, by the end of 1982 unexplained immunodeficiency related conditions were also found among Haitian immigrants (CDC, 1982), haemophiliacs, transfusion recipients, sex partners of risk-group members and children born to mothers at risk (Kreiss JK, et al. 1985; Chamberland ME, et al. 1984). A common feature among all of these cases was a decreased *in vitro* T cell response to mitogens and antigens. The epidemiological evidence suggested that the etiological agent was infectious and was spread via blood, blood products or genital secretions. The first identifications of a human retrovirus (which was termed lymphadenopathy associated virus LAV) isolated from a patient with AIDS was by Luc Montagnier's group in 1983 (Barre-Sinoussi F, et al. 1983; Chermann JC, et al. 1983). In 1984 Robert Gallo (Gallo RC, et al. 1984; Popovic M, et al. 1984)

and Jay Levy (Levy JA, et al. 1984) also isolated a retrovirus (which they named human T-lymphotrophic virus type III, HTLV-III and AIDS-associated retrovirus, ARV, respectively) from individuals with AIDS. With increasing epidemiological evidence and the acceptance that retroviruses often have extremely long clinical latency periods, it became generally accepted that this human retrovirus (renamed human immunodeficiency virus type 1, HIV-1) was the etiologic agent of AIDS. In 1986 a similar virus (termed HIV-2) was isolated in West Africa (Clavel F, et al. 1986) and shown to cause AIDS, although the virus was less infectious and clinical latency was longer (Marlink R, et al. 1994). The most important scientific accomplishment in dealing with the AIDS pandemic was the development of a serological test to determine who was infected (Sarngadharan M, et al.1984).

C. Transmission of HIV-1 and the Disease AIDS:

HIV-1 is transmitted horizontally through blood and blood products (transfusions, clotting factors and contaminated intravenous needles), genital secretions (sexual intercourse) and vertically from mother to child *in utero*, during delivery or by breast milk (Goedert JJ, et al. 1988; Rossi R., 1992). Although the earliest cases of AIDS in the U.S. were predominantly found among homosexual men, today the virus is primarily transmitted through heterosexual contact (DeVincenzi I., 1994). Protection against the acquisition of HIV-1 is increased by the screening of blood donors, the use of condoms during sex, and the use of clean needles. If a pregnant women is infected, administration of 3'-azido-3'-deoxythymidine (AZT) during pregnancy as well as the avoidance of breast

feeding, if practical, may reduce the risk of transmitting the virus to the child.

After infection with HIV-1, many patients (50-70%)(Pantaleo G, et al. 1993b) experience an acute infection which is characterized by mononucleosis-like symptoms (fever, rigors, malaise, anorexia, nausea, swollen glands or sore throat) that persist for a week or two before disappearing completely (Ho DD, et al. 1985). This acute infection occurs before a humoral immune response to HIV-1 can be generated (Cooper DA, et al. 1987). The time from infection until antibodies can be detected is called the "window" period. During the window period, the level of virus in the blood (viraemia) starts to increase (Cooper DA, et al. 1987). For this reason, the window period represents a significant problem for blood banks. Virus levels continue to rise until cellular and humoral immune responses develop and viraemia declines, often below the level of detection using the most sensitive methods. This is followed by clinical latency, which is the time that HIV-1 infected patients display no overt symptoms or signs of disease. The clinical latency period can last from months to years with a median of 8-10 years in industrialized nations (Bacchetti P, et al. 1989) and only 3.5 years among women of the Nairobi Prostitute Cohort (Anzala OAO, et al. 1994). The predominant marker of HIV-1 infection is the gradual loss of CD4+ T lymphocytes from the peripheral circulation, which may account for the subsequent immunodeficiency and opportunistic infections. Pantaleo G, et al. have hypothesized that the structural integrity of the lymph node decreases gradually until it can no longer filter virus out of the blood and viraemia once again increases. The definition of AIDS has evolved to include three requirements a) the presence of HIV-1 or HIV-2 antibodies, b) less than 200 CD4+ T

lymphocyte per ml blood and c) a least one AIDS defining illness. AIDS defining illnesses include *Pneumocystis carinii* pneumonia (PCP), mucosal candidiasis, disseminated cytomegalovirus infection, chronic perianal herpes simplex virus ulcers, tuberculosis, Kaposi's sarcoma, and neurological manifestations such as dementia. Death is characterized by severe wasting and an eventual overwhelming of the immune system by an opportunistic infection (Chaisson RE, et al. 1990).

D. HIV-1 the Virus:

HIV-1 is a member of the retroviridae family of viruses and was the third human retrovirus discovered. Human T lymphotrophic virus types I and II were discovered earlier by Gallo and colleagues (Poiesz BJ, et al. 1980; Kalyanaraman VS, et al. 1982). Retroviruses are characterized by two identical RNA genomes that are transcribed into DNA by a virally encoded RNA dependent DNA polymerase termed reverse transcriptase. The genome of retroviruses contain at least three genes *gag*, *pol* and *env*. The virion itself is about 100 nm in diameter and enveloped. There are three subfamilies of retroviruses, the Oncovirinae (eg. HTLVs), the Lentivirinae (eg. HIVs (Chiu I-M, et al. 1985)) and the Spumavirinae (eg. simian foamy virus). Lentiviruses are characterized by having extremely long incubation periods before disease onset (clinical latency). They also have complex genomes that include additional genes other than *gag*, *pol* and *env*, many with regulatory functions. HIV-1 has six additional genes that will be described in detail later.

HIV-1 is 110 nm in diameter and contains an outer lipid membrane that it

acquires from the cell during budding. The lipid membrane not only contains HIV-1 proteins but also carries cellular proteins such as MHC class I and II proteins (Arthur LO, et al. 1992). Like all other retroviruses, HIV-1 has three major structural genes (*gag*, *pol*, and *env*) that produce proteins which are assembled to form a mature virion. The *env* gene produces two glycosylated proteins, gp41, which inserts itself into the lipid membrane, and gp120, which forms a spike on the outside of the virus and is non-covalently bound to gp41. The *gag* gene product is translated as a single polyprotein that is cleaved into four smaller nucleocapsid proteins (p17, p24, p9 and p7) after budding from the infected cell. The nucleocapsid is composed of an inner and outer protein core. The outer core is composed of the myristylated matrix protein p17 that binds to the inner surface of the envelope. The inner core consists of three proteins; p24, p7 which binds directly to the RNA genome, and p9. These components are termed the nucleocapsid core. Also found within the inner core are the products of the *pol* gene; reverse transcriptase (RT), protease and integrase. These proteins cleave viral polypeptides (protease), transcribe the RNA genome into DNA (RT) and then integrate the DNA provirus into the chromosome of the newly infected cell (integrase). HIV-1 has six additional non-structural genes; *vif* (viral infectivity factor), *vpr* (viral protein R), *vpu* (viral protein U), *tat* (trans-activator of transcription), *rev* (regulator of virion expression), and *nef* (negative effector function). *Vif* increases viral infectivity (Strebel K, et al. 1987), *tat* regulates viral transcription (Arya SK, et al. 1985), *rev* regulates viral mRNA splicing (Feinberg MB, et al. 1986), and *nef*'s function as a regulator of transcription (Luciw PA, et al. 1987) is not observed in all cell types tested (Hammes

SR, et al. 1989). Although the exact mechanism of action is unknown, *vpr* may play a transcriptional regulatory role (Cohen EA, et al. 1990). *vpu* is thought to be important in viral assembly and/or release and may function through an intracellular association with the CD4 molecule (Klimkait T, et al. 1990).

E. Species Specificity:

Humans are the only known species that after infection with HIV-1 disease develops. Chimpanzees can be infected but they do not develop significant immunological abnormalities or disease (Desrosiers RC, et al. 1987). The only experimental animal models are immunodeficient mice reconstituted with aspects of the human immune system (SCID/HU mice) (McCune JM, et al. 1988; Aldrovandi GM, et al. 1993), and other mammals (cats and macaques) that can be infected by their own retroviruses (feline immunodeficiency virus, FIV, and simian immunodeficiency virus, SIV, respectively) which may cause a disease similar to AIDS (Gardener MB, et al. 1989; Bennett M, et al. 1992; Miller CJ, et al. 1989).

F. The Life Cycle:

The initial events in the life cycle of HIV-1 are to bind to a receptor and enter the susceptible cell. Although alternative cellular receptors for HIV-1 have been identified (Bhat S, et al. 1991; Schneider-Schaulies J, et al. 1992) the CD4 molecule has the highest affinity, (10^{-9}M), and is believed to be the major receptor (Deen K, et al. 1988). CD4, a 55 kilodalton (kDa) member of the immunoglobin supergene family, consists of

an N-terminal extracellular region containing a leader sequence and four immunoglobin-like domains, a transmembrane region, and a cytoplasmic region that is highly charged and known to interact with the intracellular tyrosine kinase $p56^{lck}$ (Maddon PJ, et al. 1987; Veillette A, et al. 1988). CD4 is found in high concentrations on T lymphocytes but is also found at lower levels on macrophages (Levy JA, 1993). The first experimental evidence suggesting that the CD4 molecule was the major cellular receptor for HIV-1 originates from the observation that a non-susceptible CD4- human cell line (HeLa) was made susceptible to HIV-1 infection by the transfection of a plasmid containing the CD4 cDNA (Maddon et al. 1986). CD4 amino acid residues 41-55, a region homologous to complementary determining region two (CDR2) of immunoglobin light chains, are responsible for the high affinity binding to gp120 (Arthos J, et al. 1989). The region of gp120 that interacts specifically with CD4 is in the fourth conserved region near the carboxy terminus (amino acid residues 413-447) (Lasky LA, et al. 1987; Pollard SR, et al. 1991). Although the interaction with CD4 is specific, it is not sufficient to permit entry of the virus into a cell. The requirement for a second surface protein was first suggested by the observation that transfection of a CD4 plasmid into a mouse cell would not render it susceptible to HIV-1 infection (Maddon PJ, et al. 1987). Recently, CD26 has been suggested as this second necessary molecule (Callebaut C, et al. 1993), however, this has been disputed (Broder CC, et al. 1994; Patience C, et al. 1994; Camerini D, et al. 1994; Alizon M, et al. 1994). One hypothesis suggests that after binding to CD4, gp120 undergoes conformational changes that result in the cleavage of the third variable region (V3). This cleavage exposes the fusogenic region of gp41 to

a cellular fusion protein (CD26?) and membrane fusion is initiated, resulting in the entry of the viral core into the cytoplasm of the cell (Sattentau QJ, et al. 1991; Gallaher WR, 1987). Once inside the cell viral reverse transcriptase (RT) transcribes the viral RNA genome into a single strand of DNA. However, the fidelity of this process is low and the misincorporation rate is thought to be 1 in 10,000 base pairs. It is this high error rate that gives HIV-1 its immense sequence diversity, especially in the envelope region (Coffin JM, 1990). After reverse transcription, the RNAse H function of RT digests the RNA from the RNA/DNA hybrid allowing RT to complete the complementary DNA strand. Viral integrase then inserts the DNA copy of HIV-1 into a random location on the chromosome (Vaishnav Y, et al. 1991). HIV-1 is now in its proviral form and may remain latent until the cell becomes activated. After activation, cellular and viral transcriptional regulatory proteins initiate transcription with RNA polymerase II. The transcriptional regulation of HIV-1 is extremely complex and involves viral and cellular transcriptional regulators. An in depth description is beyond the scope of this review. However, a brief description of the functions of the viral regulatory protein is necessary to appreciate the complexity of the regulation in this virus.

Tat protein is essential for transcription of HIV-1 and achieves this by binding to a 5' region of the transcript called the *trans*-activation response (TAR) element. Tat serves to destabilize a stem loop structure that, in the absence of Tat, impedes RNA polymerase II elongation. Full length viral transcripts are translated into structural proteins while multiply spliced viral transcripts are translated into regulatory proteins. The Rev protein functions by binding to a region of the HIV-1 transcript (Rev responsive

element, RRE) and removing it from the nucleus before splicing takes place thus increasing the amount of structural protein translated. Cellular factors are also important in the regulation of viral transcription (NF-KB, Sp1, HIVEN 86A, NFAT-1, and AP-1) (Vaishnav Y, et al. 1991). The function of Nef is not known, however, some have postulated that its role is in establishing viral latency (Luciw PA, et al. 1987; Folks TM, et al. 1992). If latency is not established viral transcription by the above factors is initiated.

After transcription of full length viral mRNA, the structural proteins are translated. The *gag* gene product is the 55 kDa capsid polyprotein that is proteolytically cleaved into its constituent parts (p17, p24, p9 and p7) during viral budding. The viral genome (unspliced viral mRNA) is included in the virion by a specific interaction between its 5' end and the capsid polyprotein (p9) which contains the RNA packaging or PSI sequence (Aldovini A, et al. 1993). The *pol* gene products are produced from the same mRNA species that produce the core polyprotein. The *gag* and *pol* gene products are in two separate translational reading frames. When read in the *gag* frame stop codons at the 5' end of the *pol* gene terminate translation. However, at the 3' end of the *gag* mRNA a ribosomal frame shift event takes place (estimated frequency 5%), as a result of a ribosomal slippage sequence (AAAUUUUAU) that causes the ribosome to start reading the -1 reading frame (relative to *gag*) thus permitting the complete translation of the 160 kDa *gag-pol* polyprotein (Wilson W, et al. 1988; Jacks T, et al. 1988). This mechanism results in the production of more capsid proteins than the enzymatic proteins of the *pol* gene (20:1). Both the *gag* and *gag-pol* polyproteins are

closely associated with the cell plasma membrane by virtue of an interaction between the cell membrane lipid and the highly hydrophobic myristillated amino terminal p17. The product of the *env* gene is a glycosylated 160 kDa polyprotein that is cleaved in the golgi apparatus generating gp41 and gp120. The gp41 protein is inserted into the plasma membrane and gp120, non-covalently associated with gp41, extends from the membrane like a spike. The complex of *gag, gag-pol* polyproteins and viral RNA assemble into a closed spherical particle which buds through the gp41/gp120 studded plasma membrane. During the budding process the viral polypeptides are cleaved into the individual proteins (p17, p24, p9, p7, protease, reverse transcriptase and integrase) by the viral protease and assemble into the core proper. This mature viral particle is now capable of infecting a susceptible cell (Vaishnav Y, et al. 1991).

G. The Immune Response to HIV-1 Infection:

Gi. Humoral Immune Response:

After infection by HIV-1, the vast majority of individuals respond by developing a humoral immune response that is detected by current enzyme immunoassay (EIA) systems (Pan L-Z, et al. 1991; Bruisten SM, et al. 1992; Lee T-H, et al. 1991; Yerly S, et al. 1991; Nielsen C, et al. 1991; Willerford DM, et al. 1993). HIV-1 specific humoral IgM and IgG antibodies usually develop within 1-3 and 2-6 weeks post infection, respectively (Tindall B, et al. 1991). Many reports have described antibodies which were capable of neutralizing HIV-1 infection *in vitro* (Levy JA, 1993). Six main neutralization sites have been identified, five in gp120 and one in gp41. The best known neutralization

site is at the crown of the third variable region (V3) of gp120 (amino acids 308-322) which is also known as the principal neutralizing domain (PND). In general the V3 loop is highly variable, however, the PND portion is highly conserved among many strains (Berman PW, et al. 1992). Neutralization directed at V3 does not interfere with CD4 binding but is thought to involve inhibition of proteolytic cleavage of V3 which may be important for conformational changes that allow gp41 to initiate membrane fusion (Sattentau QJ, et al. 1991). The CD4 binding region (amino acids 413-447) is also a major neutralization site and is thought to be conformationally dependent (Kang C-Y, et al. 1991; Steimer JS, et al. 1991). Strains with different V3 regions can be neutralized by the same antibodies directed against the CD4 binding region. Neutralizing antibodies can also be generated against non-protein portions, such as carbohydrate moieties of the glycosylated gp120. Their effect is thought to alter the conformation of gp120 thus interfering with binding or fusion events. The second conserved region and the second variable region of gp120 have also been reported to be sites of antibody neutralization (Levy JA, 1993). There have also been reports of neutralization sites on gp41 (Robinson WE Jr, et al. 1991) that are made more accessible after soluble CD4 binding. It has been hypothesized that the conformational alteration, after CD4 binding, is necessary for gp41 initiated fusion (Sattentau QJ, et al. 1991). HIV-1 specific serum antibodies are effective at neutralizing viral strains *in vitro* that were isolated from the same individual (autologous) at an earlier time point. They are also effective at neutralizing isolates from other individuals (heterologous). However, neutralizing serum antibodies are not effective against autologous virus isolated at the same time as the serum is collected (von

Gegerfelt A, et al. 1991). This observation draws into question the clinical significance of neutralizing antibodies. It has been hypothesized that HIV-1 is able to alter its envelope structure to escape detection by the immune system and the antibodies are always one step behind. Vaccination with a single gp120/160 molecule would theoretically protect only those individuals who came into contact with a virus that had a very similar envelope structure. It has been observed that individuals given a recombinant gp120 vaccine in efficacy trials have subsequently become infected by HIV-1, drawing into question the protective value of a humoral immune response to a single gp120 isolate. For this and other reasons, the large US clinical trials for recombinant gp120 have been cancelled (Cohen J, 1994).

Non-neutralizing antibodies may also be important in controlling HIV-1 infection. IgG antibodies that bind to gp120 and gp41 covered cells can be recognized by the Fc receptors (FcRIII or CD16) on the surface of natural killer cells (NK) or monocytes. These NK cells then lyse the infected cells through a mechanism similar to cytotoxic T cells (Yagita H, et al. 1992). Whether this antibody dependent cellular cytotoxicity (ADCC) is clinically important is not known, however, ADCC antibodies are present throughout the course of infection (Evans LA, et al. 1989) and, to date, have not been correlated with protection. In both ADCC and neutralization, the only target of the antibodies are the highly variable envelope proteins. This extreme variability makes the development of envelope based vaccines problematic.

Gii. Cellular Immune Response:

The cells of the cellular immune system are Natural Killer (NK) cells, CD4+ T lymphocytes (CD4+ T cells) and CD8+ T lymphocytes (CD8+ T cells). NK cells kill HIV-1 infected cells through a non-MHC restricted mechanism. The major mechanism of NK killing is thought to be ADCC-mediated (Abbas AK, et al. 1991). Two mechanisms of NK mediated ADCC are known. The major mechanism occurs when antibodies bound to HIV-1 envelope proteins, are recognized through Fc receptors on the surface of the NK cell. The second occurs when unbound anti-envelope antibodies first bind to the NK Fc receptor (FcRIII or CD16) then the complex recognizes and binds to an HIV-1 infected cell. In both cases the recognized cell is lysed by perforins, cytotoxins, serine esterases and proteoglycans (Abbas AK, et al. 1991). The level of ADCC specific antibodies does not correlate with disease progression, however, the function of the NK cell does decline as disease progresses (Fontana L, et al. 1986). This dysfunction can be reversed by the addition of interleukin type 12 (IL-12) which may suggest a role of regulatory type 1 helper cells (TH1) (Chehimi J, et al. 1992).

CD4+ T lymphocytes can direct the specific immune response by the release of specific cytokines (T helper function, TH) or they can become the effector cells and lyse specific targets. Although the majority of MHC restricted HIV-1 specific killing is CD8+ T cell mediated, there have been several reports of MCH class II restricted HIV-1 specific CD4+ T cells having direct cytotoxic effects on target cells (Orentas RJ, et al. 1990; Curiel TJ, et al. 1993).

In humans, the helper function of CD4+ T cells (TH) can be divided into two

types, as has been described in mice (Mosmann TR, et al. 1987). TH1 cells produce cytokines (interferon gamma (γIFN) and interleukin two (IL-2)) that enhance the cellular immune response. TH2 cells produce cytokines (IL-4, IL-6, and IL-10), that enhance humoral immune responses (Seder RA, et al. 1994). There is cross regulation within the cytokines (IL-4, IL-10 and IL-12) such that the predominance of one type of helper response will suppress the other. This is the basis for the hypothesis (Clerici M et al. 1993a) that a TH1 dominant cellular response is protective and after switching to a TH2 dominant antibody response, cellular immune responses are suppressed and the individual becomes susceptible to HIV-1 infection. Data to support this hypothesis come from several observations. TH1 responses are found predominantly among healthy asymptomatic HIV-1 seropositive individuals but this function is lost as disease progresses and a TH2 response dominates (Barcellini W, et al. 1994; Shearer GM, et al. 1991; Clerici M, et al. 1993c). This loss of TH1 function can be reversed *in vitro* by the addition of the TH1 cytokine IL-12 (Clerici M, et al. 1993b) or by the presence of antibodies against TH2 cytokines IL-4 and IL-10 (Clerici M et al. 1994c). Many groups have observed that HIV-1 exposed but uninfected individuals have TH1 CD4+ T cells that have immune memory to HIV-1 antigens, as measured by proliferation and IL-2 production. This observation has been made for gay men (Clerici et al. 1992), health care workers (Clerici M, et al. 1994b) , children born to HIV-1 seropositive mothers (Clerici M, et al. 1993; Borkowsky W, et al. 1990) and partners of HIV-1 infected persons (Kelker HC, et al. 1992). Although there is much support in the literature, some groups are not convinced that there is a TH1 to TH2 switch during HIV-1 disease

progression (Graziosi C, et al. 1994; Maggi E, et al. 1994). Considering the degree of exposure to HIV-1 in the seronegative groups was fairly low and the risk of HIV-1 infection per exposure is far less than 1.0, it is not known if the TH1 immune response is protective or if the exposure was simply insufficient to establish an infection. A study of TH1 immune responses among individuals so highly exposed to HIV-1 that they should be infected would provide a better correlate of protection. Additional evidence supporting the protective effects of a TH1 response over a TH2 response comes from animal studies. Mice vaccinated with low subinfectious dose of an infectious pathogen (*Leishmania major*) produce a TH1 response while high doses of the pathogen result in TH2 responses. Subsequent challenge by high doses of the live pathogen show that only the low dose TH1 mice were protected (Bretscher PA, et al. 1992). Recently these experiments have been repeated in macaques (Clerici M, et al. 1994). Infection with low subinfectious and high infectious doses of simian immunodeficiency virus (SIV) result in the animals developing TH1 or TH2 responses, respectively. After subsequent challenge with high doses of live SIV, only those exposed to low dose were protected. Additional evidence for CD4+ mediated cellular response in the absence of antibody production has recently been described for another human retrovirus HTLV-I (Nishimura M, et al. 1994). CD4+ T cells provide important help to the two effector arms of the immune response (cellular and humoral) and themselves have the ability to become effectors.

Like the CD4+ T cell, CD8+ T cells have two distinct functions in HIV-1 infection. The first is the ability of CD8+ T cells to repress HIV-1 replication without lysing the infected cell (Walker CM, et al. 1986; Mackewicz C, et al. 1992). A soluble

factor, that has not been identified, is thought responsible for the inhibition (Walker CM, et al. 1989). The second activity of CD8+ T cells is to directly kill HIV-1 infected cells in a MHC class I restricted manner. CD8+ cytotoxic T cells (CTL) achieve killing by either the perforin pathway that lyse the cell by putting holes in the membrane, or the recently discovered *fas* pathway which induces programmed cell death (apoptosis) and results in chromosomal breakdown and membrane permeability (Kojima H, et al. 1994). CTL activity is known to be very important in the clearance of certain viral infections such as lymphocytic choriomeningitis virus (LCMV) and influenza A virus (Byrne JA, et al. 1984; Lin YL, et al. 1981). The first HIV-1 specific CTL activity was described in 1987 (Walker BD, et al. 1987; Plata F, et al. 1987). The HIV-1 antigens presented to the CD8+ CTL are in the form of peptides, about 9 amino acids long, that are bound in the peptide binding grove of MHC class I proteins on the surface of the infected cell (Falk K, et al. 1991). Since the HIV-1 antigens are produced from the proteolytic cleavage of any internally produced protein (endogenous pathway) non-surface exposed proteins of the HIV-1 virus can become targets for CTL activity. This provides a tremendous advantage over antibodies which can only functionally interfere with virus binding and fusion (gp120 and gp41). CTL epitopes have been described from *gag*, *pol*, *env*, *nef*, and *vif* encoded proteins (Nixon DF, et al. 1992). Despite the general immunosuppression associated with HIV-1 infection, anti-HIV CD8+ CTL activity is exceptionally high. This activity is initially observed during acute infection and remains at a high level during the asymptomatic stage. Some groups feel the HIV-1 specific CTL activity declines with disease progression (Carmichael A, et al. 1993) , however, others

are able to detect HIV-1 specific CTL activity even at an advanced disease stage when the number of CD4+ T cells is low (Grant MD, et al. 1992). HIV-1 is the only known infection that the CTL response can be detected in fresh PBMC without the need for *in vitro* restimulation step (Walker BD, et al. 1994; Grant MD, et al. 1992). In addition to the TH1 vs TH2 hypothesis, evidence that cellular immune responses may be important in preventing HIV-1 infection comes from the acute stage of HIV-1 infection. At this time the level of viraemia increases and then declines drastically with the onset of humoral and cellular immune responses. David Ho has evidence to suggest that cellular immune responses develop before neutralizing antibodies and are associated with the decline in virus levels (reviewed by Walker BD, 1994). Cellular responses may therefore control HIV-1 during the asymptomatic period until CD4+ cell number declines and immune dysfunction sets in or until the virus escapes CTL detection (Phillips AN, et al. 1991). There is suggestive evidence that CTL may be protective against HIV-1 infection. Recently, the presence of HIV-1 specific CTL in HIV-1 exposed but uninfected individuals has been demonstrated. Two groups (Cheynier R, et al. 1992; Rowland-Jones, et al. 1993) reported the identification of HIV-1 specific CTL in uninfected children born to HIV seropositive mothers. The children were shown to be uninfected by HIV-1 by the sensitive polymerase chain reaction (PCR). Another study has shown that the regular sex partners of HIV infected persons had HIV-1 *nef* specific CTL although they were seronegative and PCR negative (Langlade-Demoyen P, et al. 1994). However, much like the CD4+ studies, the degree of exposure to HIV-1 is not known and therefore it is difficult to differentiate between protection and insufficient

exposure to cause an infection.

In addition to CD4+ MHC class II and CD8+ MHC class I restricted CTL there also is an HIV-1 envelope specific non-MHC restricted cytotoxic response (McChesney M, et al. 1990).

Giii. Allo Immune Responses and HIV-1:

Individuals can become exposed to non-self HLA antigens by maternal exposure to fetal blood, transfusions, organ transplantation or exposure to sperm (Mavligit GM, et al. 1984). Molecular analysis has demonstrated portions of HIV-1 envelope proteins share sequence similarity with HLA antigens suggesting the presence of molecular mimicry (Dorak MT, et al. 1994; Hounsell EF, et al. 1991). This similarity has resulted in antibodies raised against the non-self HLA antigens (allo antigens) cross reacting with envelope proteins (Golding H, et al. 1989; Young JAT, 1988; Bjork RL Jr., 1991; Lopalco L, et al. 1993). In the macaque system, immunization with whole human cells has been shown to provide protection from subsequent challenge of SIV (Stott EJ, 1991). The protection was correlated with antibodies to HLA class I antigens on the human cell line but only if the challenge SIV was grown in the same human cell line as that used as a vaccine (Chan WL, et al. 1992). HIV-1 cross reacting cellular allo immune responses have also been described (Clerici M, et al. 1993d). These cross reacting allo responses are particularly interesting considering the envelope of HIV-1 contains cellular proteins including HLA class I and II proteins (Arthur LO, et al. 1992; Meerloo T, et al. 1993). An HIV-1 cross reacting allo response has the potential of recognizing HLA molecules

on both the infecting cell and free virus. Alloimmunization has even been suggested as an AIDS vaccine (Shearer GM, et al. 1993). Conversely, if HIV-1 induced antibodies recognize self HLA antigens, the potential exists for those cells to be eliminated by ADCC or their function blocked and result in immune dysfunction.

Currently it is not known whether these immune responses are important in preventing the acquisition of HIV-1 infection or disease progression (Walker BD, 1994). Until protective immune responses to HIV-1 are characterized, vaccine development will be based on an inadequate scientific and biologic foundation.

H. The HLA Antigens and HIV:

Class I and II genes of the major histocompatibility complex (MHC) encode the human leukocyte antigens (HLA). These genes are the most polymorphic genes within the human genome and the HLA proteins are essential for specific immune recognition. The function of the HLA molecules is to bind to peptides of proteolytically cleaved self and foreign proteins and present them to T lymphocytes of the immune system (Abbas AK, et al. 1991). Class I HLA antigens present peptides to CD8+ T lymphocytes and class II HLA antigens present peptides to CD4+ T lymphocytes. T lymphocyte education in the thymus ensures that only the foreign peptides are recognized, except in cases of autoimmunity. There are three types of HLA class I proteins designated A, B and C and three main types of HLA class II proteins called DR, DQ, and DP. Each of these six HLA genes is polymorphic and may have as few as five (HLA DP) or as many as 59 (HLA B) alleles (Abbas AK, et al. 1991). The number of alleles has been defined

serologically but with highly sensitive genetic screening methods the number of alleles is growing continuously. Each one of these alleles binds to a different group of peptides defined by specific anchor residues. For this reason, two cell populations with different HLA haplotypes will present two different sets of peptides to their T cells. Geneticists have argued that this varying ability to present different portions of the same protein to the immune system ensures that all individuals in a population do not respond in the same manner to the same pathogens. This may provide a selective advantage to the population as a whole (Doherty PC, et al. 1975). An example of an infectious pathogen that has exerted selective pressure on the polymorphism of the HLA is malaria. Hill AVS, et al., 1991 have described particular HLA antigens (HLA Bw53 and the DRB1*1302-DQB1*0501 haplotype) whose frequencies are highly associated with a lack of severe malarial disease. The authors suggest that these alleles are associated with less severe infection because the HLA antigens they encode are able to present particular *Plasmodium falciparum* (the etiological agent of malaria) epitopes to CD8+ CTL and CD4+ helper cells that result in a more efficient cellular immune response, hence significantly reducing the severity of disease and increasing survival. The associated alleles are common in West Africa but rare in parts of the world where malaria is not endemic. The authors conclude this supports the view that the extreme polymorphism seen in the MHC class I and II genes is the result of selective pressure from infectious pathogens.

HLA antigens are not only associated with infectious pathogen but also with autoimmune diseases (Lepage V, et al. 1993). Insulin dependent diabetes mellitus

(IDDM), rheumatoid arthritis (Paul WE, 1993) and ankylosing spondylitis (Ebringer H, 1990) have been associated with HLAs DR3-DR4, DR1-DR4-DR6, and B27, respectively. Although the HLA association between these and many other autoimmune diseases have been determined, the molecular mechanisms of HLA involvement is still largely unknown.

Due to the potential for variable immune responses from different HLA alleles, the role of HLA in HIV-1 disease has been investigated. Much like the malaria association, researchers first determined if there was an HLA association with progression to disease in HIV-1 infected individuals. It was observed among Edinburgh haemophiliacs given a single lot of HIV-1 contaminated factor VIII or IX, that there was a differential rate to HIV-1 disease (Simmonds P, et al. 1988). Simmonds and co-workers HLA typed HIV infected haemophiliacs to investigate the role of HLA on disease progression. They found that a particular haplotype (A1, B8, DR3) was associated with rapid progression to disease (Steel CM, et al. 1988). This haplotype has also been associated with disease progression in a cohort of American homosexual men (Kaslow RA, et al. 1990). Other groups have found associations between specific AIDS defining illnesses and HLA haplotypes. DR5 showed a strong association with the development of Kaposi's sarcoma (De Paoli P, et al. 1986; Pollak MS, et al. 1984; Prince HE, et al. 1984) and lymphadenopathy syndrome (Jeannet M, et al. 1989; Enlow RW, et al. 1983; Raffoux C, et al. 1987) especially in people of Mediterranean or Ashkenazi Jewish backgrounds. The B35, CW4, DR2 haplotype and DR3 allele have been associated with opportunistic infection in patients with AIDS (Jeannet M, et al 1989;

Pollack MS, et al. 1984). HLA B35 and DR1 have been independently associated with disease progression (Itescu S, et al. 1991; Jeannet M, et al. 1989; Mann DL, et al. 1988).

Several studies have investigated HLA associated susceptibility to infection by HIV-1. Some have not found associations (Jeannet M, et al. 1989; Enlow RW et al, 1983; Steel CM, et al. 1988) while others have. There was a positive association between seroconversion and children (born to HIV-1 infected mothers) who possessed certain HLA DPB1 alleles, ie. susceptible alleles. These alleles were defined by asp-glu-ala-val amino acids at positions 84-87. There was a negative association with HIV-1 infection and the HLA DQA1 allele 0102, ie. a protective allele. The presence of susceptible alleles and the absence of the protective allele resulted in a much higher risk of infection, odds ratio 16.25 (Just J, et al. 1992). In an Italian study of 61 haemophiliacs, 50 heterosexual partners of HIV-1 seropositive individuals and 36 children born to HIV-1 positive mothers HLA B52 and B44 were associated with resistance to infection with HIV-1 while B51 was associated with susceptibility to infection (Fabio G, et al. 1992). The mechanism of HLA associated resistance to HIV-1 infection or disease progression remains unknown. Due to the biological function of the HLA antigens, to present foreign peptides to the immune system, it is possible that particular HLA antigens are better able to present protective epitopes to the immune system. However, it is also possible that the HLA antigen is acting as a marker for a protective factor, such as complement proteins or cytokines which are in linkage disequilibrium with the HLA allele (Cameron PU, et al. 1990; Jacob CO, et al. 1990).

Many apparently unrelated HLA antigens are involved in the HLA associations with susceptibility to infection and progression to disease. This is not surprising since the frequencies of HLA alleles found among various populations varies greatly (Paul WE, 1993). It has been suggested that the immune system applies selective pressure on viral isolates such that those not recognized become predominant (Callahan KM, et al. 1990; Phillips RE, et al. 1991). If the HLA allele frequencies vary among populations, it can be hypothesized that over time the viral isolates will evolve to survive among the predominant HLA alleles of that population. Theoretically, a given HLA allele may be able to present an effective CTL epitope from one sub-species of HIV-1 but a geographically separate sub-species may not contain that epitope and, therefore, the presence of that HLA allele would not be protective. Regardless of the mechanism, it seems clear that susceptibility to HIV-1 infection or disease progression is at least in part controlled by HLA associated factors or the HLA antigens themselves.

I. Examples of Resistance in Other Systems:

There are many other examples of resistance to infectious agents. Some of the resistance mechanisms are natural or innate while others are learned or acquired through the immune system. Three innate resistance mechanism have been well characterized in the murine system.

The first example of resistance has recently been described in mice for *Mycobacterium species* (Vidal SM, et al. 1993). Inbred strains of mice often differ in their susceptibility to infection by *Mycobacterium sp.* The resistance trait has been

mapped to a dominant locus on chromosome 1, *Bcg*, and has been shown to function in the macrophage early in infection (Skamene E, et al. 1991). A 400 kbp portion of the genome known to include *Bcg* contained six open reading frames. One of the open reading frames, *Nramp*, expressed solely in macrophages, was the leading candidate for the resistance gene. *Nramp* is an integral membrane protein that shares structural homology with other known transport systems. The association between *Nramp* and resistance was strengthened because susceptible strains of mice all possess a gly→asp change at position 105. Resistance to infection by *Mycobacterium sp.* seems to be controlled by a membrane transport protein in the susceptible cell, the macrophage. Williams LM, et al. have described differential susceptibility of CD4+ lymphocytes from separate individuals to infection by HIV-1 but only at low multiplicities of infection.

The second example of innate resistance in the murine system involves the retrovirus murine leukemia virus (MuLV) which causes murine acquired immunodeficiency syndrome (MAIDS) (Hartley JW, et al. 1989). Variability to infection by MuLV can be controlled at the level of the susceptible cell, the CD4+ T lymphocyte. This genetically inherited trait is polymorphic and has been mapped by linkage analysis and has been called *Fv-1*. It is believed that *Fv-1* prevents the cell from becoming infected by not allowing proper integration of the proviral form of the virus (Pryciak PM, et al. 1992). Williams LM, et al. 1991 have observed genetically inherited resistance patterns at low multiplicities of infection of HIV-1 in CD4+ lymphocytes. They suggest the existance of a human equivalent of the mouse *Fv-1* gene, but to date have not offered a candidate gene.

The final example of innate resistance is also from a mouse retrovirus, the mouse mammary tumour virus (MMTV). MMTV exists as an exogenous infectious virus transmitted through breast milk and exists in proviral form in the germ line and is passed on by classical Mendelian genetics. MMTV has been shown to encode a superantigen in the 3' long terminal repeat (LTR) region (Choi Y, et al. 1991). The minor lymphocyte stimulating (*Mls*) antigens have been known for a long time, but only recently have they been shown to be proviral form of MMTV (Frankel WN, et al. 1991). The function of the superantigen is to stimulate CD4+ T cells containing a specific variable region of the Beta chain of the T cell receptor (TCR Vß). This results in the activation of 5-10% of all CD4+ T cells, which is several orders of magnitude higher than antigen specific activation. The activated CD4+ T cells release cytokines, some of which cause B cells to proliferate. B cells are the natural target cell for MMTV and this superantigen activation results in the expansion of the provirus located within the activated B cells. With increased numbers of infected B cells the virus has a greater chance of reaching the mammary gland where it resides. In order for MMTV to achieve a successful life cycle the superantigen must amplify a specific subset of Vß CD4+ T cells. There are many different sub-types of MMTV, each with a superantigen that has a specificity for a different TCR Vß. Resistance to MMTV is achieved in baby mice that have *Mls* or the endogenous proviral MMTV. Held W, et al. have presented the following model to explain the resistance. In the fetal thymus the entire superantigen protein is produced in antigen presenting cells and will associate with MHC class II proteins. This association is different from classical antigen presentation because the

superantigen associates as a whole protein, not peptides, and the location of the binding to the MHC class II protein is not in the peptide binding groove. The MHC class II associated superantigen binds to those CD4+ T cells with the appropriate TCR Vß. The result is the deletion of all CD4+ T cells with TCR Vß that recognize the superantigen. After exposure to exogenous MMTV via the mother's milk, the virus can not establish a successful infection because there are no CD4+ T cells with the Vß chain that the superantigen recognizes, they have all been deleted in the thymus. This hole in the TCR Vß repertoire does not allow the superantigen to stimulate CD4+ T cells and, therefore, the MMTV infected B cells are not expanded and the infectious cycle is broken. However, this resistance is dependent on the endogenous and exogenous superantigen having the same Vß specificity. Infection with a MMTV with a superantigen of a different Vß specificity would result in completion of the MMTV life cycle.

There have been conflicting reports whether HIV-1 contains a superantigen and is deleting certain Vß T cells (Hodara VL, et al. 1993; Posnett DN, et al. 1993). If HIV-1 is shown to contain a superantigen this may partially explain the loss of CD4+ T cells and would suggest that persons lacking the particular Vß repertoire that HIV-1 requires to complete its life cycle could be resistant to infection. However, there is yet to be any convincing evidence that HIV-1 specifically requires CD4+ T cells with particular Vß TCR to complete its life cycle. Therefore the specific deletion of certain Vß bearing CD4+ T cells could not account for resistance.

Resistance to infectious agents can be achieved through the acquired immune response. Several examples of immune regulated resistance to infection have been

previously mentioned. Bretscher et al. and Clerici et al. have described that prior exposure to low subinfectious doses of *Leishmania major* and SIV, respectively, have resulted in a TH1 response and protection from subsequent challenges with high infectious doses of the pathogen. Stott et al. has described resistance to SIV infection mediated though a humoral response specific for human HLA molecules. Each of these examples of immune mediated resistance to infection depends on prior natural immunization to low doses of pathogen or immunizing antigen.

The evidence for resistance to HIV-1 in humans is less convincing. This is mainly because challenge experiments are not possible and any evidence must come from associations with lack of infection and a negative association is always difficult to prove. Farzandegan H, et al. have described a group of four HIV-1 seropositive homosexual men who had subsequently lost all detectable antibodies; seroreversion. Some of these individuals have also become HIV PCR negative and the authors suggest they may have cleared the infection. The phenomenon of seroreversion is controversial and, at best, is found at a very low frequency (Holmberg SD, et al. 1988). As described, HLA alleles have been associated with resistance to HIV-1 infection, however, the association is never absolute. Reports among highly HIV-1 exposed prostitutes (Fischl MA, et al. 1987) and homosexual men (Schechter MT, et al. 1986) indicate that the duration of exposure is inversely related to the likelihood of seroconverting. The best characterized of these studies is our own Nairobi prostitute cohort where it was observed that the duration of prostitution reduced the risk of seroconversion by an odds ratio of 0.39, CI 95% =0.23-0.65 $p < .004$ (Simonsen JN, et al. 1990). The mechanism of resistance in all of these

examples of human resistance to HIV-1 infection is unknown. The identification of protective immune responses among highly exposed yet uninfected individuals would be very significant and could be used to direct vaccine development.

J. Statement of Objectives and Approach:

The goal of this thesis work is to determine if the HIV-1-exposed but persistently seronegative women of the Nairobi prostitute cohort are resistant to HIV-1 infection and if so describe the mechanism of that resistance. The approach to be taken will be to 1) confirm the origin of the sera from the persistently HIV-1 seronegative women, 2) establish if the women are truly HIV-1 uninfected, 3) attempt to explain the resistance by statistical or epidemiological methods, 4) conclude whether there is resistance and if so, 5) determine if the resistance is innate or 6) determine if the resistance is acquired.

MATERIALS AND METHODS:

I. MATERIALS:

A. Source of Biological Material:

Female prostitutes attending the Majengo sexually transmitted disease (STD) clinic in the slum district of Pumwani in Nairobi, Kenya were enrolled into the Nairobi Prostitute Cohort and were the source of the biological material for this study (the Malaya (ML) study, Malaya is the KiSwahili word for prostitute) (Simonsen JN, et al. 1990; Plummer FA, et al. 1991). In 1985 the cohort was initiated with a baraza (a public community meeting) for prostitutes residing in Pumwani. At that meeting, the planned project was discussed with the women, and community representatives were elected to interact with the community and to act as community health workers. Enrollment criteria were that the women were Pumwani residents and earned money through prostitution. Free treatment of their STDs and general health care concerns were exchanged for their informed and willing participation in intervention programs and various research projects, one of which was this study (Simonsen JN, et al. 1990). Participation of the women was not only of benefit to the researcher but more importantly to the women themselves, who found treatment of their STDs through the Kenyan medical system cost prohibitive, inaccessible or often ineffective (Moses S, et al. 1992). Each woman, having been assigned a unique study number (a ML number), was interviewed on each visit to the clinic and were asked to return to the clinic on certain

dates for reassessment. A physical examination included vaginal speculum and bimanual examinations. The interviews collected basic demographic information and prostitution, sexual practice, medical, obstetric and contraceptive histories (Simonsen JN, et al. 1990). Sexually transmitted diseases were confirmed by culture of endocervical and genital ulcer swabs for *Neisseria gonorrhoeae*, *Haemophilus ducreyi*, and *Chlamydia trachomatis* cultures which were performed as described (D'Costa LJ, et al. 1985) and rapid plasma reagin test (RPR) and *Treponema pallidum* hemagglutination (TPHA) tests for syphilis. In the later years of the study, *C. trachomatis* was detected from endocervical secretions using an enzyme immunoassay (Chlamydiazyme). The presence of HIV-1 antibodies was detected using commercial kits described in detail later in Methods. The women averaged 2-4 visits to the clinic per year for reassessment and treatment of STDs. To date over 1800 women have been enrolled in the cohort.

HIV-1 seronegative individuals who were at low risk for acquiring HIV-1 infection (negative controls) were Winnipeg laboratory staff and Kenyan mothers attending an antenatal clinic (the maternal child health (MCH) study) (Datta P, et al., 1995).

B. General Chemicals:

Unless otherwise mentioned the sources of general chemicals were Fisher Scientific, Sigma Chemical Company, and Mallinckrodt Speciality Chemical Company.

C. Tissue Culture Solutions:

Lymphocyte Media (LM) - RPMI 1640 plus 10 mM HEPES and L-glutamine pH 7.2

(Gibco), 50μM ß-mercaptoethanol (Sigma), 10 units/ml Penicillin, 0.1mg/ml Streptomycin, .25μg/ml Amphotericin B, and 10 % fetal calf serum (Intergen) which had been heat inactivated at 56°C for 40 minutes.

Freezing Media (FM) - heat inactivated fetal calf serum (Intergen) and dimethyl sulfoxide (DMSO) at a 9:1 ratio (Sigma).

Phosphate Buffered Saline (PBS) - 137mM NaCl, 2.7 mM KCl, 10.1mM Na_2HPO_4, 1.76mM KH_2PO_4, pH 7.0, heat sterilized.

Tris Buffered Saline (TBS) - 144mM NaCl, 25mM Tris-HCl, pH 7.5.

T-TBS - TBS plus 0.5% Tween 20.

TMT/SS - 2% non-fat dry milk, 20% fetal calf serum and 0.5% Tween 20 in TBS

$T_{10}E_1$ - 10mM Tris-HCl 1mM EDTA pH 7.5

D. Reagents for Molecular Biology:

10x SSC - 1.5M sodium chloride, 0.15M sodium citrate

10% SDS (sodium dodecylsulfate) - 100g SDS, ddH_2O up to one litre.

20x SSPE - 3M NaCl, 172.5mM Na_2HPO_4, 20.0 mM EDTA, pH 7.5 heat sterilized.

SOC - 2 g tryptone, 0.5g yeast extract, 1ml 1M NaCl, 0.25ml 1M KCl, 1ml 2M $MgCl_2$, 1ml 2M $MgSO_4$, bring up to 99ml with ddH_2O and autoclave. Add to cooled media 1ml 2M glucose and filter sterilized.

LB broth - 10g tryptone, 5g yeast extract, 10g NaCl bring up to 1 litre and heat sterilized.

pBS plates - 4g tryptone, 2.5g yeast extract, 2.5g NaCl, 7.5 g agar, ddH_2O up to 500ml,

autoclave for 20 minutes at 121°C and allow to cool but not solidify (50°C). To make pBS plates add the following: 50mg ampicillin (final concentration of 100μg/ml), 20mg X-gal dissolved in 1 ml dimethyl formamide (final conc. 40μg/ml) and 590 mg IPTG (isopropyl-ß-D thiogalactopyranoside) dissolved in 2ml sterile ddH_2O (final concentration 5mM). Pour into sterile plates and once hardened store in foil, to prevent light damage, at 4°C.

Boiled Prep Buffer (BPB) - 8% sucrose, 0.5% Triton X-100, 50mM EDTA pH 8.0, 10mM Tris-HCl pH 8.0 - filter sterilized and stored at room temperature.

E. Plasmids and DNA Molecular Weight Standards Used:

E1. Plasmids:

pBH10R-3 - HIV-1_{HXB2} minus a portion of the 5' long terminal repeat (LTR), making integration impossible, was cloned into pSP64 (Ratner L, et al. 1987).

pT4B - The full length CD4 cDNA cloned into the lambda cloning vector gt10 (Maddon PJ, et al. 1985).

pBluescribe (Stratagene) - A cloning and sequencing phagemid (plasmid with a phage origin) with many advantages. It has a multiple cloning site (MCS), with 21 unique restriction enzyme sites, located in the N-terminal portion of the *lacZ* gene that encodes for ß-galactosidase. This allows recombinants cloned into the MCS to be detected by the interruption of the B-galactosidase activity making colour selection on pBS plates

possible. Selection for the presence of the phagemid is by the ampicillin resistance gene. The MCS is flanked by T3 and T7 DNA polymerase promoter sites for the generation of RNA transcripts of the insert from either orientation. Oligonucleotide primers complementary to these promoters facilitate sequencing of inserted foreign DNA.

E2. DNA Molecular Weight Standards:

Molecular Weight III (Boehringer Mannheim) - Lambda phage DNA digested with EcoRI and HinDIII: fragment sizes in base pairs - 21,226 5,148 4,973 4,277 3,530 2,027 1,904 1,584 1,330 983 831 564 125.

Molecular Weight V (Boehringer Mannheim) - pBR322 digested with HaeIII: fragment sizes in base pairs - 587 540 504 458 434 267 234 213 192 184 124 123 104 89 80 64 57 51 21 18 11 8.

One kilobase Ladder (Gibco BRL) - fragment sizes in base pairs - 12,216 11,198 10,180 9,162 8,144 7,126 6,108 5,090 4,072 3,054 2,036 1,636 1,018 517/506 396 344 298 220 201 154 134 75.

pBR322 Hpa II Hot Marker - *Hpa II* digested pBR322 end filled with α^{32}PdCTP and dGTP, fragment sizes in base pairs - 622 527 404 309 242 238 217 201 190 180 160 147 123 110 90 76 67 34 26.

II. METHODS:

A. GENERAL METHODS:

A1. Data Entry and Statistical Methods of Analysis:

Epidemiologic and laboratory data were computer coded and entered on a Filemanager database. Data were analyzed using the SPSS and STATA statistical packages. Standard parametric and non-parametric statistical tests were used for analysis of numeric and ordinal data. Odds ratios with 95% confidence intervals and incidence rate ratios with 95% confidence intervals were used to measure the strength of associations for cross sectional and longitudinal data, respectively. Standard survival analysis (Kaplan-Meier survival plots and log-rank tests) were used for univariate analysis of time dependent data. Multivariate analysis of survival data was performed by Cox proportions hazards modelling, with and without time dependent independent variables. Survival modelling was performed in consultation with Dr. N.J.D. Nagelkerke. Postulated survival models were fitted to the data using non-parametric regression modelling with maximum likelihood.

A2. HIV-1 Polymerase Chain Reaction (PCR):

A2i. Prevention of PCR Contamination:

Efforts were taken to prevent amplification products from one set of PCR reactions from contaminating subsequent rounds of PCR. Physically separate room were used for PBMC lysis, PCR preparations, and post PCR analysis. Nothing from the post

PCR analysis room (PCR dirty) was used in any pre-PCR procedures. Positive displacement micropipettors with disposable pistons (Microman pipettes, Mandel Scientific Ltd.) were used for peripheral blood mononuclear cells (PBMC) lysis and PCR preparations.

A2ii. Lysis of PBMC for PCR:

Three to six x 10^6 ficoll isolated PBMC (see Methods: Isolation of PBMC from Whole Blood) were resuspended in one ml of PBS and transferred to a sterile 1.5 ml microcentrifuge tube and pelleted in a microcentrifuge (Sorvall Microspin 24S) for one minute at 12,000 x g (12,000 rpm). The supernatant was removed and the cells resuspended in 25μl double distilled water (ddH_20). Twenty five μl of 2% triton X-100 (Fisher Scientific) in $T_{10}E_1$ pH 7.5 was added and the tube mixed briefly. The sample was then boiled for 15 minutes, placed on ice for 5 minutes and centrifuged for 1 minute to pellet cellular debris. The supernatant containing the DNA was removed, transferred to a new tube and stored at -20°C until needed.

A2iii. PCR Protocol:

HIV-1 PCR was only performed if the quality and quantity of DNA in the lysate was determined, by HLA DQA1 PCR (see Methods HLA DQA1 PCR), to be sufficient for HIV-1 PCR.

The oligonucleotide primers and probes in Table 1 were chosen for HIV-1 PCR because of the high degree of sequence conservation among the various strains of HIV-1,

Table I: HIV-1 PCR Primers and Probes

Oligo Name and Function	Oligonucleotide Sequence (5'→3')	Nucleotide Position $HIV\text{-}1_{HXB2}$
Vif-A' Primer	ATTGTGTGGCAAGTAGACAGGATGA	5064-5088
Vif-B Probe	AGTTTAGTAAAACACCATATGTATGTTTCA	5106-5135
Vif-C' Primer	CTAGTGGGATGTGTACTTCTGAACT	5217-5193
SK68' Primer	AGCAGCAGGAAGCACIATGG	7795-7814
SK70' Probe	ACGGTACAGGCCAGACAITTATTGTCTGGTATAGT	7835-7869
SK69' Primer	CCAGACIGTGAGTTGCAACAG	7936-7916
Nef-A' Primer	ACCTCAGGTACCTTTAAGACCAATG	9008-9032
Nef-B Probe	GATCTCAGCCACTTTTTAAAAGAAAAGGGGGGACTG	9051-9086
Nef-C' Primer	TGTGTAGTTCTGCCAATCAGGGAA	9179-9156
Rev-A' Primer	AATAGAAGAAGAAGGTGGAG	8414-8438
Rev-B Probe	GAACCTGTGCCTCTTCAGCTACCACCGCTTGAGAGACTT	8506-8544
Rev-C' Primer	CCTGCGTCCCAGAAGTT	8589-8571

especially the African isolates, at these positions. The primer positions are in reference to HIV-1_{HXB2} (Ratner L, et al. 1987). Novel primers and probes were developed to the regulatory genes *vif*, *nef* and *rev* (Dawood MR, et al. 1992). The structural gene *env* was targeted for amplification with primers and probes based on published sequences (Ou C-Y, et al. 1988) with a single inosine being substituted in each oligonucleotide at positions of sequence variation.

To minimize pipetting errors one common cocktail containing all of the PCR reagents, with the exception of the water and lysate DNA, was prepared for all samples to be tested on a given day. This cocktail was mixed and 36.5 μl aliquoted into each individual test 0.5 ml microcentrifuge tube (BioRad). The final concentration of the cocktail reagents, after the addition of water and DNA, were as follows: 1x PCR buffer (1mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin), 0.2 mM deoxynucleotide triphosphate (dNTP) (Pharmacia), 0.5 μM of the primers, and 2.5 units AmpliTaq DNA polymerase (Perkin Elmer). The volume of PBMC lysate estimated to contain 1 μg of DNA was determined by HLA DQA1 PCR. Enough ddH_20 was added to each tube to bring the volume up to 100 μl once the lysate DNA was added. After the lysate DNA was added, the entire reaction mixture was overlaid with two drops of mineral oil, the tubes were sealed and placed into the thermal cycler (Perkin Elmer Cetus). For each set of PCR reactions one positive and two negative controls were included. The positive control was either the plasmid HIV-1 containing pBH10R-3, lysed PBMC from a known HIV-1 positive individual or tissue culture cells infected *in vitro* with HIV-1_{IIIB}. The negative controls were a reagent control consisting of all of the PCR

reagents with the exception of the cellular DNA and lysed PBMC from a known HIV-1 seronegative low risk individual.

The amplification protocol for HIV-1 PCR was as follows: Denaturation for one minute at 94°C, primer annealing for one minute at 56°C, extension for one minute at 72°C repeated for 30 cycles followed by 7 minutes at 72°C to complete all extension. The annealing temperature was five degrees below the estimated melting temperatures (Tm) of the primers which were all designed to have Tm's at 61±1°C.

A2iv. Post PCR Analysis:

After amplification the tubes were removed to the PCR dirty room, opened and the aqueous layer removed from beneath the mineral oil and transferred to a fresh 0.5 ml microcentrifuge tube. To maximize the amount of PCR product that could be loaded into a single well of an agarose gel the amplification products were ethanol precipitated by adding 10 μl 3 M sodium acetate (NaOAc) and 200 μl cold 95% ethanol to the tube containing the 100 μl amplification product. The tube was kept at -70°C for 15 minutes then spun in a microcentrifuge at 4°C for 15 minutes at 12,000 rpm (12,000x g). The supernatant was then poured off, the pellet allowed to dry and then resuspended in 20 μl $T_{10}E_1$.

Half of the precipitated amplification products, 10 μl, and 2 μl tracking dye (0.1% bromophenol blue plus 10% glycerol in $T_{10}E_1$) were loaded onto an ethidium bromide stained 3.5% agarose (3% NuSieve agarose, Mandel Scientific company Ltd.; 0.5% SeaKem GTG agarose, Mandel Scientific company Ltd.) gel and electrophoresed until

the dye reached the bottom of the gel. Molecular weight standards (DNA Molecular Weight Standards V, Boehringer Mannheim) were loaded onto each gel to permit size determination of the amplification products. The gel was placed onto a ultraviolet (UV) transluminator and photographed. The determination whether a sample was PCR positive or negative was made after the gel was transferred to a support matrix and probed with the appropriate radiolabelled internal oligonucleotide.

A3. Southern Transfer:

DNA was transferred from an agarose gel to a nylon membrane, GeneScreen*Plus* (NEN DuPont), using the capillary transfer method as described by the manufacturer. To denature the DNA the gel was incubated in 0.4N NaOH - 0.6M NaCl for 30 minutes at room temperature with gentle agitation. The gel was neutralized by incubating in 1.5 M NaCl - 0.5 M Tris-HCl, pH 7.5 for 30 minutes at room temperature with gentle agitation. Before the completion of the neutralization step a piece of nylon was cut to the dimensions of the gel, wetted in ddH_2O, and soaked for 10 minutes in 10x SSC (1.5M sodium chloride - 0.15M sodium citrate). The gel was then placed face down onto a 10x SSC soaked filter paper wick (Whatman 3MM) whose ends were resting in a reservoir of 10x SSC. The nylon membrane was then laid onto the gel followed by two pieces of filter paper soaked with 10x SSC and cut to the same dimensions as the gel. A glass rod was then used to roll out any bubbles. A stack of absorbent paper towel, cut to the dimensions of the gel, was then laid on top of the filter papers. A small weight, usually a book, was placed on top of the filter papers to compress the paper towel and

ensure good capillary movement up through the various layers. The transfer was allowed to occur to 16-24 hours after which time the paper towel layer was removed, the gel flipped over and the locations of the well were marked onto the nylon membrane with an HB pencil (Venus). To ensure the DNA bound to the nylon membrane was denatured it was immersed in 0.4N NaOH for one minute and neutralized in 0.2M Tris-HCl, pH 7.5 - 2 x SSC. The DNA was then permanently bound to the membrane by drying it at 37°C for one hour or 80°C for 15 minutes.

A4. Hybridizations:

A4i. Radiolabelling Oligonucleotides:

Oligonucleotide probes were resuspended at 1pmol/ul in ddH_2O or $T_{10}E_1$. Ten μl (10 pmol) of probe, 2μl 10x polynucleotide kinase buffer (Pharmacia LKB Biotechnology), 7μl γ-^{32}P Adenosine 5' triphosphate (ATP) (3000 Ci/mmol, 10 mCi/ml, NEN DuPont) and 1 μl T4 polynucleotide kinase (5.3 units/μl) (Pharmacia LKB Biotechnology) were added to a microcentrifuge tube and incubated for 45 minutes in a 37°C sand bath. Following the kinase reaction the free γ^{32}P-ATP was removed by bringing the volume of probe up to 100μl with $T_{10}E_1$ and passing it through a one ml tuberculin syringe spin column of Sephadex G-50 (Pharmacia) by centrifuging on a clinical table top centrifuge for two minutes. The specific activity of the purified ^{32}P labelled oligonucleotide was determined by placing 1μl of the probe into 1-5ml scintillation cocktail (Econolume, ICN) and counting on a liquid scintillation counter (LS 500 CE, Beckman). Probes were used only if their specific activity was greater than 1

x 10^8 counts per minute (cpm)/μg of oligonucleotide.

A4ii. Hybridization of ^{32}P Labelled Probes to Membrane Bound DNA:

The dried nylon membrane was sealed in a plastic hybridization bag and hybridization solution (5 x SSPE, 1% SDS, 10 x Denhardts solution (50 x Denhardts - 1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin (BSA)) was added based on one ml of solution for every 15cm^2 of membrane. This prehybridization solution was incubated for one hour, with gentle shaking, at a hybridization temperature 10 degrees below the melting temperature (Tm -10°C) of the probe. For the HIV-1 PCR probes the hybridization temperatures were 48°C for Vif-B, and 56°C for Nef-B and SK-70'. After prehybridizing 1-2 x 10^6 cpm of labelled probe was added per ml of hybridization solution in the bag. The bag was incubated, with shaking, at the hybridization temperature overnight. After hybridization the excess probe was removed by washing with 1% SDS - 1x SSC at the hybridization temperature. The first wash was for 10 minutes followed by two washes for 30 minutes. After the third wash the nylon membrane was sealed into a plastic bag to prevent the probe from binding permanently. The membrane was then placed onto a filter paper support with radioactive orientation markers, exposed to X-ray film (Kodak X-O-Mat AR5) and placed between two intensifying screens (NEN DuPont) at -70°C for 6-48 hours. The films were developed with an automated film processor (MiniMed/90 X-ray Film Processor, AFP Imaging Corp.).

A5. Plasmid Purification:

A5i. Small Scale Preparations:

White colonies representing potential recombinant plasmids with inserts were picked from pBS plates and grown overnight, at 37°C with shaking, in 5ml Brain Heart Infusion (BHI) broth (Gibco) plus 100mg/l ampicillin. One and a half ml of culture was removed the next day to a 1.5 ml microcentrifuge tube and spun for one minute (12,000 x g) in a microcentrifuge. The supernatant was removed and the pellet of cells resuspended in 350μl Boiled Prep Buffer (BPB) (8% sucrose, 0.5% Triton X-100, 50mM EDTA, 10mM Tris-HCl pH 8.0 - filter sterilized and stored at room temperature). Twenty-five μl of fresh lysozyme (Sigma) solution (10mg/ml) in BPB was added to the resuspended cells were mixed briefly. The tube was incubated for one minute at room temperature then placed into a 100°C water or sand bath for one minute. The tube was then spun for 10-15 minutes. A sterile toothpick was then used to remove the insoluble pellet from the bottom of the tube (this contained most of the cellular debris and genomic DNA). To precipitate the plasmid 200μl of 7.5M NH_4OAc and 600μl isopropanol was added to the supernatant and mixed briefly. After a 15 minute incubation at room temperature the sample was centrifuged at 12,000 x g for 15 minutes, the supernatant removed and the pellet allowed to air dry for 10 minutes. The pellet was resuspended in 50μl $T_{10}E_1$ supplemented with 10μg/ml RNase A and 2mM spermidine to remove the contaminating RNA. The isolated plasmid DNA was of sufficient quality to permit restriction digestions to identify plasmids with inserts.

A5ii. Large Scale Plasmid Preparations:

The method of large scale plasmid preparations was that of Sambrook J, et al. 1989.

A5iii. Purification of Plasmid DNA for Sequencing:

The DH5α *E.coli* with the plasmid to be sequenced was grown overnight in LB broth plus ampicillin (100mg/l) at 37°C with shaking. One and a half ml was transferred to a 1.5ml microcentrifuge tube and spun at 12,000 x g for 2 minutes. As much of the supernatant was removed as possible and the cells were resuspended in 100μl ice-cold 50mM glucose, 10mM EDTA and 25mM Tris-HCl pH 8.0 and incubated at room temperature for 5 minutes. Two hundred μl of freshly prepared 0.2N NaOH, 1% SDS was added, mixed gently by inversion and incubated on ice for 5 minutes. One hundred and fifty μl ice-cold potassium acetate solution pH 4.8 (60 ml 5M KOAc, 11.5ml glacial acetic acid, 28.5ml H_2O) was added, mixed by inversion, and returned to ice for 5 minutes. The sample was then spun for 5 minutes and the supernatant transferred to a new 1.5ml tube. RNAse A was added to the sample to a final concentration of 20μg/ml and then incubated at 37°C for 20 minutes. An equal volume of 1:1 Tris saturated phenol:$CHCl_3$, about 500μl, was added, mixed for 2 minutes, centrifuged for 3 minutes, and the upper layer transferred to a new 1.5ml tube. The plasmid DNA was then precipitated by adding two volumes of 100% ice-cold ethanol, mixing, incubating for 15 minutes at -70°C and pelleted by centrifuging for 15 minutes. The supernatant was removed, the pellet allowed to air dry and then resuspended in 50μl $T_{10}E_1$ and 30μl

polyethylene glycol (PEG) - 2.5M NaCl, mixed and incubated on ice for 20 minutes. The plasmid was then precipitated by centrifuging for 10 minutes. The supernatant was discarded and the excess salts removed from the pellet by rinsing with 70% ethanol, centrifuging for 2 minutes and discarding the ethanol. The pellet was allowed to air dry and finally resuspended in 20μl H_2O. A typical yield of pBluescribe was 9-15μg/1.5ml culture.

A6. Serology:

A6i. HIV-1 Serology:

All individuals in the study were tested for the presence of HIV-1 antibodies with commercial enzyme immunoassays (HTLV-III Elisa, DuPont from 1985 through 1988; Vironostika, Organon Technika, from 1988 through 1990; Detect HIV, IAF Biochem, from 1990-1992; and Enzygnost HIV-1/2 EIA, Behring from 1991 to present). All seroconversions were confirmed by immunoblot (Novapath Immunoblot, BioRad) until 1991 when they were confirmed by Recombigen HIV-1/2 EIA, Cambridge Biotech. All women testing negative by enzyme immunoassay were confirmed negative by immunoblot on at least one occasion. These commercial systems were used to detect the presence of HIV-1 antibodies according to the manufacturer's instructions.

A6ii. Rubella Serology:

Samples were sent to the Cadham Provincial Laboratory for the quantification of IgG antibodies to Rubella virus using the Rubella IgG Antibody IMX kit (Abbott

Laboratories).

A7. Protein Analysis:

A7i. Lysis of Cells for Protein Analysis:

To determine if the recombinant vaccinia/HIV-1 vectors supplied through the AIDS Research and Reference Reagent Program, AIDS Program, NIAID, NIH produced the HIV-1 proteins, immunoblots of whole cell lysates from B cells infected with these viruses were prepared. The method of infecting the Epstein-Barr virus (EBV) transformed B cells with these vaccinia viruses is described in the Methods: Cell Mediated Cytotoxic Responses to HIV-1 section. In brief, 1 x 10^7 B cell blasts, sample KRF, were infected with the recombinant vaccinia/HIV-1 viruses at a multiplicity of infection (MOI) =3.0. After an overnight infection the cells were washed two times in cold PBS, resuspended in 0.5ml of lymphocyte lysis buffer (McDougal JS, et al. 1986) and incubated on ice for 45 minutes with occasional gentle mixing. The sample was then centrifuged in a microcentrifuge at 4°C for 15 minutes. The supernatant was removed from the nuclear pellet and stored at -20°C.

A7ii. Polyacrylamide Gel Electrophoresis (PAGE):

Protein quantification was performed using the Bio-Rad Protein Assay kit as per the manufacturer's instruction. The cellular lysates had a total protein concentration of 4mg/ml. Polyacrylamide gel electrophoresis (PAGE) (Sambrook J, et al. 1989) was performed with standard methods using a stacking gel and a resolving gel. A total of

50μg total protein was loaded into each well of the Bio-Rad mini gel apparatus. A 12% polyacrylamide gel was used for detecting the p24 and reverse transcriptase (RT) proteins, while a 7.5% PAGE gel was used to detect gp160.

A7iii. Protein Transfer:

The proteins were transferred from the polyacrylamide gel to a nylon membrane (Immobilon, Millipore) using a semi-dry protein transfer apparatus (Bio-Rad) as per the manufacturer's instructions. In brief, the stacking gel was removed and the resolving gel soaked for 15 minutes with shaking in Tris/Glycine blot buffer (48mM Tris-HCl, 39mM glycine, 0.0375% SDS). The membrane, cut to the dimensions of the gel, was wetted in methanol, transferred to ddH_2O for 5 minutes and then soaked in blot buffer for 15 minutes with shaking. Two thick filters (Bio-Rad) cut to the dimensions of the gel were wetted in blot buffer. One filter was placed onto the transfer apparatus, the filter laid on top followed by the gel and finally the last filter. A glass rod was rolled overtop the stack to ensure no air bubbles remained between layers. The apparatus was reassembled and transfer was achieved at 15 volts for 30 minutes.

A7iv. Immunoblot of Recombinant Vaccinia/HIV Infected B cells:

Standard immunoblotting methods were used (Sambrook J, et al. 1989). The following antibodies, Table II, were received through the AIDS Research and Reference Reagent Program, AIDS Program, NIAID, NIH.

Table II: Primary Antibodies Used to Detect HIV-1 Proteins

Antibody	Specificity	Dilution Used	Source
NIH # 384	recombinant p24/25	1:500	rabbit polyclonal
NIH # 634	recombinant reverse transcriptase	1:1000	rabbit polyclonal
NIH # 188	recombinant gp160	1:1000	goat polyclonal

The secondary antibodies used for immunoblotting were anti rabbit/goat IgG conjugated to horse radish peroxidase (HRP) and purchased commercially (Chemicon).

After the proteins were transferred to the nylon blots, they were blocked in 5% skim milk at 4°C overnight or at 37°C for two hours with shaking. The primary antibodies were diluted in PBS plus 1% BSA, added to the blots and incubated for 120 minutes at room temperature with shaking. The blots were washed three times in T-TBS, with 15 minute shaking at room temperature. The secondary antibodies were diluted to 1:500 in PBS - 1% BSA, added to the blots and incubated at room temperature with shaking for one hour. The blots were washed three times in T-TBS. Colour development of the HRP was achieved by combining 1.5 ml DAB (diamino benzidine) stock (1.25mg/ml in ddH_2O), 750μl 1% $CoCl_2$, 20μl H_2O_2 and 35.25ml PBS and adding it to the blot. Development was for five minutes and stopped by washing with water.

B. HLA ANALYSES:

B1. HLA DQA1 PCR:

The primers and probes (Erlich HA, et al. 1986) used (Table III) are complementary to or correspond to the second exon of the polymorphic HLA DQA1 locus. The 5' sense primer, GH26, and the 3' antisense primer, GH27, are located in conserved regions that flank a highly polymorphic region. The universal probe RH54 binds in that region and is able to hybridize all known alleles of HLA DQA1. The reagents for HLA DQA1 amplification were the same as those for HIV-1 PCR with the exception that the final concentration of primers was 0.25μM. The amplification protocol consisted of denaturation at 94°C, annealing at 50°C, and extension at 72°C, each step for 30 seconds, for 30 cycles and ended by 7 minutes at 72°C to complete extension. The products of amplification were analyzed by agarose gel electrophoresis either by visual inspection or by subsequent Southern blotting and hybridizations with radiolabelled probes (see Methods: Southern Transfer and Hybridizations).

B2. DNA Isolation from Sera:

The Bio-Rad Prep-A-Gene DNA purification kit was used for DNA isolation from serum. In brief, 50 μl of serum or plasma was diluted with an equal volume of sterile dd$H_2$0 in a 1.5 ml microcentrifuge tube and 300 μl of binding buffer (50mM Tris-HCl, 1mM EDTA, 6 M $NaClO_4$, pH 7.5) was added and mixed. Ten μl of resuspended silica gel matrix was added and incubated for 15 minutes at room temperature with occasional mixing. After incubation the suspension was centrifuged for ten seconds to pellet the

Table III: Oligonucleotide Primers and Probes used in HLA DQA1 Amplification and Genetic Typing.

Oligonucleotide Name	Sequence 5'→ 3'	HLA DQA1 sequences recognized
GH26	GTGCTGCAGGTGTAAACTTGTACCAG	conserved 5' region
GH27	CACGGATCCGGTAGCAGCGGTAGAGTTG	conserved 3' region
RH83	GAGTTCAGCAAATTTGGAG	1 allele
GH88	CGTAGAACTCCTCATCTCC	1.1 allele
GH76	GTCTCCTTCCTCTCCAG	all but 1.3
GH89	GATGAGCAGTTCTACGTGG	1.2,1.3,4 alleles
GH77	CTGGAGAAGAAGGAGAC	1.3 allele
RH71	TTCCACAGACTTAGATTTGAC	2 allele
GH67	TTCCGCAGATTTAGAAGAT	3 allele
GH66	TGTTTGCCTGTTCTCAGAC	4 allele
HE46	CATCGCTGTGACAAAACAT	4.2, 4.3 alleles
RH54	CTACGTGGACCTGGAGAGGAA GGAGACTGCCTG	all alleles

silica matrix. The matrix was washed twice in binding buffer then three times in wash buffer (20 mM Tris-HCl, 2 mM EDTA, 0.4 mM NaCl, pH 7.4 in 50% ethanol) by resuspending the pellet in 500 μl of the appropriate buffer and centrifuged for 10 seconds to pellet. The DNA was eluted from the matrix by resuspending the silica matrix in 20 μl of sterile ddH_2O and incubating at 50°C for 15 minutes. The sample was then centrifuged for 30 seconds and the DNA containing supernatant was removed and saved. The elution step was repeated once and the supernatants pooled.

B3. HLA DQA1 Product Analysis:

To ensure amplification of the HLA DQA1 gene was successful 20 μl of the post-amplification reaction mixture was size separated on a ethidium bromide stained 3.5% agarose gel where the quantity and size of the amplification products was verified visually. Replicate blots were prepared and each probed with a different ^{32}P labelled allele specific oligonucleotide (ASO). Hybridizations were performed as described in Methods: Hybridizations. The probe RH54 binds to all alleles of HLADQA1 and was used at a hybridization temperature of 65°C. The hybridization temperatures for probes GH66, RH83, GH88, and GH89 were 50°C and 47°C for probes GH67, RH71, GH77, and HE46 (see Table III for oligonucleotide sequences). Blots were washed at the specified hybridization temperatures with 1X SSC and 1% SDS (sodium dodecylsulfate) for 10 minutes followed by two 30 minute washes. The blots were exposed to x-ray film (Kodak X-O-Mat AR 5) for 2-24 hours with intensifying screens at -70°C and then the films developed.

B4. HLA Serological Typing:

Standard serological tissue typing was used (Vartdal F, et al. 1986) to determine the HLA haplotype of the test and control individuals. In brief, 5-10 ml whole blood was collected in acid citrate dextrose (ACD) vaccutainers (Becton-Dickinson), spun down and the buffy coat removed and brought up to 5 ml with PBS (phosphate buffered saline) plus 0.6% Na-citrate. Immunomagnetic beads (Dynal) specific for the CD8 molecule were used to purify cells for HLA class I determination. HLA class II determinations were made on cells purified by immunomagnetic beads specific for B cells. To the diluted buffy coat 50 μl of immunomagnetic bead suspension was added and mixed gently for five minutes at room temperature. The tube was then placed against a rare earth magnet for 2-3 minutes to allow the bead bound cells to adhere against the magnet. With the magnet in place the supernatant was poured off and after four washes with PBS the bead bound cells were resuspended into 0.2-0.4 ml RPMI. One μl of cell suspension was placed into each well of a 72 well Terasaki tray (One Lambda Inc. or Canadian Red Cross, Ottawa) and incubated at room temperature for 30 minutes. Five μl of rabbit complement (Cedar Lane Laboratories Ltd.) was then added to each well and incubated for 30 minutes at room temperature and would cause cellular lysis in any well that contained antisera which recognized the HLA antigens expressed on the surface of those cells. Lysis of the cells was detected by the addition of one μl of a mixture of fluorescent dyes, acridine orange and ethidium bromide, which are taken up differentially by viable and dead cells respectively. Five μl 2.5% Indian Ink (Sheaffer's Script) in PBS

was added to provide a black background against which the fluorescing cells can be seen. The plates were read on an inverted fluorescent microscope at 450 nm and each well was scored for percent cell lysis.

C. CD4 ANALYSES:

C1. Isolation of CD4 mRNA

Total cellular mRNA was isolated from ficoll-hypaque (Sigma) purified PBMC that were frozen in Nairobi at 1-3 x 10^6/ml in PBS, and mRNA was isolated at a later date in Winnipeg using the Micro-FastTract kit (Invitrogen Corp.), as per manufacturer's instructions. Briefly, the cells were pelleted, resuspended in an SDS/proteinase K buffer to the lyse the cells and incubated for 15 minutes in a 45°C water bath. An oligo deoxythymidine triphosphate (oligo dT) cellulose tablet was added and binding of the poly A tail of the cellular mRNA was allowed to occur during a 15 minute room temperature incubation with shaking. The oligo dT was then pelleted in a microcentrifuge at 4,000 x g and the supernatant removed. The oligo dT was then washed several times with initially a binding buffer and subsequently with a low salt wash buffer which removed the cellular debris, protein, DNA, tRNA and rRNA. The mRNA was eluted off the oligo dT with two 100μl elutions of $T_{10}E_1$. The total mRNA was concentrated by ethanol precipitation and resuspended in 10μl $T_{10}E_1$.

C2. First Strand cDNA Synthesis of CD4 mRNA:

First strand cDNA synthesis of the CD4 mRNA was primed with a complementary 3' oligonucleotide (3'CD4-Nest) (5'-CTGGGGAGGCTGCAAGTGGGA TCTGCCTGG-3') located at position 1491-1462 (relative to Human T-cell surface glycoprotein T4 mRNA, complete coding sequence (Maddon PJ, et al. 1985)). Reverse transcription was performed using standard methodologies (Sambrook J, et al. 1989). Briefly 200 units Moloney Murine Leukemia Virus RNase H$^-$ reverse transcriptase (MMLV-RT)(Superscript^{TM}, Gibco BRL) was incubated with 5μl of isolated mRNA, 1pmol 3'CD4-Nest primer, 1 x RT buffer (Gibco, BRL), 10mM dithiothreitol, 0.5mM each deoxyribonucleotide triphosphate, and 32.4 units human placental RNase inhibitor (RNAguard, Pharmacia) for 30 minutes at 42°C.

C3. Amplification of the CD4 cDNA:

Half of the CD4 cDNA (10μl) was amplified using a nested polymerase chain reaction (PCR) strategy. The more distal set of primers used for the initial amplification consisted of oligonucleotide 5'CD4-Nest (5'-ACTGCTCAGCCCCTTCCTCCCTCGG CAAGG-3' which corresponded to position 41-70 of the mRNA) and the complementary 3'CD4-Nest used to prime the first strand cDNA synthesis. After the initial round of thirty cycles, one tenth (10μl) of the total reaction volume was removed and served as the template for the second round of amplification with the internal primer pair consisting of the primer CD4-5'PCR (5'-CCGAATTCATATGAACCGGGGAGTCCCT-3' corresponding to position 76-93) and the complementary primer CD4-3'PCR (5'-

CCAAGCTTCATATGGTGCCTCAAATGGGGCTACA-3' position 1457-1438). These latter primers incorporated both the initiation and termination codons such that the entire coding sequence of the CD4 message (1.4 kilo base pairs, kbp) was represented in the amplification product. Eco RI and Hind III restriction sites were engineered onto the 5' ends of the CD4-5'PCR and CD4-3'PCR primers, respectively, to facilitate directional cloning into the sequencing vector pBluescribe (Stratagene).

The reagents for the amplification were identical to those for HIV-1 PCR with the exception that the primer concentration was 0.25μM each and 1.0 unit of DNA gyrase (Perfect Match^{TM}, Stratagene) was added to increase the specificity of the primer binding.

The conditions of amplification were as follows:

First round of PCR - denaturation 94°C 1 minute, annealing 65°C 1 minute and elongation 72°C 2 minutes for thirty cycles followed by a ten minute extension at 72°C. Second round of PCR - denaturation 94°C 1 minute, annealing 53°C 1 minute and elongation 72°C 2 minutes for 7 cycles then an annealing temperature of 60°C for an additional 23 cycles after which a ten minute period of extension at 72°C. The annealing temperatures were five degrees below the melting temperature (Tm-5°) of the primers used. The internal set of primers required two annealing temperatures due to the tailing of restriction enzyme sites onto the ends of the primers. These tails are not complementary with the CD4 cDNA sequence therefore the first annealing temperature of 53°C was Tm-5° below that portion of the primer that was complementary to the CD4 cDNA. After the first few cycles the template for elongation was primarily the PCR

product, not the CD4 cDNA, therefore the entire length of the primer was complementary and the annealing temperature was raised to 60°C.

C4. Analysis and Purification of the 1.4 kbp CD4 Product:

After the completion of the second round of PCR, 10 μl of the amplification product (one tenth of the total) was loaded onto a diagnostic 1% agarose gel to determine the quantity and size of the amplification products. Although smaller fragments were present, the 1.4 kbp fragment was the predominant band. To ensure that only the full length 1.4 kbp product was being further manipulated, 70μl of the amplification product was resolved on a 1% agarose preparative gel from where the 1.4kbp fragment was excised. The 1.4 kbp fragment was purified from the gel matrix using the Prep-A-Gene DNA isolation kit (BioRad) as per manufacture's instructions.

C5. Cloning of CD4:

To confirm the amplification products were of CD4 origin and not an amplification artifact, a 1% agarose preparative gel was performed, transferred to a support matrix and probed with a ^{32}P labelled CD4 oligonucleotide 5'CD4 (Clonetech) (GTGAACCTGGTGATGAGAGC position 1009-1031).

The sequencing plasmid pBluescribe (Stratagene) and the 1.4kbp CD4 amplification products were digested with the restriction enzymes (Sambrook J, et al. 1989) EcoRI and HindIII (Gibco, BRL), resolved on a 1.0% agarose gel (to remove the digested ends), excised from the gel and purified by a silicon gel matrix (Prep-A-Gene,

BioRad). The quantity of digested, gel purified product was estimated by comparing the intensity of staining of an aliquot (1/10 total volume) of purified product to known DNA standards when resolved on a 1% ethidium bromide stained agarose gel.

The ligation of the amplified CD4 cDNA (insert) into the sequencing plasmid pBluescribe (vector) was performed using standard methods (Sambrook J, et al. 1989). In brief, 50ng of the vector was incubated with the CD4 cDNA from each individual being tested (at the molar ratios of vector to insert of 1:1 and 1:3), 0.5 units T4 DNA ligase (Gibco, BRL), 1 x ligase buffer, and .25mM adenosine triphosphate (ATP) at 16°C for 40 hours. The ligation products, 20μl in total, were then precipitated by adding 0.5μl tRNA (10mg/ml), 1.8μl 3M NaOAc, and 41 ul 95% cold ethanol, mixed, incubated for 15 minutes at -70°C, spun for 15 minutes at 12,000 x g in a microcentrifuge and resuspended in 10μl $T_{10}E_1$. Two μl was then transformed into competent *E.coli* DH5α^{TM} (Gibco, BRL) for identification and amplification of the vectors with ligated inserts. The method of transformation is described below.

C6. Transformation of DH5α cells:

One tube of competent DH5α^{TM} cells (Gibco, BRL), stored at -80°C, was thawed on ice and 20μl transferred to a pre-chilled 1.5 ml microcentrifuge tube. One μl of precipitated ligation mixture (maximum of 5ng DNA) was added to the cells and incubated for 30 minutes on ice. The cells were then heat shocked by incubating at 42°C for 60 seconds then returned to the ice. Eighty μl of SOC (see Media and Solutions) was added and incubated for one hour at 37°C with shaking. Ten and 90μl were plated

separately onto selective plates (pBS plates with ampicillin, see Solutions and Media) and incubated at 37°C overnight. Clones with inserts were identified as white colonies and were picked for further plasmid analysis. Positive control transformations included 1.6ng of uncut pBluescribe.

C7. Single Strand Conformation Polymorphism (SSCP) Analysis:

The method used for SSCP analysis was originally described by Orita M, et al. 1989 and modified by Dr. Barbara Triggs-Raine, Department of Biochemistry and Molecular Biology, University of Manitoba. In brief, the CD4 cDNA 1.4 kbp amplification product from each individual was reamplified in three sections (450, 624 and 454 bp in length), using a different reaction tube for each section. The amplification conditions were previously described in Methods: CD4 PCR, with the exception that α-^{32}P deoxyadenosine 5' triphosphate (dATP) (3000 Ci/mmol, 10 mCi/ml, NEN DuPont) was added so that the amplification product could be detected by autoradiography after resolution by polyacrylamide gel electrophoresis (PAGE). Since the SSCP technique is most sensitive with DNA fragments less than 300 bp, the three amplification products were digested with specific restrictions enzymes such that each fragment was less than 300 base pairs in length. The fragments, the primers used to amplify them, the restriction enzyme used and the size of the resulting fragments are summarized in Table IV.

Table IV: Generation of SSCP Fragments

Amplification Product (Size)	Amplification Primers	Restriction Enzymes	Size of Fragments (bp)
5' Product (450 bp)	CD4-5'PCR 3'CD4-492	RsaI	a) 129 b) 321
Central Product (624 bp)	5'CD4-461 3'CD4-1054	NheI Sau3a	a) 223 b) 150 c) 251
3' Product (454 pb)	5'CD4-1025 CD4-3'PCR	AvaI	a) 180 b) 274

Note: For sequence of amplification primers see Methods: Sequence Analysis of CD4.

The samples were diluted 1:5 with water and 10μl of this dilution combined with 2μl sequencing stop solution (95% formamide, 0.1% bromophenol blue and 0.1% xylene cyanol). The samples were heated at 95°C for 3 minutes to denature the double stranded DNA. A non-denatured control sample was run to illustrate the migration of double-stranded products as reannealing may occur after heating. The radiolabelled fragments and pBR322 Hpa II hot markers were resolved under non-denaturing conditions on a 6% acrylamide (29% acrylamide, 1% bis acrylamide)/ 10% glycerol gel. The gels were run at 12mA overnight to prevent warming and denaturation of the secondary structure of single DNA strands. Gels were dried on filter paper and exposed to x-ray film for several hours before development.

C8. Sequence Analysis of CD4:

C8i. Sequencing of the CD4 cDNA:

Sequence data of the cloned CD4 cDNAs were generated with the dideoxynucleotide triphosphate chain termination Sequenase™ kit from Stratagene as per the manufacturer's instruction. Sequencing through the multiple cloning site and into the cloned CD4 cDNA was achieved using primers (T3 and T7) which are complementary to the T3 and T7 promoter sites which flank the cloning site on the pBluescribe vector. Two primers 5'CD4-461 (5'-CCGAATTCTGACACCCACCTGCTTC-3' corresponding to positions 461-478) and 5'CD4-1025 (5'-CCGAATTCTGAGAGCCACTCAGCTCC-3' corresponding to position 1025-1042) and two complementary primers 3'CD4-492 (5'-CCAAGCTTCTCTCCAAGGTCAGGGTC-3' positions 509-492) and 3'CD4-1054 (5'-CCAAGCTTCCCCACACCTCACAGGT-3' positions 1071-1054), were designed to prime sequencing from within the cloned product. Direct sequencing of PCR products from the second round of amplification (Gibco PCR sequencing kit as per manufacturer's instructions) was performed to ensure that any discrepancies between the cloned products and the published sequences were not due to AmpliTaq™ DNA polymerase (Cetus) incorporation errors in the clone.

C8ii. Analysis of the Sequencing Reactions:

The sequencing reactions of both the plasmid and PCR products were resolved on single load 8% and double load 4% acrylamide gel using standard methods (Sambrook J, et al. 1989). Sequences were digitally entered into the MicroGenie DNA sequencing

program (Beckman) for alignment and analysis.

D. CELLULAR ANALYSES:

D1. Isolation of PBMC from Whole Blood:

Blood was drawn by venepuncture into sodium heparin vacutainers (Becton Dickinson) or into CPDA (citrate, phosphate, dextrose, and adenine) blood collection bags (Red Cross Society International) and mixed to prevent coaggulation. The blood was either diluted with an equal volume of PBS or layered undiluted onto ficoll-hypaque 1.077 separation gradient as per manufacturers instructions (Sigma), the volume being not less than 20% that of the blood, and centrifuged for 30 minutes at 20°C at 500 x g (2000 rpm) in a Beckman GPR table top centrifuge. After centrifugation the PBMC layer was removed aseptically in a sterile laminar flow hood and added to a second sterile tube. PBS was used to wash the cells and added at a volume of 4-5 times that of the PBMC layer. The tube was then centrifuged at 400 x g (1,500 rpm) at 20°C for 5 minutes. Following centrifugation the supernatant was discarded and the PBMC washed twice with 10 ml LM and centrifuged at 400 x g (1,500 rpm) at 20°C for 5 minutes. After the final wash the cells were resuspended in LM and used as described for various procedures.

D2. Long Term Storage of Cells:

D2i. Freezing of PBMC or Tissue Culture Cells:

The cells to be frozen were counted on a haemocytometer and then pelleted by

centrifuging at 400 x g (1,500 rpm) for five minutes. The supernatant was discarded and the cell pellet was resuspended in ice cold freezing media to yield a final cell concentration of 1-5 x 10^6/ml. The resuspended cells were transferred to pre-chilled labelled cryovials, with silicon gaskets, (Nalgene) in one ml aliquots. The lid of the cryovials was tightened and then the cryovial transferred to a pre-chilled cell freezing apparatus (Stratacooler, Stratagene) where cells were stored overnight at -80°C. The next day the cryovials were removed from the cell freezer, placed into canes and stored in liquid nitrogen (LN_2).

D2ii. Thawing of Frozen PBMC:

The cryovial containing the cells of interest was removed from the LN_2 and placed into the final position of a LN_2 storage cane. The cryovial was lowered into a 37°C water bath where it was shaken until almost completely thawed. The cryovial was taken to a laminar flow hood where it was opened while wrapped in an ethanol swab. The contents of the cryovial were gently resuspended and transferred to a tube containing 10ml cold LM and mixed. A small aliquot of the mixed cells was removed, stained with trypan blue for viability estimations and counted on a haemocytometer. The remainder of the cells were pelleted by centrifuging at 400 x g (1,500 rmp) for five minutes at 20°C. The supernatant was poured off, the cells resuspended and washed once in 10ml LM. After the final centrifugation, the supernatant was removed and the cells resuspended in variable volumes of LM to yield a known cell concentration.

D3. HIV-1 Infection of PBMC from Uninfected Resistant and Control Subjects:

PBMC were purified from 11 persistently seronegative prostitutes (MLs 1356, 1376, 1275, 887, 935, 1025, 1250, 893, 1260, 889, 1358) and 9 HIV-1 seronegative controls with no known exposure to HIV-1 (one laboratory personnel, KRF, and 8 Kenyan women attending an antenatal clinic, MCHs 1:5452, 1:5451, 1:5503, 1:12499, 1:8404, 1:6527, 1:180 and 1:7007) as described in Materials: Source of Biologic Material. The isolated PBMC were washed twice with PBS and once with lymphocyte media (LM) and resuspended at 2 x 10^6/ml in LM. For each individual tested, duplicate wells of 1.5 x 10^6 cells were prepared in a sterile 24 well tissue culture plates (Corning) and stimulated with 5 μg/ml phytohemagglutinin-P (PHA) (Sigma). Three days later the stimulated PBMC were pelleted by centrifugation, the supernatant removed, and the cell pellet exposed to 1.2 x10^4 infectious units (IU) of HIV_{IIIB} (100 μl of 1.2 x 10^5 IU/ml)(multiplicity of infection MOI=.03 assuming 25% of PBMC were susceptible to HIV-1 infection) for 2 hours at 37°C with occasional shaking. The cells were resuspended in 1.5 ml LM and incubated at 37° overnight. The cells were pelleted by centrifugation, the supernatant was aspirated, and the cells were washed twice with 2 ml PBS. The washed cells were resuspended in 2 ml LM supplemented with 5 units/ml recombinant human interleukin 2 (IL-2) (Genzyme). After one hour, 0.5 ml of supernatant was removed and stored at -70°C (Day 0 post infection). The cells were returned to the incubator. At days 3, 6, and 9 post infection 0.75 ml supernatant was removed for storage at -70°C and replaced with 0.75 ml fresh LM supplemented with 10 U/ml IL-2. The supernatants were later analyzed for production of the HIV-1 protein

p24.

D4. Titration of Infecting Virus on Uninfected PBMC:

Titrations of the infecting virus were performed on two controls and three persistently seronegative women. The preparation, plating, and analysis of cells for the titration experiment are identical to the infection experiment with the exception that duplicate wells of PBMC from each test individual were infected at MOIs of 0, .0003, .003, and .03.

D5. HIV-1 p24 Quantitation:

The HIV-1 core protein p24 was detected by a sandwich ELISA using reagents obtained through the AIDS Research and Reference Reagent Program, AIDS Program, NIAID, NIH. Flat bottomed 96 well plates were coated with 5 μg/ml of anti-p24 polyclonal antibody D7320 in 100 mM $NaHCO_3$ at 20°C overnight. D7320 is a mixture of three sheep polyclonal antibodies raised against the following highly conserved HIV-1 peptides:

Peptide sequences	p24 Amino Acid # ($HIV\text{-}1_{LAV}$)
SALSEGATPQDLNTML	173-188
GQMREPRGSDIA	226-237
LDIRQGPKEPFRDYV	283-297

The plates were washed two times with 300 μl of Tris Buffered Saline (TBS) (144 mM NaCL, 25 mM Tris, pH 7.5). One hundred μl of dilutions of test culture supernatant,

or a standard control supernatant with a known amount of p24 were added to the wells of the coated plates and incubated at 20°C for 2.5 hours. Live virus was inactivated by the addition of Empigen zwitterionic detergent (Calbiochem) (1% by volume), to the culture supernatants and standard controls. Unbound p24 was washed away with two washes of 200 μl of TBS. Bound p24 was reported by the addition of 100 μl of a 1:2000 dilution of the alkaline phosphatase conjugated monoclonal antibody BC 1071-AP in TMT/SS (2% non-fat dry milk, 20% fetal calf serum and 0.5% Tween 20 in TBS) for 45 minutes at 20°C. Antibody BC 1071-AP has been mapped to a complex epitope consisting of the following HIV-1 p24 peptides sequences GHQAAMQMLKETINEEAAEWDRVHPVHAGPIAPGQ (amino acids 193-227) and NPPIPVGEIYKRWII (amino acids 253-267). Unbound BC 1071-AP was removed with four washes of 300 ul of T-TBS (TBS plus 0.05% Tween 20) and bound BC 1071-AP was detected using the AMPAK kit (Dako Diagnostics Inc.) according to the manufacturer's instructions. The AMPAK system amplifies the signal from the alkaline phosphatase with a molecule of red formazan being produced every cycle. The reaction was stopped by the addition of 50 μl of sulphuric acid and the plates were read on an automated ELISA reader at 492 nm. A standard curve was produced for each plate and the concentration of p24 per ml of unknown culture supernatant was determined by linear regression analysis using the software "Curve Fitter-PC" (Interactive Microware, Inc.).

D6. Immune Memory to HIV-1 Peptides:

D6i. Cellular Proliferation:

The method of detecting cellular activation to HIV-1 peptides and the selection of peptides was based on work of Clerici M, et al., 1992. Isolated PBMC were resuspended at 3 x 10^6/ml in LM. Eight separate test solutions (100μl/well) were added in triplicate to the wells of a sterile 96 well plate. The test solutions used were lymphocyte media (LM)(negative control), recombinant soluble gp120 (Intracel Corporation) 2μg/ml, five different HIV-1 *env* peptides (NIH Reagent Program) 2.5μg/ml and PHA-P 5μg/ml (positive control). The HIV-1 *env* peptides, their NIH catalogue numbers, the T cell epitopes they represent and their location on the gp120 protein are described in Table V. Three x 10^5 PBMC were incubated, at 37°C, with each the 8 test solutions (triplicate wells per test solution). Six days later one μCi of ^{3}H-methyl thymidine (6.7 Ci/mmol, 1mCi/ml NEN, Dupont) was diluted in 24μl LM and added to each well and incubated at 37°C overnight. Cells were harvested by filtering the resuspended cells onto a glass filter membrane held in a 96 well dot blot apparatus (Bio-Rad). Cells were lysed by washing three times with ddH_2O. The membrane was left to dry, a wax scintillation material (MeltiLex, Wallac) applied and then sealed in a plastic bag. The filter was placed in a filter cassette (Wallac) and the amount of incorporated ^{3}H in each well was detected using a Microbeta liquid scintillation counter (Wallac).

D6ii. Interleukin 2 (IL-2) Production:

Detection of IL-2 production was performed as described for the peptide

Table V: HIV-1 Peptides Used in Proliferation and IL-2 Assays

NIH Cat. #	T cell epitope	Location in HIV-1_{MN} gp120	Peptide Sequence and T cell Epitope (**bold**)
2007	T1	421-440	**KQIINMWQEVGKAMYA**PPIE
1929	T2	101-120	EQM**HEDIISLWDQSLK**PCVK
864	P18	306-327	YNKRK**RIHIQRGPGRAFYTTKN**IIC
1991	P23	361-380	TIVF**NQSSGGDPEIVMHSF**N
1589	T1	418-441 HIV_{IIIB}	CRI**KQIINMWQKVGKAMYA**PPISG

proliferation experiment with the exception that 25μl of a 1:100 dilution of anti-Tac monoclonal antibody (a generous gift from Dr. Gene M. Shearer) was added to all wells. This antibody blocked the high affinity IL-2 receptor and thus prevented consumption of secreted IL-2 by the cells. Samples were incubated at 37°C for seven days. On day seven the supernatant from each well, approximately 200μl, was removed and placed into a second sterile 96 well plate and frozen at -70°C until assayed. The cellular pellets on the original plates were frozen for future analyses.

IL-2 and other cytokines were detected by commercial ELISA kits (R&D Biosystems) as per the manufacturer's instructions.

D7. Bulk B Cell Transformations:

Five to ten x 10^6 PBMC were resuspended in 1-2 ml of B95-8 supernatant and incubated at 37°C. The supernatant was derived from the marmoset cell line B95-8 (AIDS Research and Reference Reagent Program, AIDS Program, NIAID, NIH) which chronically produce Epstein Barr Virus (EBV) and has been filtered through a .45 μ filter to remove B95-8 cells. After four hours the volume of the original supernatant was doubled with fresh LM and incubated overnight. Supernatant was removed and the cells were resuspended in 3ml LM supplemented with 15% fetal calf serum (FCS)(LM-15) and 1μg/ml cyclosporin A (Sandoz), which inhibited CD8+ cytotoxic T cells directed against EBV infected cells. The cells were then split equally into two separate 24 well plates and incubated at 37°C. At weekly intervals half of the supernatant was removed from each well and replaced with fresh LM plus cyclosporin A. Cyclosporin A

supplementation was used for the first three weeks after which most anti-EBV CD8+ cells would have died. The progress of the transformation was monitored by microscopy and after 3-5 weeks, the culture was expanded into T25 tissue culture flasks (Corning). Each B cell bulk culture was frozen in LN_2 in aliquots and maintained in culture. Cells were split twice weekly.

D8. Cell Mediated Cytotoxic Responses to HIV-1:

D8.i ^{51}Cr Release Assay on Fresh PBMC:

The following method was adapted from a procedure established in Dr. K. Rosenthal's laboratory (Grant MD, et al. 1992). On day one, 6-10 x 10^6 of fresh purified PBMC from test and control individuals were established at 1 x 10^6/ml in LM. One tenth the total cell number was removed and stimulated with 5 μg/ml PHA for three days at 37°C (stimulator cells). The remainder of the cells (90%) were incubated unstimulated at 37°C (effector cells). After three days the PHA stimulated cells were pelleted by centrifugation, the supernatant removed, and the cells resuspended in 100 μl of HIV-1_{IIIB} supernatant (1.2 x 10^5 infectious units per ml). The cells were incubated for 2 hours at 37°C with occasional mixing followed by the addition of 1 ml of LM. The cells were incubated overnight. On day four the PHA stimulated HIV-1 infected cells were treated with 50 μl mitomycin C (0.5 mg/ml) (Sigma) for 20 minutes at 37°C. Mitomycin C treatment cross links the DNA of the stimulator cells which prevents them from proliferating (back stimulation). Stimulator cells were washed four times in 10 ml PBS, resuspended in 1 ml LM, and added to the effector cells. Three days later (day

seven of the assay) an equal volume of LM supplemented with 10 units/ml recombinant human interleukin 2 (IL-2) (Genzyme), was added to the culture to expand proliferating cells. At day ten 1 x 10^6 autologous Epstein Barr Virus (EBV) transformed B cells (the targets) were infected with one of four recombinant vaccinia/HIV-1 viral vectors by resuspending the cell pellet in 1.5 x 10^7 plaque forming units (MOI=15) for one hour, 1 ml LM was added and incubated overnight at 37°C. The recombinant viruses have HIV-1 structural genes inserted into a bacterial ß-galactosidase gene located within the vaccinia genome (Chakrabarti S, et al., 1985). The recombinant vaccinia/HIV-1 viruses include vSC8 (wild type vaccinia with the ß-galactosidase gene), vDK1 (vSC8 HIV-1 *gag* inserted into vSC8), vCF21 (HIV-1 *pol* inserted into vSC8) and vPE16 (HIV-1 *env* inserted into vSC8). The viral vectors were obtained through the AIDS Research and Reference Reagent Program, AIDS Program, NIAID, NIH.

On day 11 of the assay the effector cells were counted and resuspended at 5 x 10^6/ml in LM. The recombinant vaccinia/HIV-1 infected autologous B cells were pelleted and resuspended in 200 μl of a 1:1 dilution of LM and ^{51}Cr sodium chromate for 90 minutes at 37°C. The target cells were washed four times with 5 ml of 37°C PBS supplemented with 1% fetal calf serum (FCS). The target cells were counted on a haemocytometer and resuspended at 1 x 10^5/ml in LM. The experimental wells (Exp) were set up in duplicate with 5 x 10^3 targets in each well with effector to target ratios of 50:1, 25:1 and 12.5:1 and LM was added to a final volume 200 μl. One set of wells contained only the target cells and another set contained target cells plus 1% SDS (sodium dodecyl sulphate) to determine the spontaneous (Min) and maximal release

(Max), respectively, of ^{51}Cr from the labelled cells. Once established the plates were centrifuged at 400 x g for five minutes to maximize cell to cell contact and incubated for 4-5 hours at 37°C. At the end of the incubation 100 μl of supernatant was removed from each well and combined with 1 ml of scintillation cocktail (Ecolume, ICN). ^{51}Cr release was detected using a liquid scintillation counter (LS 5000 CE, Beckman). Percent spontaneous release was calculated as Min/Max x 100. Percent specific lysis was calculated by Exp - Min/Max - Min x 100. Lysis of the B cells infected with the recombinant vaccinia/HIV-1 (vDK1, vCF21 and vPE16) viruses was considered significant if lysis was 10% or greater than the lysis of the vaccinia control (vSC8).

D8.ii ^{51}Cr Release Assay on Frozen PBMC:

The methods for the detection of HIV-1 specific cellular lysis using frozen PBMC was identical with the procedure for fresh PBMC with the exception that a second cryovial of PBMC cells was thawed on day 3 and cultured overnight to serve as the effector cells. This decreased the length of time the effector cells were cultured without stimulation from four days to one day.

RESULTS:

OVERVIEW:

Over 1800 women have been enrolled in the Nairobi Prostitute Cohort since 1985. Despite an intense level of exposure to HIV-1 over several years some women have not seroconverted to HIV-1. Part I of the results section addresses the possibility that these persistently seronegative women are resistant to HIV-1 infection and Part II examines the potential mechanisms of this HIV-1 resistance.

PART I - DETERMINATION OF RESISTANCE

A. HIV-1 Serology:

The presence of HIV-1 specific humoral IgG antibodies was detected using commercial ELISA and Western blot kits as described in Methods. Table VI shows the initial and most recent ELISA and Western blot results for many of the women who have remained seronegative for at least three years. We define any woman who is actively engaged in prostitution and who has remained seronegative for three years or more, some up to 9 years, as being persistently seronegative (PSN).

Table VI: HIV-1 Serological Results for the Persistently Seronegative Prostitutes

Study No.	Date	Elisa	Blot	Study No.	Date	Elisa	Blot
ML	28.3.85	-	-	ML	21.6.88	-	ND
466	3.5.94	-	ND	1025	28.6.88	-	-
					26.4.94	-	ND
ML	12.9.85	-	ND	ML	16.5.89	-	ND
546	3.3.88	-	-	1070	21.9.90	-	-
	18.11.93	-	ND		18.5.94	-	ND
ML	11.11.86	-	ND	ML	14.5.90	-	ND
630	1.9.87	-	-	1250	6.11.90	-	-
	15.11.93	-	ND		26.4.94	-	ND
ML	2.3.87	-	ND	ML	2.4.90	-	ND
825	10.12.91	-	ND	1260	21.8.90	-	-
	10.11.92	-	ND		26.4.94	-	ND
ML	14.4.87	-	ND	ML	24.4.90	-	ND
851	10.7.90	-	-	1275	30.1.93	-	-
	26.4.94	-	ND		26.4.94	-	ND
ML	28.4.87	-	ND	ML	22.5.90	-	ND
857	31.5.93	-	-	1286	3.5.94	-	ND
	28.4.94	-	ND				
ML	28.4.87	-	ND	ML	16.10.90	-	ND
858	13.10.87	-	-	1327	22.4.94	-	ND
	26.4.94	-	ND				
ML	26.5.87	-	ND	ML	19.2.91	-	-
870	17.7.90	-	-	1356	16.5.94	-	ND
	15.2.93	-	ND				
	21.5.93	+	ND				
ML	23.6.87	-	ND	ML	6.3.91	-	ND
887	16.7.87	-	-	1358	3.5.94	-	ND
	26.4.94	-	ND				
ML	23.6.87	-	ND	ML	19.3.91	-	ND
889	28.4.94	-	ND	1362	28.4.94	-	ND
ML	20.6.87	-	ND	ML	7.5.91	-	ND
893	26.4.94	-	ND	1371	28.4.94	-	ND
ML	7.7.87	-	ND	ML	22.5.91	-	ND
896	3.7.90	-	-	1376	26.4.94	-	ND
	17.11.93	-	ND				
ML	25.8.87	-	ND	ML	11.1.91	-	ND
923	1.9.87	-	-	1378	26.4.94	-	ND
	17.11.93	-	ND				
ML	8.9.87	-	ND				
935	20.3.90	-	-				
	26.4.94	-	ND				

ND = Not Done

B. The Humoral Immune Response of the Persistently Seronegative Women:

To determine if the absence of HIV-1 antibodies represented a general inability of the immune system to mount a humoral response to any viral infection, sera from the PSN women were tested for the presence of antibodies to Rubella virus. Antibody titres above 10 international units was indicative of past exposure to Rubella virus. Table VII shows the Rubella antibody titres were significantly above 10 international units. The antibody titres were performed by the Cadham Provincial Laboratory using the Rubella IgG Antibody IMX kit (Abbott Laboratories).

C. HLA DQA1 Genetic Typing of DNA from Serum and Plasma:

It was possible that, due to a clerical error in the patient study codes or a labelling error of the blood samples, the PSN phenomenum was actually the result of mixing samples from two or more individuals and that no single individual had remained HIV-1 seronegative for at least three years. To determine that sequential serum or plasma samples collected under each PSN patient study code actually were from the same individual, genetic typing of DNA isolated from the samples was performed. Control experiments were first performed to demonstrate that DNA could be isolated and amplified from serum or plasma.

Ci. Control Experiments:

Before valuable biological material from Nairobi was used, the conditions of DNA isolation and HLA DQA1 amplification were established using serum or plasma from

Table VII: Antibody Titres to Rubella Virus

Study Number	HIV-1 Status	Sample Date	IgG Titre (IU/ml)
546	PSN	20.3.91	181.0
825	PSN	22.11.90	92.6
857	PSN	26.3.91	349.7
870	PSN	22.11.90	271.6
887	PSN	22.3.91	85.1
893	PSN	20.11.90	104.0
923	PSN	9.7.91	213.3
935	PSN	18.7.91	204.4
1025	PSN	2.7.91	274.8

IU = International Units

Winnipeg laboratory workers. A silica gel matrix was used to isolate DNA from serum. The DNA was then detected by the amplification and subsequent probing of the HLA DQA1 locus. The HLA DQA1 locus was chosen because it is highly polymorphic and using allele specific oligonucleotides (ASO) the HLA DQA1 genotype of one sample can be compared to a second sample. Figure 1 shows that amplifiable DNA was successfully isolated from 50μl of serum or plasma.

Cii. Test Experiment:

After establishment of experimental conditions, serum or plasma isolated from 13 PSN individuals in 1985/86 and again in 1990/91 were subjected to DNA extraction, amplification (Figure 2 top) and probed with allele specific oligonucleotides (Figure 2 bottom). Table VIII is a summary of all of the ASO typing data and shows that for each study code the serum or plasma samples, collected years apart, share the same HLA DQA1 genotype. The entire DNA extraction, HLA DQA1 amplification, and ASO typing process was performed on a sample of water (DNA-H_20) to ensure that no cross contamination occurred at any point in the procedure.

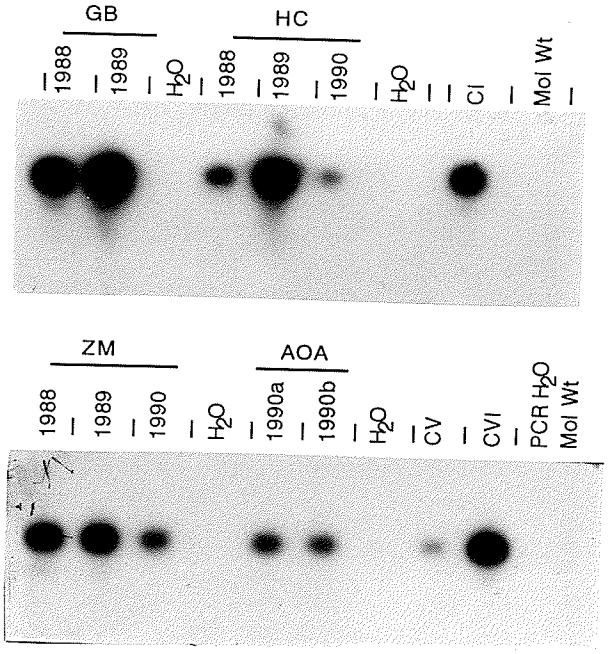


Figure 1: Isolation of DNA from Serum.

Figure 1. Isolation of DNA from Serum or Plasma.

The samples used to establish conditions of DNA isolation from serum or plasma were from Winnipeg laboratory workers GB, HC, ZM and AOA. DNA was isolated using a silica gel matrix and amplified with HLA DQA1 primers GH26 and GH27. The amplification products were resolved on a 3.5% agarose gel and Southern blotted onto a nylon membrane where they were probed with ^{32}P labelled GH76 oligonucleotide which recognized all alleles of HLA DQA1 except 1.3. The figure is the autoradiogram after the blot was exposed to X-ray film for 24 hours. The lanes are marked by the laboratory worker and the year the serum was isolated. The negative controls were water (H_2O) that had been carried through the entire DNA isolation and HLA DQA1 amplification procedure and PCR H_2O which was the reagent control for the PCR reaction. Positive controls CI, CV and CVI were PBMC lysates from laboratory workers GB, AOA and KRF, respectively.

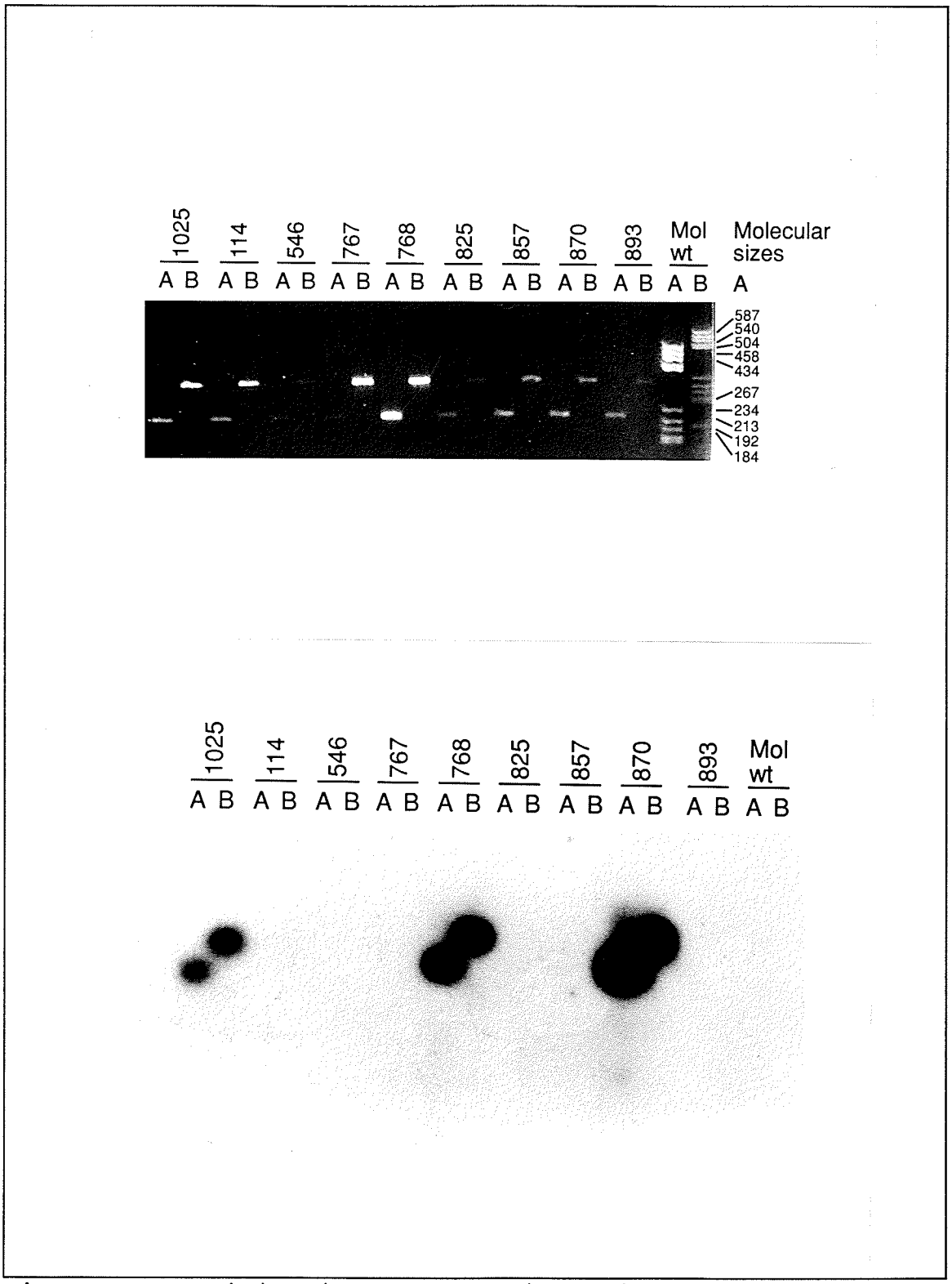


Figure 2: Amplification and Genetic Typing of HLA DQA1 Locus using DNA Isolated from Serum or Plasma:

Figure 2. Amplification and Genetic Typing of HLA DQA1 Locus using DNA Isolated from PSN Serum or Plasma.

Top: Amplification products were resolved on an ethidium bromide stained 3.5% agarose gel. To prevent inter-well leakage and to increase spacing between samples while maximizing gel space the gel was loaded in a staggered way. The "A" series of samples and molecular weight standards were loaded into every second well and electrophoresis was begun. After 15 minutes the electrophoresis was stopped, the "B" series of samples, including the molecular weight standards, were loaded into the empty alternate wells and electrophoresis was resumed until completion. The predicted length of the amplification product of HLA DQA1 locus is 242 base pairs. The amplification product for the "A" series is 242 base pairs when compared to the "A" molecular weight standard and similarly with the "B" series of amplification products when compared to the "B" molecular weight. Only the "A" molecular weight standards are labelled (in base pairs).

Bottom: The amplification products loaded onto the agarose gel described in Figure 3A were transferred onto a nylon membrane and probed with ^{32}P labelled allele specific oligonucleotide HE46 which identifies the HLA DQA1 allele 4 subtypes 4.2/4.3.

Table VIII: Binding Patterns of HLA DQA1 Allele Specific Oligonucleotides to Amplification Products of DNA Isolated from Plasma and Serum.

Study Number	Sample Number	Collection Date	Binding Allele Specific Oligonucleotide (ASO)	HLA DQA1 Alleles
114	114a	21/2/85	RH83,GH89	1.2/1.3
114	114b	26/4/88	RH83,GH89	1.2/1.3
546	546a	14/11/85	RH83,GH89	1.2/1.3
546	546b	20/3/91	RH83,GH89	1.2/1.3
767	767a	13/1/87	GH67,RH71	2;3
767	767b	8/1/91	GH67,RH71	2;3
768	768a	9/1/87	RH83,GH88,RH71,HE46	1.1;2;4.2/4.3
768	768b	25/1/91	RH83,GH88,RH71,HE46	1.1;2;4.2/4.3
825	825a	24/2/87	GH66,GH89,RH71	2;4.1
825	825b	22/11/90	GH66,GH89,RH71	2;4.1
857	857a	6/10/87	RH83,GH89,RH71	1.2/1.3;2
857	857b	26/3/91	RH83,GH89,RH71	1.2/1.3;2
870	870a	30/6/87	GH66,RH83,GH88,HE46	1.1;4.2/4.3
870	870b	22/11/90	GH66,RH83,GH88,HE46	1.1;4.2/4.3
893	893a	7/8/87	GH66,GH89	4.1
893	893b	20/11/90	GH66,GH89	4.1
896	896a	11/9/87	GH66,GH89	4.1
896	896b	21/1/91	GH66,GH89	4.1
923	923a	29/9/87	GH66,GH89	4.1
923	923b	9/7/91	GH66,GH89	4.1
935	935a	8/9/87	GH66,RH83,GH89	1.2/1.3;4.1
935	935b	18/7/91	GH66,RH83,GH89	1.2/1.3;4.1
968	968a	18/2/88	RH83,GH88,GH89,HE46	1.1;4.2/4.3
968	968b	9/11/90	RH83,GH88,GH89,HE46	1.1;4.2/4.3
1025	1025a	28/6/88	GH66,RH83,GH88,GH89,HE46	1.1;4.2/4.3
1025	1025a	2/7/91	GH66,RH83,GH88,GH89,HE46	1.1;4.2/4.3
DNA-H_2O	DNA-H_2O	-	-	-

D. DEGREE OF EXPOSURE:

Of 424 initially seronegative women followed for 1 to 9 years, 239 have seroconverted to HIV-1, in spite of comprehensive prevention efforts (Ngugi EN, et al. 1988). The overall HIV-1 seroincidence was 42 per 100 person years. The magnitude of sexual exposure to HIV-1 in this cohort can be estimated by $U = a \cdot b \cdot c \cdot d$, where U=unprotected sexual exposures per year, a=average number of clients per day, b=estimated work days per year, c=HIV-1 prevalence among clients, and d=proportion of clients not using condoms. In 1986, 12% of the clients were HIV-1 seropositive (Simonsen JN, 1988) and this prevalence has since increased substantially. Using cohort averages (Simonsen JN, et al. 1990; Plummer FA, et al. 1991) (a=4, b=200) and adjusting condom use and HIV-1 prevalence among clients for observed increases (Ngugi EN, et al. 1988), the average number of unprotected sexual exposures to HIV-1 per year for these women increased from 24 in 1984 to 60 in 1993. Since the number of unprotected exposures to HIV-1 infected partners has increased over time, the risk of seroconversion should also have increased, in a homogeneously exposed population. However as shown in Figure 3, after a very high HIV-1 seroincidence over the first two years of follow up, HIV-1 prevalence began to plateau and the incidence declined markedly, which was contrary to the expected increasing risk of seroconversion. Among women remaining seronegative for three years, the seroincidence declined markedly and a fraction of women remained persistently seronegative for periods of up to nine years.

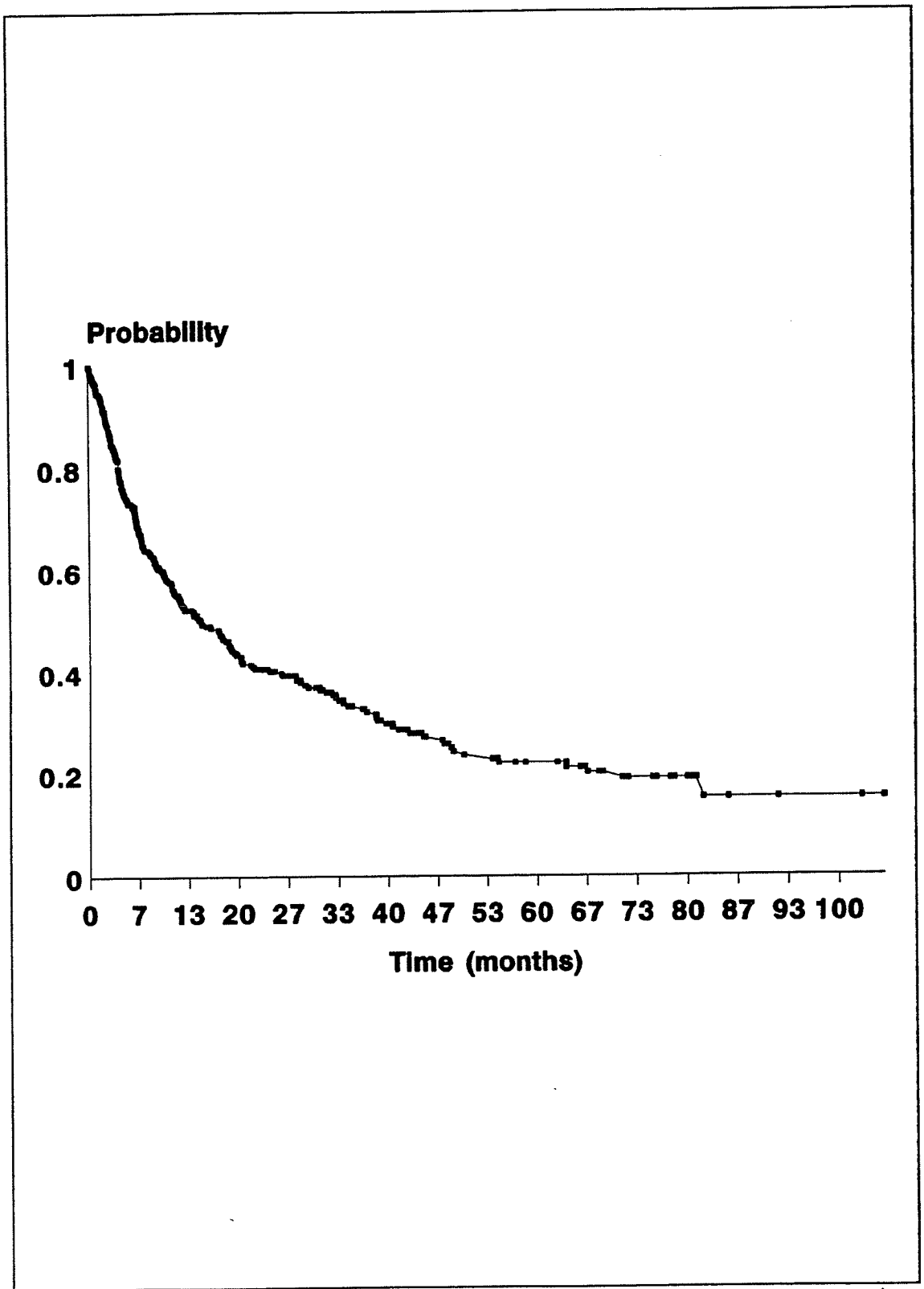


FIGURE 3: Survival Curve of the Time to HIV-1 Seroconversion Among 424 Prostitutes in Nairobi.

FIGURE 3. Survival Curve of the Time to HIV-1 Seroconversion Among 424 Prostitutes in Nairobi.

Kaplan-Meier plot of the time to HIV-1 seroconversion is shown with the marked line. Women were followed at six month intervals and blood was taken for HIV-1 serology. Seroconversion was defined as a positive HIV-1 enzyme immunoassay and a positive immunoblot or confirmatory enzyme immunoassay, among women previously seronegative. The time of seroconversion was estimated as the mid-point between the last seronegative and the first seropositive date.

E. Survival Modelling of Time to HIV-1 Seroconversion:

With the assistance of Dr. N. Nagelkerke three mathematical models commonly used in survival analyses (Elandt-Johnson RC) were fitted to the cohort time to seroconversion data. The first model was an exponential model that assumes that all individuals are equally susceptible to HIV-1 infection and that given time all individuals will become infected. The second model was a Weibull model that assumes that susceptibility to HIV-1 infection declines over time, by the factor b, to the point that some individuals become resistant to infection. The third model, called the mixture model, assumes that a portion of the cohort was susceptible to HIV-1, as described by the exponential model, but a fraction of the cohort (f) were totally resistant to HIV-1 infection from Day 1. The models were fitted to the survival data using maximum likelihood with interval censoring. Log-likelihoods were 1772.9, 1758.6 and 1753.7 for the exponential, Weibull and mixture models respectively. As seen in Figure 4, the mixture model described the survival curve better than Weibull model (Chi-square 38.4, $p < .00001$) which was itself a better description than the exponential model.

F. Declining Risk of Infection vs. Increasing Exposure:

If the risk of HIV-1 infection declines over time as a result of the selection of resistance to HIV-1 infection, the degree of protection should be related to the extent of exposure. To examine this, we compared the incidence of HIV-1 infection among women with a duration of prostitution of more than three years with that of women with a duration of prostitution of less than three years, for calendar years 1989 through 1993.

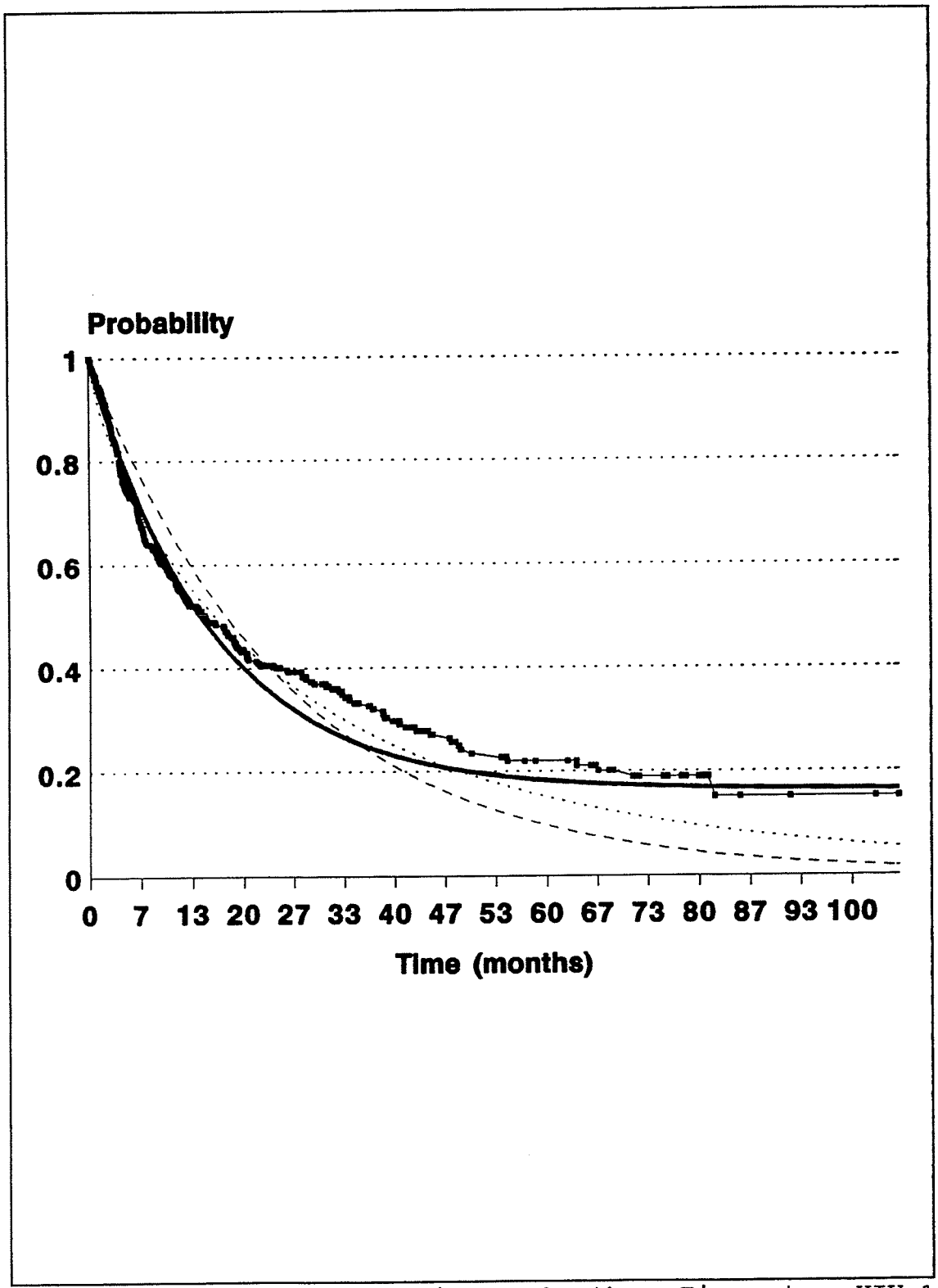


FIGURE 4: Survival Modelling of the Time to HIV-1 Seroconversion Among 424 Prostitutes in Nairobi.

FIGURE 4. Survival Modelling of the Time to HIV-1 Seroconversion Among 424 Prostitutes in Nairobi.

The survival curve described in Figure 3 is shown with the marked line. Three models were fitted to the data using maximum likelihood with interval censoring. The dashed line shows the expected time to seroconversion if the seronegative survival time (S_t) is exponentially distributed ($S_t=e^{at}$ or $S_t=\exp(at)$, model parameter a=-0.0013, in this instance). The dotted line shows expected time to seroconversion under a Weibull distribution ($S_t=\exp(at^b)$ with a=-0.00589 and b=0.77). The solid unmarked line shows the expected time to seroconversion from a mixture model ($S_t=f+(1-f)e^{at}$, f being the fraction of the population which is resistant to infection, f=.164, a=-0.0021).

An increasing protective effect for each seronegative year of prostitution was observed (1989: Odds Ratio(OR)=1.7, 95% confidence intervals (95%CI) 0.6-4.84, p=0.33, 1990: OR=3.5, 95% CI 1.26-9.45, p=0.02, 1991: OR=4.43, 95% CI 1.1-17.46, p=0.03, 1992: OR=4.76, 95% CI 1.36-16.5, p=0.01, 1993: OR=12.8, 95% CI 3.81-43.3, $p<0.0001$). However, the extent of exposure is proportional not only to the duration of prostitution, but also to HIV-1 prevalence among clients (increasing), the number of clients (constant) and condom use (increasing), with the net effect being an increase in overall infection pressure during the HIV-1 epidemic in Nairobi. To account for this effect, each year of prostitution was weighted and examined the relationship between weighted year of exposure as a time-varying independent variable and the risk of seroconversion by Cox regression modelling (Cox DR, et al. 1972). As shown in Figure 5, women who initiated prostitution in 1984 and who remained HIV-1 uninfected through 1984 had a relative risk of HIV-1 seroconversion in 1985 of .93, compared to women who began prostitution in 1985. Similarly, women who became prostitutes in 1992 and escaped infection in that year had a .35 relative risk of seroconversion during 1993, compared to women who became prostitutes in 1993. The estimated cumulative protective effect, for women practising prostitution from 1984 through 1993 and remaining seronegative, compared to women beginning prostitution in 1994, was approximately a 100-fold reduction in the risk of infection.

Thanks to Dr. F. A. Plummer for performing the above analyses.

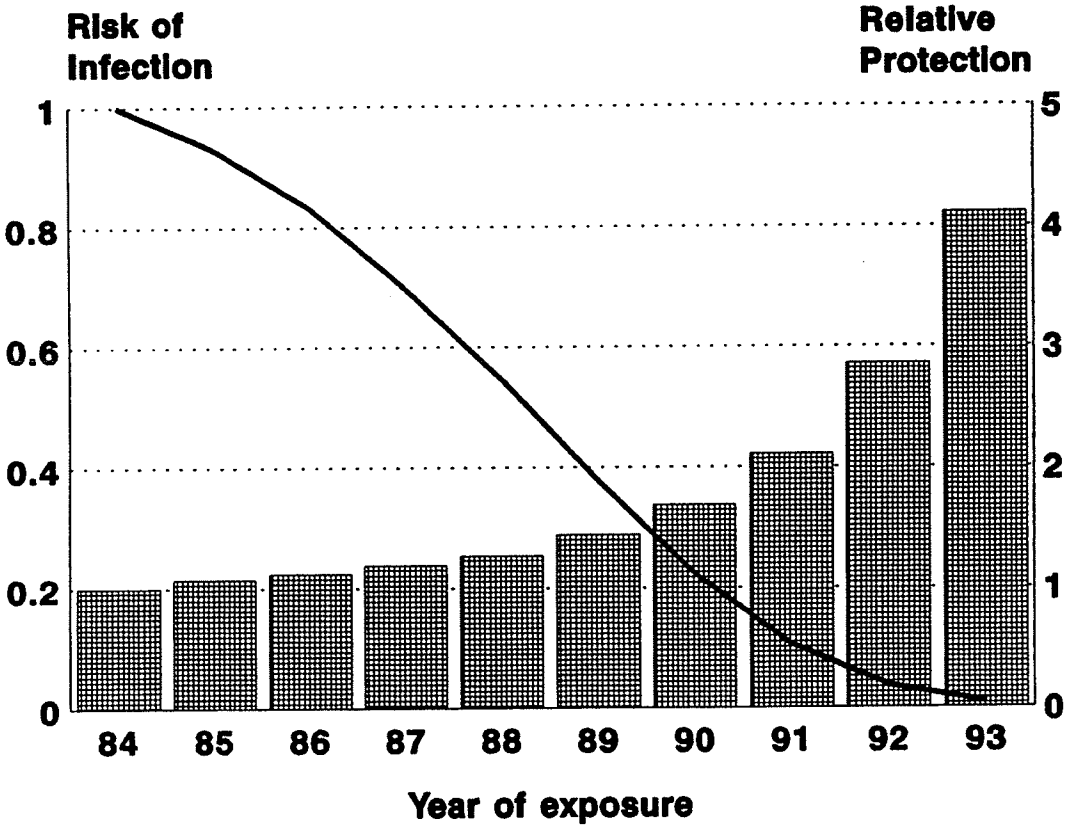


FIGURE 5: Protective Effect of Exposure to HIV-1 Through Prostitution on Subsequent Risk of Seroconversion.

FIGURE 5. Protective Effect of Exposure to HIV-1 Through Prostitution on Subsequent Risk of Seroconversion.

Although the risk of HIV-1 seroconversion has declined with time in this cohort, simply using years of exposure as a measure of any protection conferred does not take into consideration the changes in infection pressure resulting from increases in client HIV-1 prevalence and increasing use of condoms. To account for this effect, we calculated an exponentially weighted duration of prostitution for each, with each year of prostitution weighted by $e^{(\pi x \text{calendar year})}$, π being a measure of the increase in the infection pressure per year. Cox proportional hazard modelling with the weighted duration of prostitution as a time varying independent variable was used to analyze the relationship between this variable and HIV-1 incidence. π was estimated by maximizing the Cox likelihood with respect to π, yielding a value for π of .12 which was significantly larger than 0, $p<0.001$. This value corresponds to a doubling in infection pressure every $\ln 2/.12=5.8$ years. The cumulative reduction in risk is shown with the continuous line (left Y axis) and the relative protective effect for each year relative to the subsequent year is shown with the histogram (right Y axis).

G. Analysis of Risk Factors for the Acquisition of HIV-1:

Although the risk of HIV-1 infection decreased with the time of exposure, the decline in risk may have been related to the selection of women with safer sexual behaviours or immunity to STD which facilitate HIV-1 transmission, as well as to resistance to HIV-1. However, the duration of prostitution remained significantly associated with a reduction in HIV-1 incidence (hazard ratio=0.73, 95% confidence intervals 0.66-0.8, $p < .001$) when age, number of sex partners, number of regular sex partners, condom use, gonococcal and chlamydial cervicitis and the frequency of genital ulcers disease were included in the Cox modelling as independent variables. This indicated that the protective effect of increasing exposure was not explained by these factors.

Thanks to Dr. F. A. Plummer for performing the risk factor analyses.

H. Seronegative HIV-1 Infection:

It was possible that the PSN women were infected by HIV-1 but were not producing a humoral immune response, ie. a seronegative HIV-1 infection. Since HIV-1 ELISA and western blot tests detect the immune response to HIV-1 and not the virus itself, a test was needed that would directly detect the presence of HIV-1. The polymerase chain reaction (PCR) is sufficiently sensitive to detect HIV-1 provirus in infected lymphocytes and was chosen to determine if the PSN women are infected by HIV-1.

Hi. Establishment of HIV-1 PCR:

At the time this study was initiated in 1989 little data were available about HIV-1 PCR and it was decided that a novel system was required. Regulatory genes were chosen as the targets for amplification because of their high degree of sequence conservation between HIV-1 isolates. Using the sequence information made available through the Los Alamos AIDS and Human Retrovirus database, conserved regions of *vif*, *nef* and *rev* genes were selected for amplification primers and internal probes (for oligonucleotide sequences see Table I). For comparison purposes, primers SK68' and SK69' and probe SK70', which were based on published PCR oligonucleotides to *env* (Ou C-Y, et al. 1988), were used. Figure 6 shows the locations of the amplification target regions on a schematic of the HIV-1 proviral genome. Optimal conditions for HIV-1 PCR were established using HIV-1 infected PBMC from Winnipeg patients and the plasmid pBH10R-3, which has the full length HIV-1_{BH10} provirus, minus a portion of the 5' LTR, cloned into the plasmid pSP64 (Ratner L, et al. 1987). Amplification products of the correct predicted length hybridized to radiolabelled internal oligonucleotides (Figure 7).

The limit of detection of these PCR systems was determined by serially diluting a known amount of plasmid pBH10R-3 in a background of uninfected genomic DNA from the CD4+ T-lymphocyte cell line Molt. As can be seen in Figure 8 the nef PCR system can detect 3.8 viral copies in a background of DNA from 150,000 cells. Table IX summarizes the results for the limit of detection of the various PCR systems and shows that the *vif* and *nef* PCR systems were as sensitive as the published *env* system, while the *rev* system was ten-fold less sensitive.

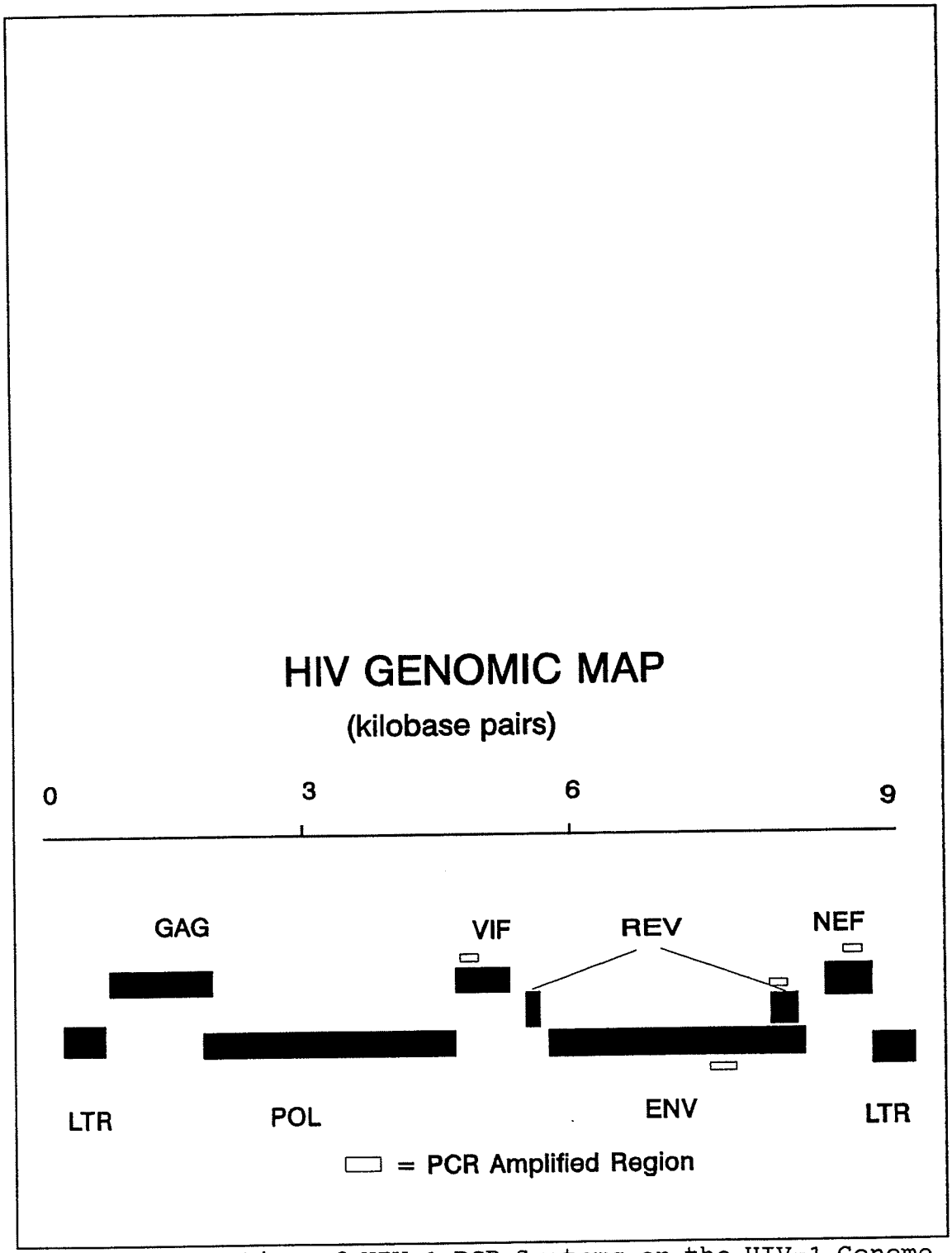


Figure 6: Location of HIV-1 PCR Systems on the HIV-1 Genome.

Closed boxes represent the open reading frames of HIV-1 and the open boxes represent the regions amplified by the various PCR systems.

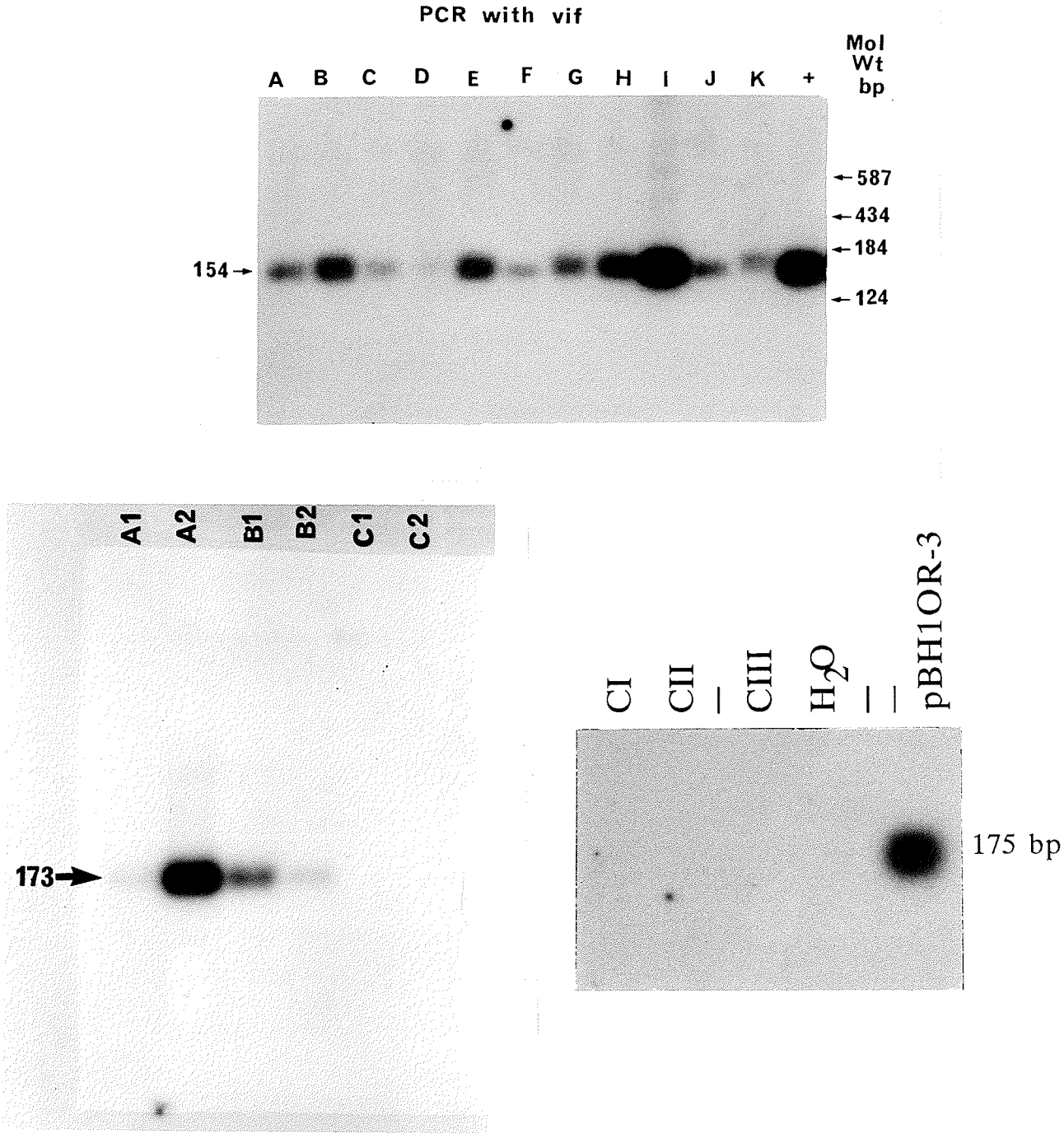


Figure 7: Production of Amplification Products that are the Predicted Molecular Weight and Hybridize to the HIV-1 PCR Probes.

Figure 7: Amplification and Detection of PCR Products from Regulatory Genes.

Figure 7 (top) is a Southern blot of the amplification products of vif PCR from the PBMC of 11 different HIV infected Winnipeg individuals (A-K) probed with the internal oligonucleotide vif-B. The predicted size of the vif PCR product is 154 bp which corresponds to the band hybridizing to the probe. The plasmid pBH10R-3 was used as a positive control (+) and the molecular weight standard used was Boehringer Mannhiem Molecular Weight V.

Figure 7 (bottom-left) is a Southern blot of nef PCR products probed with the internal oligonucleotide nef-B. Samples A1, A2, B1 and B2 are PBMC from HIV-1 infected Winnipeg individuals. Samples C1 and C2 are PBMC from HIV-1 uninfected laboratory workers (negative controls). The predicted size of the amplification products of nef PCR is 173 bp which is the size of the band hybridizing to nef-B.

Figure 7 (bottom-right) is a Southern blot of rev PCR products probed with the internal oligonucleotide rev-B. Negative controls, samples CI, CII, and CIII, are PBMC from HIV-1 uninfected laboratory workers while the positive control is plasmid pBH10R-3. The probe rev-B hybridized to a 175 bp fragment which is the predicted size of the rev amplification product.

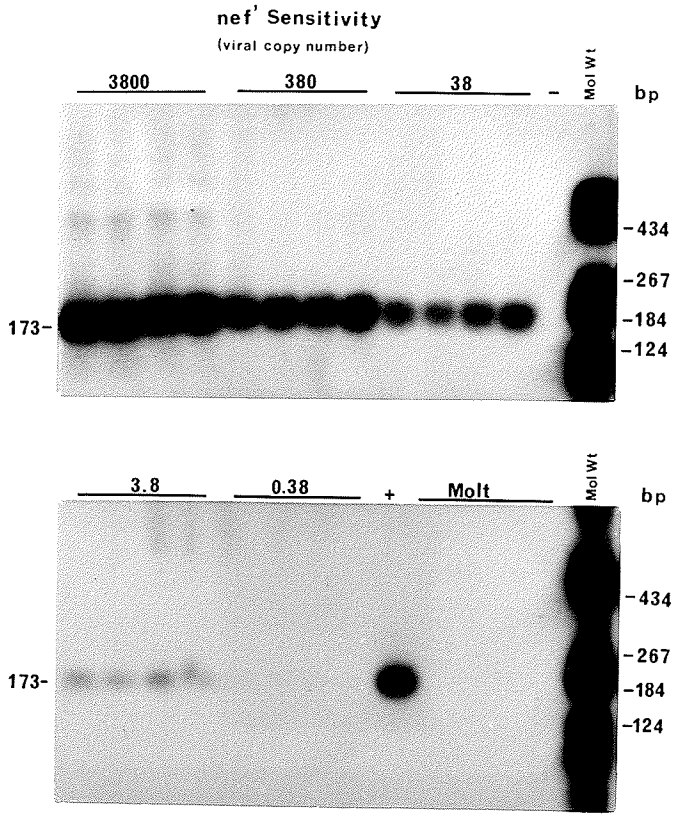


Figure 8: *nef* PCR System Limit of Detection

Figure 8: *nef* PCR System Limit of Detection

A known amount of the plasmid pBH10R-3 was serially diluted in a background of HIV-1 uninfected genomic DNA. One μg of human genomic DNA, the equivalent of DNA from 150,000 cells was maintained in each PCR reaction while the amount of plasmid varied. Each dilution was tested in quadruplicate. Negative controls consisted of the reagent control (-) and uninfected Molt DNA alone (Molt), while the positive control was undiluted pBH10R-3 in ddH_2O (+). For this experiment the molecular weight standards were 5' end labelled with ^{32}P and are labelled as shown.

Table IX: Limit of Detection of the Various HIV-1 PCR Systems.

Target Gene	Primers Used	# of Viral Copies Detected/150,000 cells
env	SK68' & SK69'	3.8
vif	vif-A' & vif-C'	3.8
nef	nef-A' & nef-C'	3.8
rev	rev-A' & rev-C'	38.0

The PCR systems were shown to be very sensitive and able to detect HIV-1 infection in Winnipeg specimens however, due to the paucity of sequence information of African isolates, especially East African isolates, it was not known if these PCR systems would detect Kenyan isolates of HIV-1. To determine this a pilot study of HIV-1 infected PBMC from 4 Winnipeg and 7 Kenyan individuals was conducted. As seen in Figure 9 the *env* (SK) PCR system was able to detect Winnipeg and Kenyan isolates. Table X is a summary of the PCR results from the same pilot study using the other PCR systems. The *env*, *vif* and *nef* PCR systems were all able to detect Kenyan isolates and were chosen for further studies. Since the *rev* PCR system had a limit of detection 10-fold less than the other systems and did not detect any of the Kenyan isolates its use was discontinued.

Hii. Sensitivity and Specificity of the HIV-1 PCR Systems:

The sensitivity and specificity of the HIV-1 PCR systems were determined in a second pilot study that examined 22 known HIV-1 seropositive and 17 seronegative individuals from the Nairobi cohort. A PCR positive sample was defined as one that reacted with at least two PCR primer sets. With this definition the sensitivity and specificity of the combined PCR systems, when compared with HIV-1 serology, were 100% and 88%, respectively (Table XI).

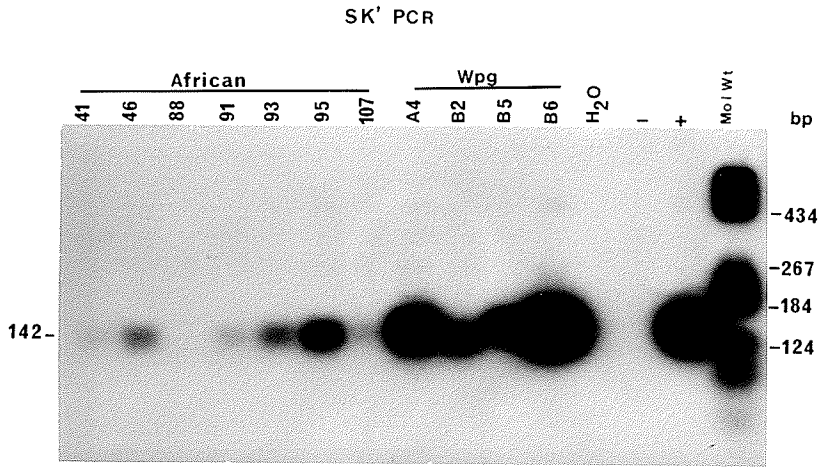


Figure 9: Detection of Kenyan HIV-1 Isolates with the *env* (SK') PCR system.

Figure 9: Detection of Kenyan HIV-1 Isolates with the *env* (SK') PCR system.

Southern blot of the amplification products from env PCR probed with the radiolabelled internal oligonucleotide SK70'. Samples 41, 46, 88, 91, 93, 95 and 107 are from Kenya, samples A4, B2, B5 and B6 are from Winnipeg. The negative controls are the reagent control (H_20) and uninfected PBMC (-) and the positive control (+) is the plasmid pBH10R-3. The molecular weight standards were 5'end labelled with ^{32}P.

Table X: HIV-1 PCR on Winnipeg and Kenyan Specimens.

	Specimens										
	African							Winnipeg			
Primers	41	46	88	91	93	95	107	A 4	B 2	B 5	B 6
env	+	+	ND	+	+	+	+	+	+	+	+
vif	+	+	+	-	+	+	+	+	+	+	+
nef	+	+	ND	ND	ND	ND	ND	+	+	+	+
rev	-	-	-	-	-	-	-	+	+	+	+

ND = Not Done

Table XI: Sensitivity and Specificity of HIV-1 PCR on Nairobi Samples.

Sensitivity	*vif*	*env*	*nef*	total
PCR+ of Antibody+	20/21=.95	20/22=.91	21/21=1.0	21/21=1.0

Specificity	*vif*	*env*	*nef*	total
PCR- of Antibody-	13/17=.76	15/17=.88	15/17=.88	15/17=.88

Hiii. HIV-1 PCR on PBMC from the Persistently Seronegative Women:

HIV-1 PCR was performed on lysed PBMC of the PSN women. Figure 10 is an example of a typical HIV-1 PCR Southern blot. Table XII summarizes the data from all of the PCR experiments performed and shows 91% (21/23) of the PSN women tested have remained PCR negative. The two who became PCR positive had only been enrolled in the cohort for 3 years; the minimum time to be included in the PSN category.

Part I Results Summary:

This section of the results has presented evidence to suggest a) the phenomena of persistent seronegativity is real, b) these women have been sufficiently exposed to HIV-1 that it is statistically improbable that they remain uninfected, c) seronegative HIV-1 infection as assayed by PCR does not explain the absence of infection and d) differences in HIV-1 exposure or acquisition co-factors can not account for the fact that they remain uninfected. Thus, by a process of elimination, it can be hypothesized that these persistently seronegative women remain uninfected because they are resistant to HIV-1. Part II of RESULTS explores potential mechanisms of this resistance.

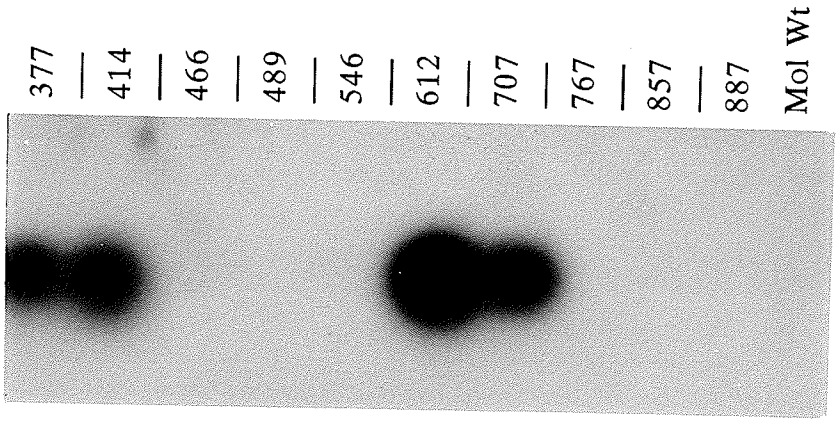


Figure 10: HIV-1 PCR on PBMC from the Nairobi Prostitute Cohort.

Figure 10: HIV-1 PCR on PBMC from the Nairobi Prostitute Cohort.

HIV-1 *vif* PCR was performed on PBMC from the Nairobi Prostitutes. The amplification products were resolved on a 3.5% agarose gel, Southern transferred to a nylon support, and probed with ^{32}P labelled internal oligonucleotide Vif-B. The samples are identified by the study codes of the individuals tested and are labelled on the figure. MLs 466, 546, 857 and 887 are PSN. MLs 377, 414, 612 and 707 are HIV-1 seroconverters. MLs 489 and 767 were HIV-1 seronegative at this time but have subsequently seroconverted. All PBMC were isolated between November 1990 and July 1991.

Table XII: HIV-1 PCR on PBMC from the Persistently Seronegative Women:

Study Number	Date Collected	HIV-1 PCR	Study Number	Date Collected	HIV-1 PCR
ML 466	12.4.91	-	ML 1025	3.7.91	-
	3.5.94	-		26.4.94	-
ML 546	20.3.91	-	ML 1070	ND	
ML 556	19.10.90	+	ML 1250	26.4.94	-
	3.5.94	-			
ML 630	26.10.90	-	ML 1260	26.4.94	-
ML 825	22.11.90	-	ML 1275	26.4.94	-
ML 851	ND		ML 1286	ND	
ML 857	26.3.91	-	ML 1327	22.4.94	-
	28.4.94	-			
ML 858	26.11.90	-	ML 1356	16.5.94	-
	26.4.94	-			
ML 870	22.11.90	-	ML 1358	3.5.94	+
ML 887	22.3.91	-	ML 1362	ND	
	26.4.94	-			
ML 889	28.4.94	-	ML 1371	ND	
ML 893	20.11.90	-	ML 1376	26.4.94	-
	26.4.94	-			
ML 923	10.7.91	-	ML 1378	26.4.94	+
ML 935	18.7.91	-	ML 1490	3.5.94	-

ND = Not Done

PART II - EXPLORING MECHANISMS OF RESISTANCE.

A. Innate Resistance:

It is possible that the resistant women are not infected by HIV-1 because their cells can not support the replication of HIV-1. We have termed this ability to resist infection innate resistance. The next section of results will explore the possibility that these women are innately resistant to HIV-1.

B. Receptor Mediated Resistance to HIV-1 Infection:

The interaction of HIV-1 membrane glycoprotein, gp120, with the major cellular receptor, the CD4 molecule, is highly specific and has been mapped to a few amino acid residues (Lasky LA, et al. 1987; Pollard SR, et al. 1991). *In vitro* experiments have shown that alterations in the protein sequence of gp120 or CD4 can have dramatic effects on the ability of HIV-1 to infect the cell (Fleury S, et al., 1991). To determine if a change in the CD4 cellular receptor accounted for the absence of infection in the resistant prostitutes, the CD4 molecule was cloned, sequenced, and the predicted open reading frames compared to published sequences and to sequences derived from seroconverting women.

Bi. Establishing Conditions for CD4 Analysis:

PBMC from Winnipeg laboratory staff were used to establish conditions for the isolation of mRNA, first strand synthesis of CD4 cDNA, amplification, and cloning of the resultant products. Figure 11 top shows the amplification products from two separate PBMC samples from which mRNA was extracted, primed for first strand cDNA

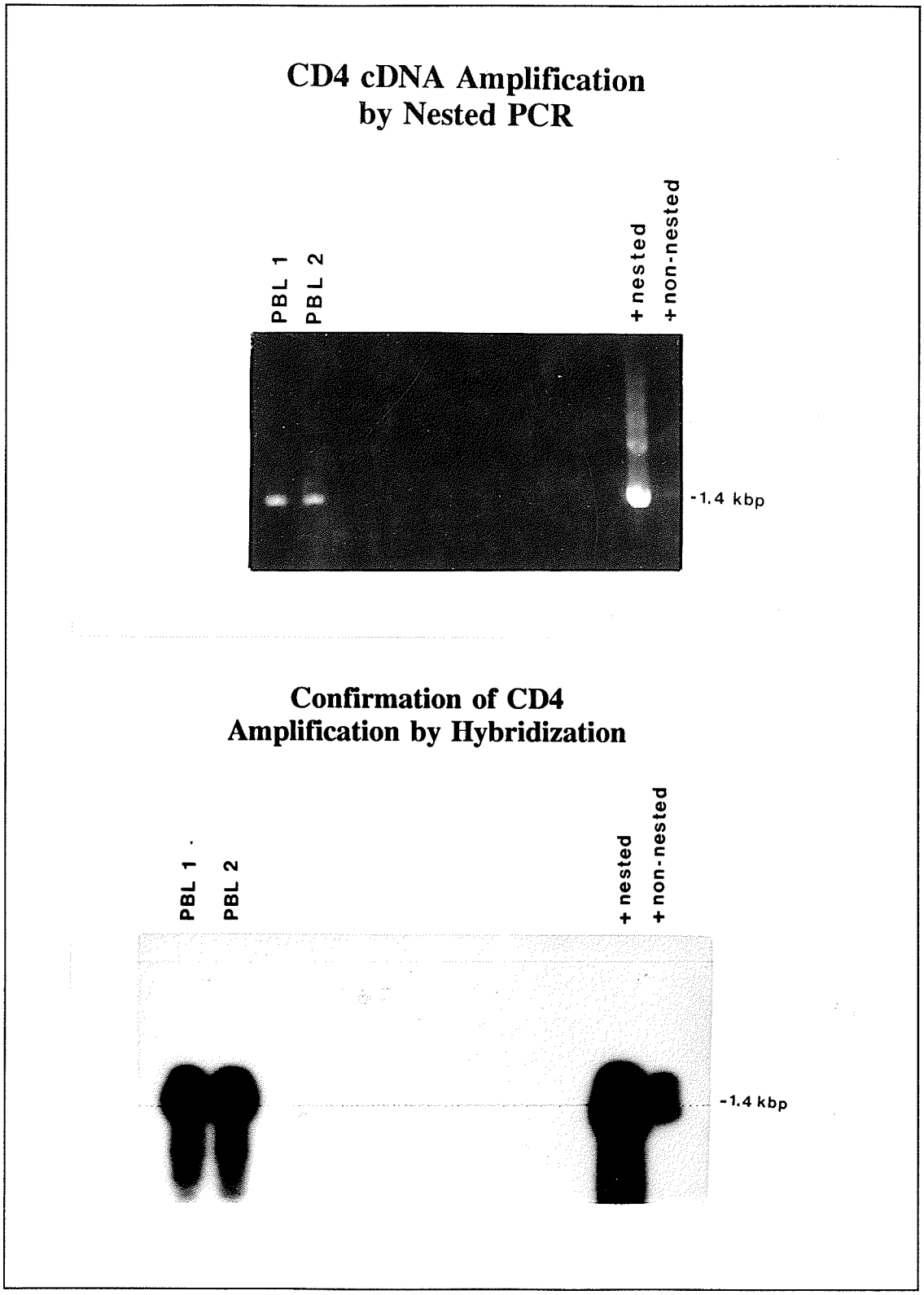


Figure 11: The Generation and Confirmation of CD4 cDNA Amplification Products.

Figure 11: The Generation and Confirmation of CD4 cDNA Amplification Products.

The mRNA was isolated from the PBMC of two Winnipeg laboratory workers KRF (PBL1) and JNS (PBL2), primed for first strand cDNA synthesis with the primer 3'CD4-Nest and amplified using the nested protocol described in Methods. As positive controls the plasmid pT4B, which includes the full length CD4 cDNA, was amplified using the nested PCR strategy (+nested) and using only the internal primers (+non-nested).

Figure 11 (top) is an ethidium bromide stained agarose gel of the amplification products of the CD4 cDNA PCR. The predicted size of the CD4 cDNA amplification is 1.4 kbp which is the size of the product generated.

Figure 11 (bottom) is a Southern blot of the above gel probed with a ^{32}P labelled internal CD4 oligonucleotide, 5' CD4.

synthesis and amplified by nested PCR as described in Methods. The predicted molecular weight of the CD4 cDNA amplification product was 1.4 kbp which was the size of the band observed. A ^{32}P labelled internal CD4 oligonucleotide, 5'-CD4 was used to confirm that the generated product was the CD4 cDNA (Figure 11 bottom). Two positive controls were included that used the plasmid pT4B (Maddon PJ, et al. 1985), containing the full length CD4 cDNA as template. The first control (+nested) was the product of the nested PCR strategy and the second (+non-nested) was the product of PCR using only the internal set of primers. The difference in the intensity of the two signals from the two amplification strategies illustrated the increased sensitivity obtained using the nested PCR strategy.

Initially PBMC from 3 resistant and 3 seroconverting prostitutes were chosen for CD4 analysis. However, after the analysis was complete two of the resistant women seroconverted (ML 825 and 870) altering the ratio to 1 resistant and 5 seropositive women. One to three x 10^6 PBMC were frozen as a pellet in PBS at -70°C in Nairobi and shipped to Winnipeg for mRNA extraction, first strand cDNA synthesis and CD4 amplification. The major product of the PCR was a 1.4 kbp band that corresponded to the band observed in the positive control, pT4B. However, as seen in Figure 12 (top) there were also minor amplification products of smaller sizes. The gel was Southern transferred and probed with the internal CD4 oligonucleotide to confirm that the products amplified were of CD4 origin, Figure 12 (bottom). The 1.4 kbp fragment was excised from the gel and purified by silica gel matrix (Prep-a-Gene, BioRad) as per the manufacturers instructions. The gel purified 1.4 kbp fragment was redigested with EcoR

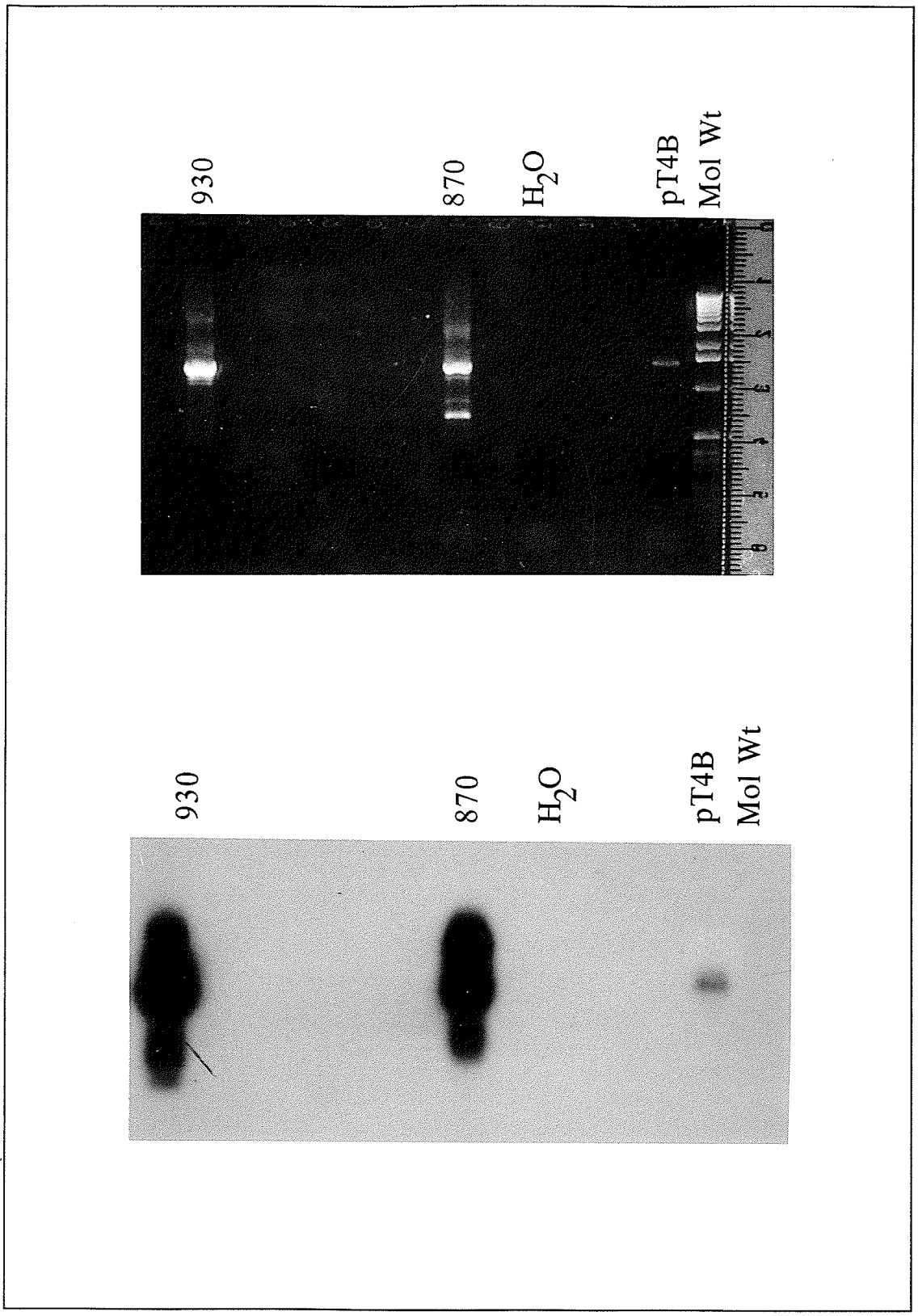


Figure 12: CD4 cDNA Amplification Product from Nairobi Specimens.

Figure 12: CD4 cDNA Amplification Product from Nairobi Specimens.

Total cellular mRNA was isolated from the PBMC of Nairobi prostitutes, first strand cDNA primed with a CD4 oligonucleotide, and amplified with the nested CD4 PCR strategy.

Figure 12 (top) is an ethidium bromide stained gel of the amplification products from prostitutes 930 (lane 1) and 870 (lane 2) and the positive control plasmid pT4B. The molecular weight marker is the 1 kb ladder.

Figure 12 (bottom) is a Southern blot of the above gel probed with the ^{32}P labelled internal CD4 oligonucleotide 5'-CD4.

I and Hind III, gel purified, and ligated into the multiple cloning site of pBluescribe. The ligated plasmids were transformed into *E. coli* strain DH5α and plated on selective medium (pBS plates). White colonies were isolated and small scale plasmid preparations were performed to isolate recombinant plasmids. Plasmids were digested with Nde I which liberated the CD4 cDNA insert due to engineered unique Nde I sites in the internal PCR primers. The 3.2 kbp pBluescribe plasmid contains a single Nde I site at position 184. As can be seen in Figure 13, when pBluescribe with no insert was cut with Nde I it linearized the plasmid, lane 11. However, when a CD4 cDNA was inserted into the plasmid and digested with Nde I, the 1.4 kbp insert was released from the multiple cloning site and the 3.2 kbp plasmid, digested at position 184 generating two fragments of 0.7 and 2.5 kbp.

Bii. Single Strand Conformation Polymorphism Analysis of CD4:

The method chosen for preliminary analysis of the CD4 cDNAs was single strand conformation polymorphism (SSCP) analysis. SSCP is sensitive enough to detect single nucleotide changes. The technique takes advantage of the observation that upon denaturation, a single strand of DNA will fold back onto itself in a conformation that is sequence dependent. The CD4 cDNAs were resolved on a non-denaturing polyacrylamide gel. The rate of migration of single stranded DNA within a non-denaturing gel is conformation, and therefore, sequence dependent. Shifts in the migration patterns of test DNA can be compared to wild type DNA to determine if nucleotide changes are present in test DNA. SSCP is most sensitive for fragments of

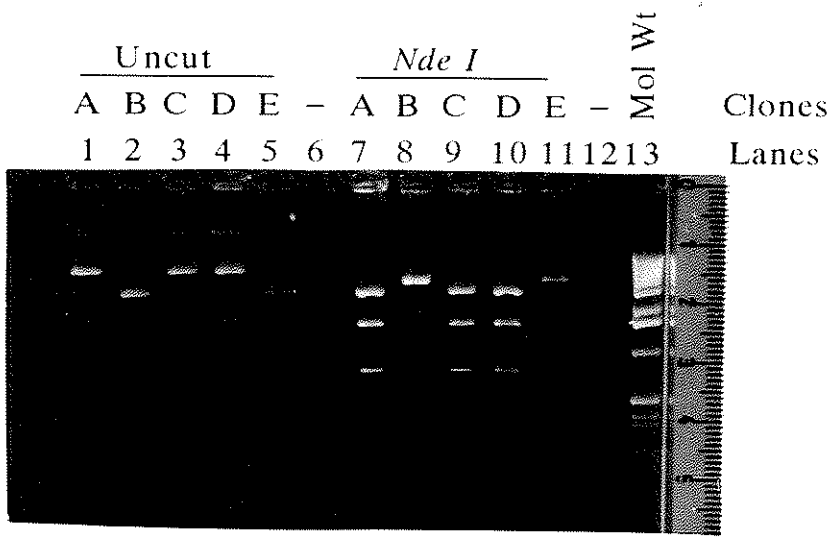


Figure 13: Liberation of the 1.4 kbp CD4 cDNA from pBluescribe by *Nde I* Digestion.

Figure 13: Liberation of the 1.4 kbp CD4 cDNA from pBluescribe by *Nde I* Digestion.

Colonies were picked from pBS plates, mini-plasmid preps prepared and *Nde I* digestion performed. The above figure is a 1% ethidium bromide stained agarose gel of digested and undigested plasmid preps. Lanes 1-5 are undigested plasmids, showing the open circle and supercoil forms, from clones A-F. Lanes 7-11 are clones A-E digested with *Nde I*. Lanes 6 and 12 are empty, lane 13 is the 1 kpb ladder. Clones A-D, derived from prostitutes 1026, 935, 870, 870 respectively, were picked from white colonies and clone E was picked from a blue colony as a negative control, to illustrate how pBluescribe appears without an insert. Clones A, C and D contain 1.4 kbp inserts while clone B did not contain an insert.

DNA less than 300 bp, accordingly, the 1.4 kbp CD4 cDNA was amplified in three separate portions (450, 624 and 454 bp in length) which were then individually digested by specific restriction enzymes to generate fragments less than 300 bp (see Table IV in Methods which summarized the fragment sizes and the restriction enzymes used). Figure 14 is an example of the SSCP analysis showing multiple banding patterns which suggest variations in the DNA sequence.

Biii. Sequencing of the CD4 cDNA:

The clones of the various CD4 cDNA were sequenced by dideoxynucleotide chain termination using the Sequenase kit from Stratagene. Any changes observed relative to the published sequence were confirmed by direct sequencing of the PCR products generated from the original amplification of the CD4 cDNA. Sequence analysis (summarized in Table XIII) revealed two non-synchronous and one synchronous change within the isolates. The first non-synchronous change occurred at nucleotide positions 351 and was conserved in all six isolates. The second non-synchronous change was at position 783 and was observed in one seroconverter (ML 069). There was one alteration that encoded an amino acid change (synchronous) at nucleotide position 868 and amino acid 240 (Figure 15). This change was observed in one of the individuals (ML 825) who subsequently seroconverted. Since none of the changes observed protected ML 825 from seroconversion it was concluded that the observed nucleotide or amino acid changes in the CD4 molecule do not explain resistance. There was insufficient evidence to suggest that resistance was at the CD4 cellular receptor level.

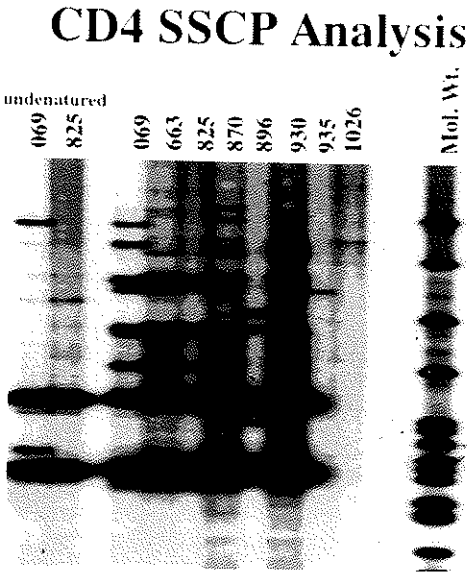


Figure 14: SSCP Analysis of CD4 cDNA

Figure 14: SSCP Analysis of CD4 cDNA.

The CD4 cDNA was amplified in the presence of $\alpha^{32}P$ dATP, into three segments, each segment digested with specific restriction enzymes, heat denatured and loaded onto a non-denaturing polyacrylamide gel. The above figure is the autoradiogram of the third segment of CD4 amplified, digested with AvaI, and resolved. Since some of the single strands reanneal after cooling lanes 1 and 2, starting from the left, show two samples, 069 and 825, that have not been denatured illustrating the location of the two undenatured DNA fragments, 180 and 274 base pairs. Lane 3 is empty while lanes 4-11 are denatured samples labelled with the appropriate study number. The right most lane is the molecular weight marker (pBR322 digested with *Hpa II* end filled with α^{32}PdCTP).

Table XIII: Sequence Analysis of CD4 cDNA from Nairobi Prostitutes.

Study Number	Nucleotide Position	Amino Acid Position
Persistent Seronegatives		
896	351 C→T	67 No Change
Seroconverters		
069	351 C→T	67 No Change
	783 C→T	211 No Change
663	351 C→T	67 No Change
	868 C→T	240 Arg→Trp
825	351 C→T	67 No Change
	868 C→T	240 Arg→Trp
870	351 C→T	67 No Change
930	351 C→T	67 No Change

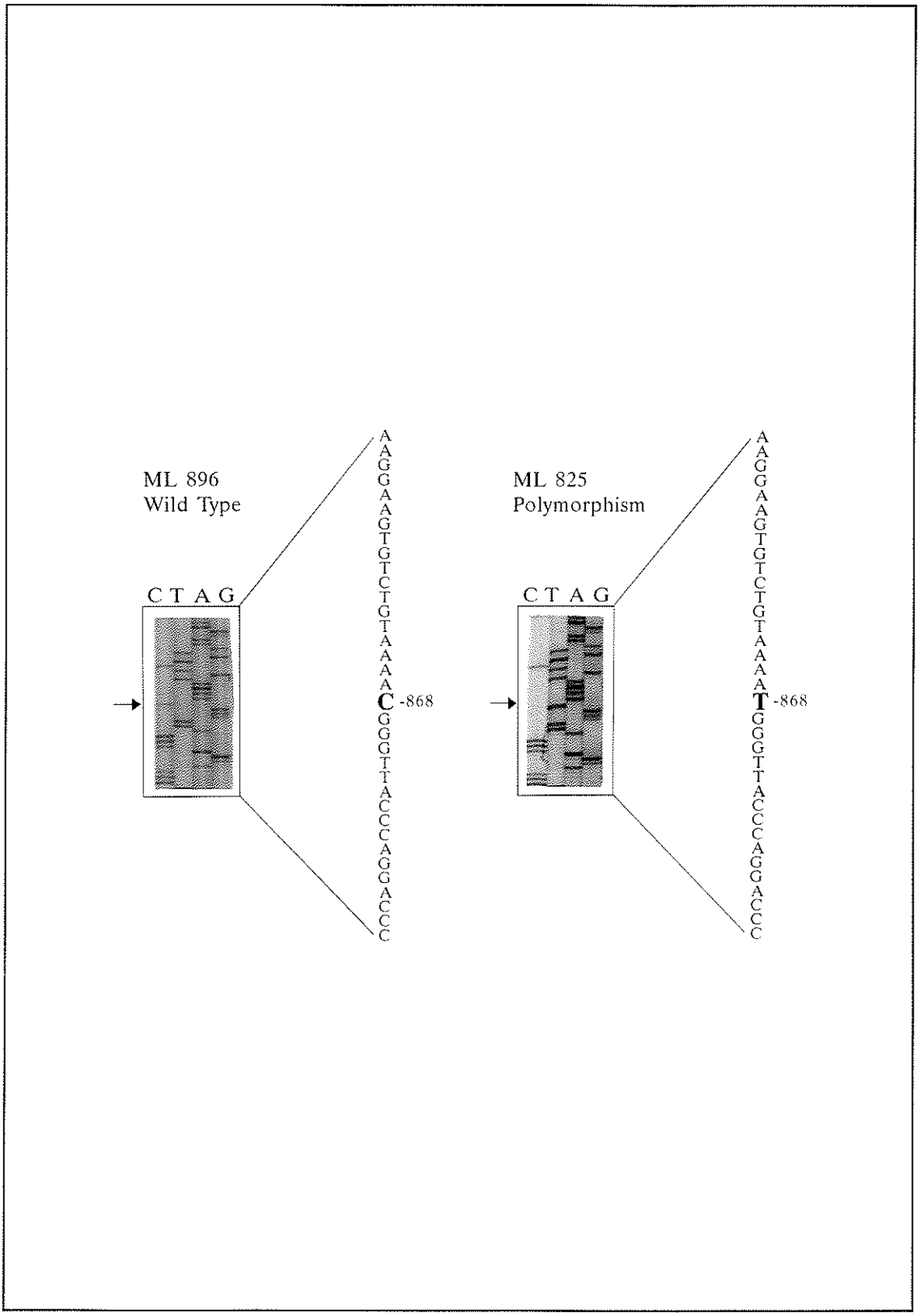


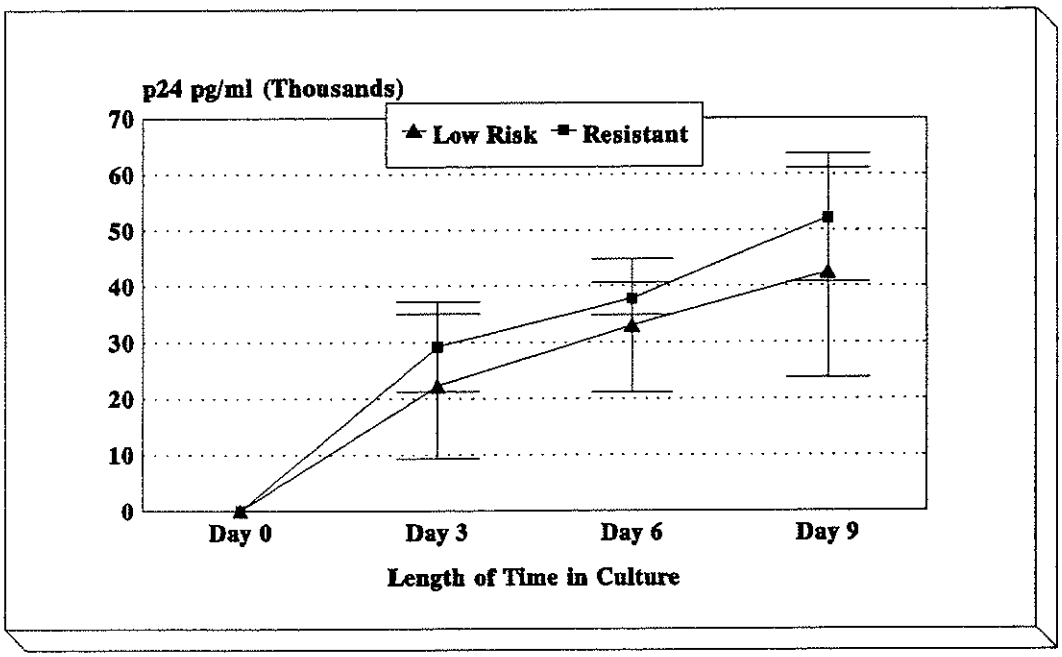
Figure 15: Synchronous Nucleotide Change at Position 868 of the CD4 cDNA

Figure 15: Synchronous Nucleotide Change at Position 868 of the CD4 cDNA.

The sequence information on the left and right sides of the figure are the CD4 cDNA of ML 896 and ML 825, respectively. The sequencing products were resolved on a 4% polyacrlyamide gel as described in Methods. Position 868, indicated by an arrow, is the site of the polymorphism. ML 896 has a cytosine at position 868 which is the wild type sequence while ML 825 has a thymidine which encodes for an amino acid change at position 240.

C. Post Receptor Resistance Mechanisms:

If the resistance to HIV-1 infection is not at the receptor level it may be the result of a post receptor blockade in viral replication. To investigate this possibility, PBMC from the resistant prostitutes were exposed *in vitro* to a laboratory strain of HIV-1, HIV-1_{IIIB}. The supernatants were monitored at intervals for the production of the viral core protein p24 which was used as an indicator of productive infection. The PBMC from 11 resistant women and 9 low risk HIV-1 seronegative persons (8 Kenyan women from the local ante-natal clinic and one Winnipeg laboratory worker) were infected as described in Methods. The PBMC from the resistant women demonstrated p24 production indicating that the cells were capable of supporting viral replication. The mean p24 production of the resistant women was statistically no different than that of the low risk controls indicating that, at an MOI of 0.03, the PBMC of the resistant women were just as susceptible to HIV-1 infection as the control (Figure 16). However, it has been suggested (Williams LM, et al. 1991) that only at low MOI are differences in susceptibility to retroviral infection apparent. To determine if lower MOIs affect the susceptibility of the PBMC to HIV-1 infection, PBMC from 2 low risk negative controls and 3 resistant individuals were exposed to serial dilution of HIV-1_{IIIB} and cultured as above. As shown in Figures 17 and 18, all individuals tested were equally susceptible to HIV-1 infection at all MOIs tested. From these results we conclude that the PBMC of the resistant women did not have a post receptor blockade to viral infection.

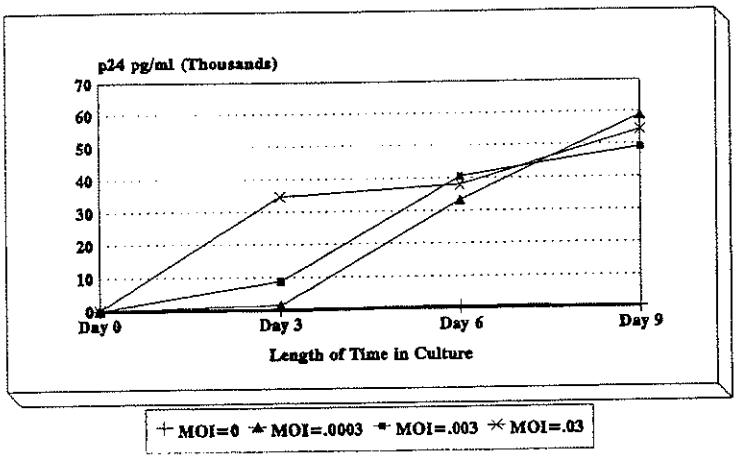


Error bars = Standard deviation around the mean

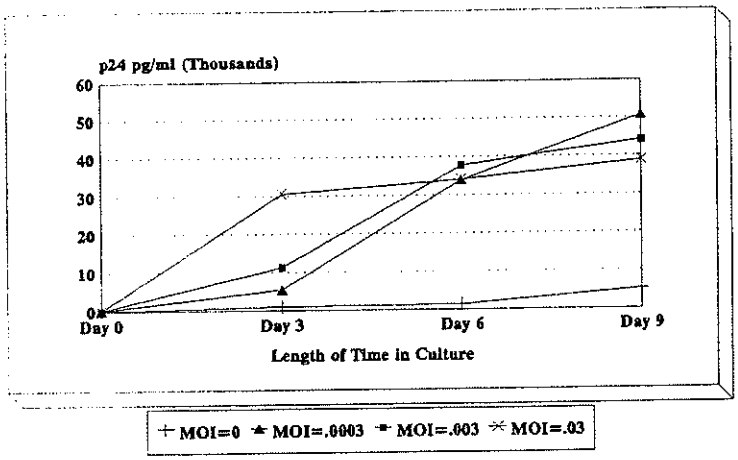
Figure 16: Mean p24 *in vitro* Production from the PBMC of Resistant and Control Individuals Infected with HIV-1_{IIIB} (MOI=.03).

Figure 16: Mean *in vitro* p24 Production from the PBMC of Resistant and Control Individuals Infected with HIV-1_{IIIB} (MOI=.03).

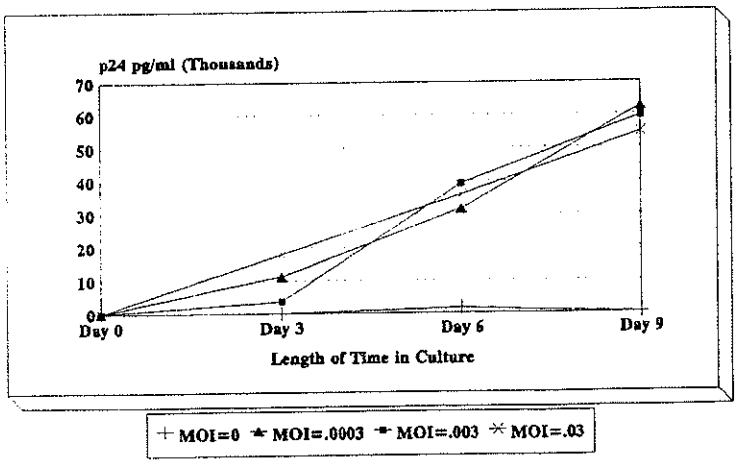
PBMC from eleven resistant and nine low risk HIV-1 seronegative control individuals were infected *in vitro* with HIV-1_{IIIB} and supernatant removed every three days to assay for p24 production. Each individual was tested in duplicate and the average used to generate the mean for the group. The mean p24 production (pg/ml) of the low risk seronegative individuals (▲) and the resistant prostitutes (■) are plotted with standard deviation error bars.



ML 887

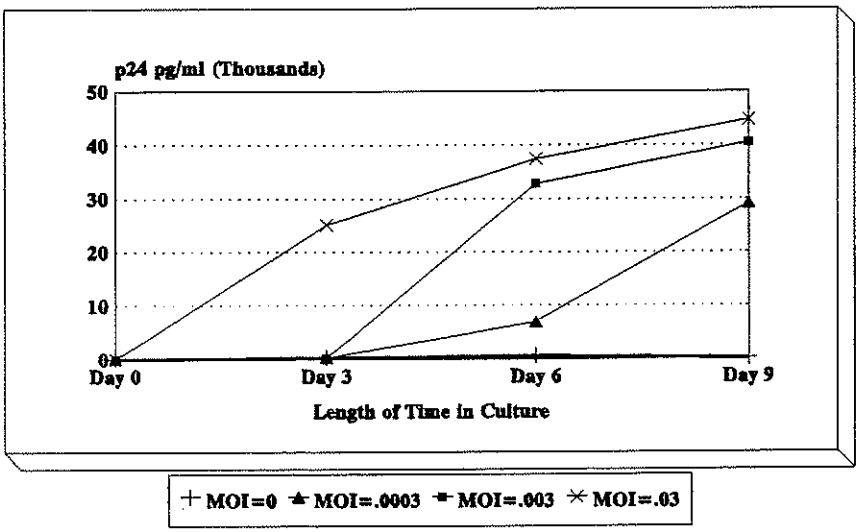


ML 893



ML 1250

Figure 17: p24 Production from PBMC of Resistant Prostitutes Infected *in vitro* at Low MOIs with HIV-1



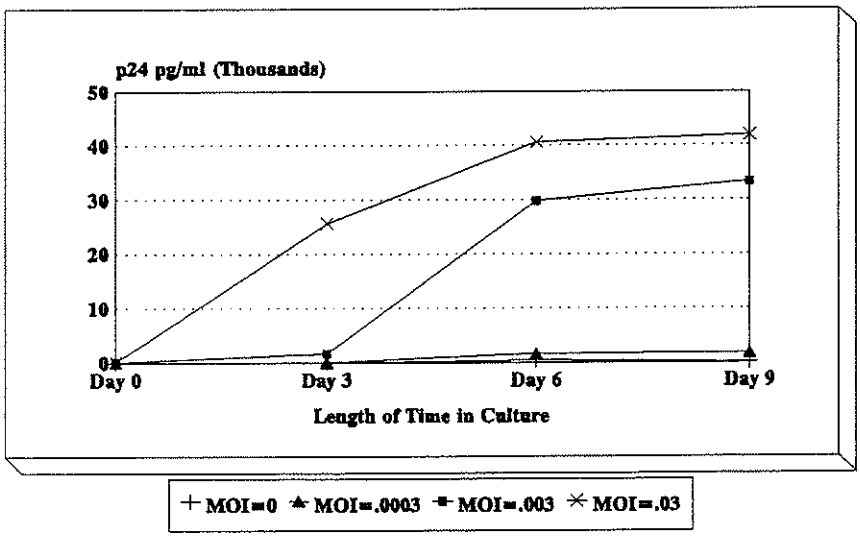


Figure 18: p24 Production from PBMC of Low Risk Negative Controls Infected *in vitro* at Low MOIs with HIV-1

Figure 17: p24 Production from PBMC of Resistant Prostitutes Infected *in vitro* at low MOIs with HIV-1.

Figure 18: p24 Production from PBMC of Low Risk Seronegative Controls Infected *in vitro* at low MOIs with HIV-1.

In vitro infection of PBMC, as described in Methods, was performed using log dilutions of the infecting virus. The MOIs used were 0 (|), .0003 (▲), .003 (■) and .03 (X). Each experiment was performed in duplicate (except MCH 1:7007 which had only one well per dilution), and each point represents the mean. Supernatants were removed from the cultures at day 0, 3, 6 and 9 post infection and assayed for p24 production (pg/ml).

D. Acquired Mechanisms of Resistance:

Results suggested that resistance to HIV-1 infection was not at the cellular receptor (CD4) or at any post receptor step that could interfere with HIV-1 establishing a productive infection. Using our cellular definition of innate resistance we concluded that resistance to HIV-1 infection was not innate. If resistance is not innate then could it be acquired?

E. HLA Associations with Resistance:

The presentation of foreign antigens to the cells of the immune system by Human Leucocyte Antigen (HLA) proteins is an essential step in the generation of an acquired immune response. Due to the importance of HLA in Major Histocompatibility Complex (MHC) restricted antigen presentation and because particular genes have been associated with certain diseases (Ebringer A, 1990; Lepage V, et al. 1993) we chose to consider the role of HLA antigens in resistance. Are certain HLA alleles found at a higher frequency, ie. associated, among resistant women? Standard serological typing (Vartdal F, et al. 1986) was used to determine MHC Class I haplotypes of 91 initially HIV-1 seropositive and 153 initially seronegative women from the cohort, 88 of whom seroconverted during follow-up. The cohort frequency of HLA alleles found among all those tested is depicted in Figure 19 (Top). Cox regression hazard modelling showed that the HLA class I alleles A69 and B18 were independently associated with a decreased risk of seroconversion (A69 hazard ratio=0.46, 95% confidence intervals 0.24 - 0.87, p=0.02; B18 hazard ratio=0.33, 95% confidence intervals 0.13 - 0.84, p=0.02). The HIV-1

seronegative survival value of these antigens is shown in the Kaplan Meier plots, Figure 19 (Middle and Bottom). Conversely, the HLA antigens A30 and A23 were associated with an increased risk of seroconversion by regression analysis (A23 hazard ratio=2.01, 95% confidence intervals 1.06-3.8, p=0.03; A30 hazard ratio=1.64, 95% confidence intervals 1.01-2.67, p=0.05). Not only were specific HLA alleles associated with remaining seronegative longer, but the rarity of the alleles within the HLA haplotype was also associated. MHC Class I alleles were determined on 244 (153 initially seronegative and 91 initially seropositive) women. The MHC rarity score was determined from the frequency distribution of Class I alleles in the prostitute cohort [score=$(F^{A1} + F^{A2})x(F^{B1} + F^{B2})x(F^{C1} + F^{C2})$ where F is the frequency of each allele]. The rarity scores were ranked and broken into quartiles, with the lowest score being quartile number 1, and compared to HIV-1 seroprevalence, Figure 20. The per cent prevalence of HIV-1 was significantly associated with increasing rarity score ($p<0.005$, Chi-square trend test) such that the more rare a person's HLA haplotype the longer that person remained seronegative. Figure 21 shows the increased seronegative survival advantage of having a rare HLA haplotype.

Thanks to Dr. F. A. Plummer and Dr. N. Nagelkerke for analyzing the data.

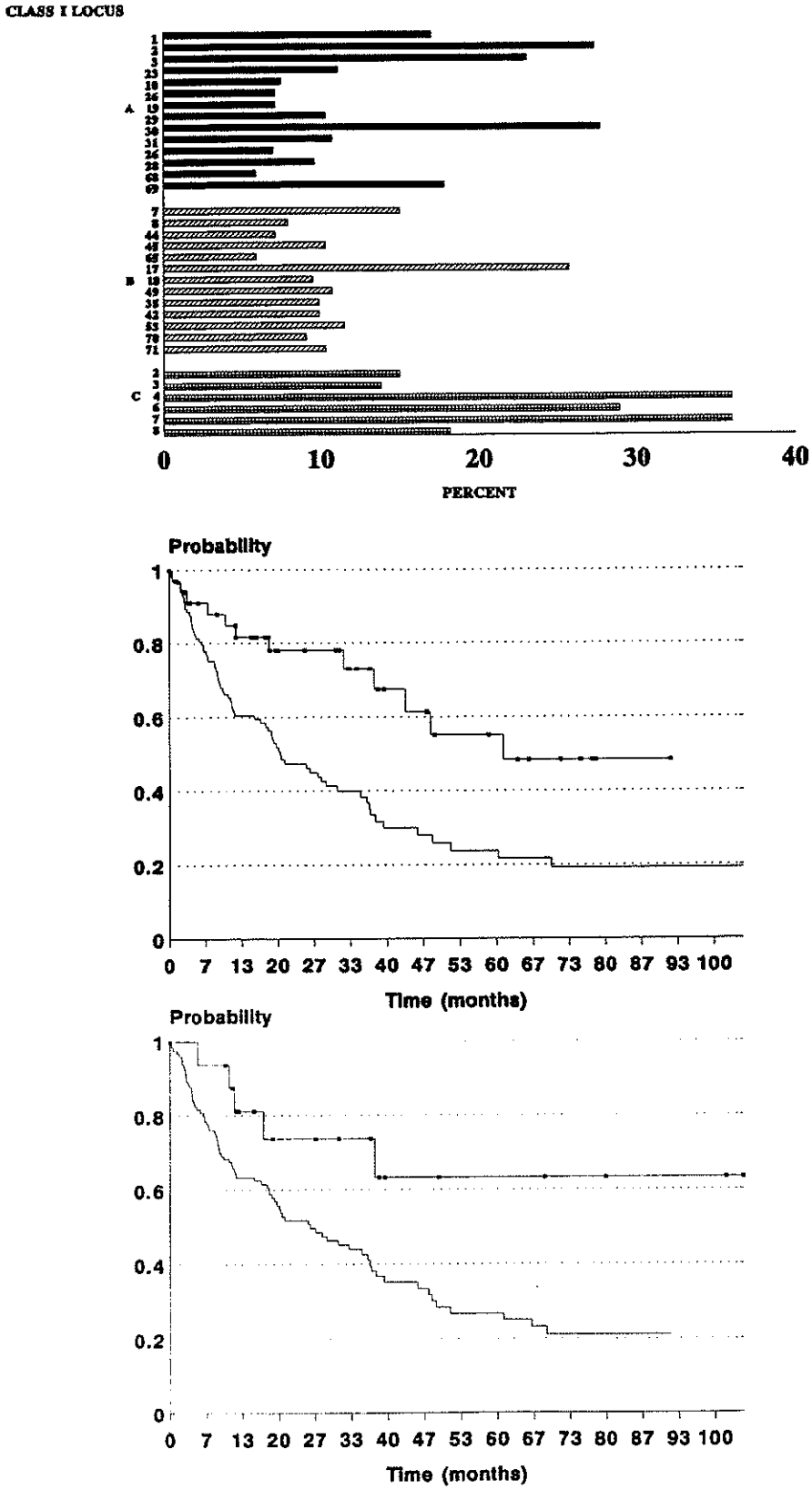


FIGURE 19: HLA Frequency and Kaplan-Meier plots of MHC restriction of resistance to HIV-1 infection among highly exposed prostitutes.

Figure 19. Kaplan-Meier plots of MHC restriction of resistance to HIV-1 infection among highly exposed prostitutes.

Top. Frequency of MHC Class I alleles in the Nairobi prostitute study. Only alleles with a frequency of greater than 5% are shown. The risk of prevalent HIV-1 infection and seroconversion was examined for alleles with a frequency of greater than 5%.

Middle. Time to HIV-1 seroconversion among prostitutes with the MHC Class I allele A69 (marked line) compared to women lacking A69. The HIV-1 seronegative survival data for the entire cohort was analyzed for the presence of certain HLA antigens. Women with the A69 allele are shown with the marked line. The HIV-1 incidence rate ratio for A69 positive women was 0.37, 95% confidence intervals 0.18 - 0.68, $p<0.0004$. A69 was also associated with a reduced risk of HIV-1 seropositivity at cohort enrollment (Odds ratio=0.27, 95% confidence intervals 0.08 - 0.96, $p<0.03$).

Bottom. Time to HIV-1 seroconversion among prostitutes with the MHC Class I allele B18 compared to women lacking B18. Women with the B18 allele are shown with the marked line. The incidence rate ratio for B18 positive women was 0.31, 95% confidence intervals 0.1 - 0.74, $p<0.003$. At entry into the cohort B18 was not associated with a reduced risk of prevalent HIV-1 seropositivity, Odds ratio=0.71, 95% confidence intervals 0.28 - 1.81, $p=0.3$.

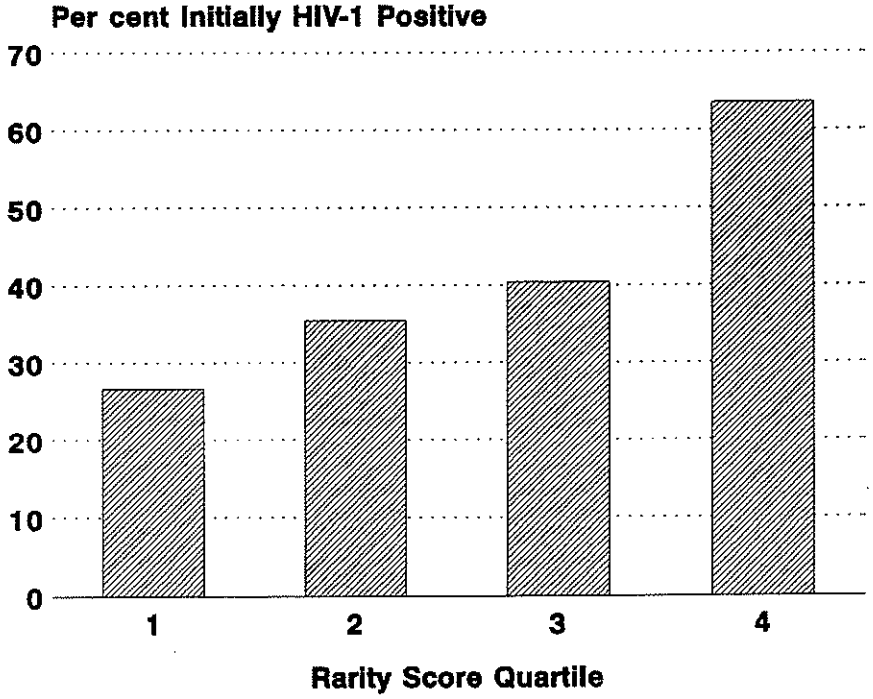


Figure 20: The Per Cent Prevalence of HIV-1 per Quartile of MHC Class I Rarity Score at Entry into the Cohort.

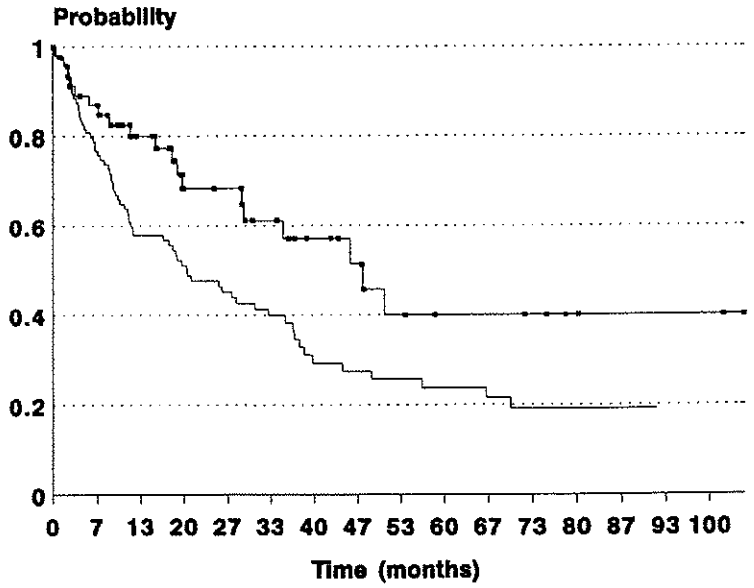


Figure 21: Kaplan-Meier Plot of Time to Seroconversion Among Prostitutes in the Lowest Quartile of the Frequency Distribution of the MHC Class I Rarity Score Compared to Prostitutes in the Upper Three Quartiles.

Figure 20: The Per Cent Prevalence of HIV-1 per Quartile of MHC Class I Rarity Score at Entry into the Cohort.

The per cent seroprevalence of HIV-1 at enrollment in the cohort is shown by quartile of the rarity score, with quartile 1 being the rarest MHC types. The per cent prevalence of HIV-1 was significantly associated with increasing rarity score ($p < 0.05$, Chi-square trend test).

Figure 21: Kaplan-Meier Plot of Time to Seroconversion Among Prostitutes in the Lowest Quartile of the Frequency Distribution of the MHC Class I Rarity Score Compared to Prostitutes in the Upper Three Quartiles.

MHC Class I rarity score was determined as described in A. The survival probability is plotted for women in the lowest quartile of the rarity score frequency distribution (marked line) compared to women in the upper three quartiles of the rarity score distribution. The incidence rate ratio for women in the lowest quartile compared to women in the upper three quartiles was 0.5, 95% confidence intervals 0.28-0.84, $p < 0.005$.

F. Resistance to HIV-1 Through Cell Mediated Immune Responses:

One mechanism through which HLA may be associated with resistance is through MHC restricted cellular immune responses. Experiments were performed to determine if there was any cellular immune response to HIV-1 in the seronegative HIV-1 resistant women.

G. Cellular Immune Response to HIV-1 *env* Peptides:

In a secondary immune response CD4+ lymphocytes are known to proliferate and produce interleukin type 2 (IL-2) if the T cell receptor recognizes its cognate antigen. The cognate antigens are linear T cell epitopes, presented as peptides, that have been inserted into the peptide binding grove of MHC class II proteins on the surface of antigen presenting cells (APC). If there are sufficient numbers of CD4+ lymphocytes that recognize a specific peptide, the activation of these cells will be detected by lymphocyte proliferation and IL-2 production, which are measured by tritiated thymidine incorporation and ELISA respectively. PBMC from 7 low risk seronegative, 14 resistant, and 7 asymptomatic HIV-1 seropositive individuals were cultured for 6-7 days in the presence of eight different test solutions, each tested in triplicate. The test solutions were lymphocyte media (LM) (unstimulated negative control), recombinant soluble gp120 (Intracel Corporation) 2μg/ml in LM, five different HIV-1 *env* peptides (NIH Reagent Program) 2.5μg/ml in LM and PHA-P 5μg/ml in LM (positive control). Separate plates, using freshly thawed PBMC, were established for proliferation and IL-2 assays as explained in Methods. Other than the end product detection systems the two

assays only differed in that the IL-2 assay required the addition of 25μl of a 1:100 dilution of anti-Tac monoclonal antibody which blocked the major IL-2 receptor to prevent consumption of secreted IL-2 by the cells. The proliferative responses of the PBMC to the eight separate solutions, as measured by tritiated thymidine incorporation into newly formed DNA, showed such variation between the triplicate wells that statistically they could not be considered related. There was neither material nor time in Kenya to determine the source of the variation. It is possible that the variation was artifactual, due to a technical problem with the harvesting of the cells or the standardization of the liquid scintillation counter for this particular assay. Therefore the results of the proliferation experiment were considered invalid. The IL-2 results, however, showed much less variation and were statistically valid. The IL-2 production (Table XV) and the stimulation indices (Figure 22) demonstrated that the low risk seronegative and the HIV-1 seropositive control groups did not respond to recombinant gp120 or the *env* peptides. However, the PBMC were capable of being stimulated and producing IL-2 as evidenced by the very strong PHA response, shown in Table XV. It should be noted that PBMC from the low risk seronegative subject 1:5451 later tested indeterminant by HIV-1 PCR (one positive and one negative PCR set) and may have been recently exposed to or infected by HIV-1 which could explain the high response to gp120 and peptide P18. In contrast to the two control groups, the PBMC from some of the resistant prostitutes (MLs 1275, 889, 1371, 1358) responded well to PHA and displayed marked IL-2 production upon stimulation by gp120 and the *env* peptides.

Table XIV: IL-2 Production (pg/ml) After Exposure to HIV-1 Antigens:

	KRF (LR)	1:5451 (LR)	1:8404 (LR)	ML 1275 (RES)	ML 887 (RES)	ML 889 (RES)
media	9.0	10.0	<6.0	18.0	15.0	34.0
gp120	9.5	18.5	<6.0	45.5	12.0	64.0
T1-2007	8.0	7.5	8.0	42.0	8.0	55.5
T2-1929	8.5	<6.0	<6.0	37.0	14.5	30.5
P18	6.0	21.0	<6.0	33.0	8.5	34.5
P23	6.0	7.5	<6.0	37.0	10.5	165.0
T1-1589	10.0	10.0	ND	36.5	9.5	340.0
PHA	2215	3825	ND	7125	4060	2785

	ML 1371 (RES)	ML 1362 (RES)	ML 1490 (RES)	ML 1358 (RES)	ML 1075 (HIV+)	ML 1359 (HIV+)
media	9.0	19.0	21.0	12.0	8.5	<6.0
gp120	10.0	14.5	22.0	27.5	7.5	<6.0
T1-2007	8.0	16.0	29.5	31.0	<6.0	<6.0
T2-1929	15.0	11.0	31.0	22.5	8.0	8.0
P18	8.5	9.5	27.0	16.5	7.0	7.0
P23	8.5	14.5	21.5	13.0	8.5	<6.0
T1-1589	16.5	10.5	25.0	14.5	8.5	<6.0
PHA	1871.5	1685	6605	467.5	550	417.5

NOTE LR = Low Risk HIV-1 Negative
RES = Resistant Prostitute
HIV+ = HIV-1 Seropositive Prostitute

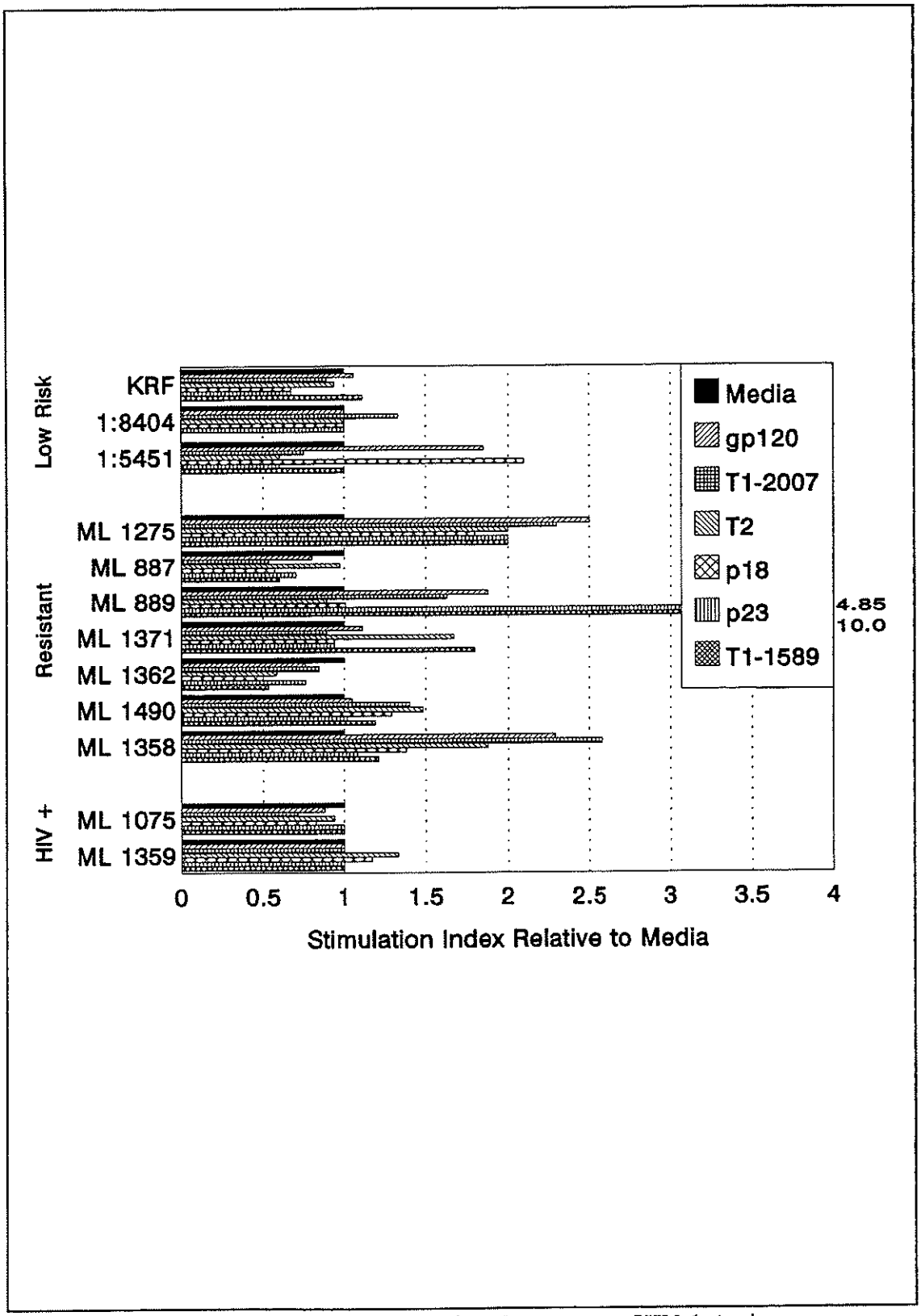


Figure 22: IL-2 Stimulation Indices After Exposure to HIV-1 Antigens

Figure 22: IL-2 Stimulation Indices After Exposure to HIV-1 Antigens

PBMC from low risk HIV-1 seronegative individuals (Low Risk), resistant prostitutes (Resistant), and HIV-1 seropositive prostitutes (HIV +) were exposed to eight different solutions. The IL-2 production was measured, by ELISA, in the supernatants after one week of culture (Table XV). The stimulation indices (SI) relative to Media are plotted above. The stimulation index of PHA was not plotted as it was often several hundred times greater than the SI of the other samples. ML 889 had two stimulation indices that were beyond the range of the ordinate: 4.85 and 10.0 for peptides p23 and T1-1589, respectively.

H. Cytotoxic Responses to HIV-1 Antigens:

The IL-2 results suggested that some of the resistant women had a CD4+ lymphocyte mediated T cell memory to HIV-1 antigens. To determine if the resistant women possess HIV-1 specific cytotoxic cells, cytotoxic assays were performed. The targets for the killing were Chromium 51 (^{51}Cr) labelled autologous Epstein-Barr Virus (EBV) transformed B cells infected with recombinant vaccinia viruses containing the HIV-1 structural genes *gag*, *pol* and *env*. To determine that the recombinant vaccinia/HIV-1 viruses produced HIV-1 proteins after infection, EBV transformed B cells (KRF) were infected with the various recombinant viruses. The lysates were resolved by polyacrylamide gel electrophoresis, the proteins transferred to a support membrane, and the immunoblot probed with polyclonal antibodies specific for HIV-1 proteins (see Table II). As seen in Figure 23, each of the recombinant viruses produced proteins recognized by the HIV-1 specific antisera that were not produced in cells infected with the control virus vSC8.

Effector cells were PBMC that had been stimulated by autologous HIV-1 infected PBMC. The two populations of cells were plated at effector to target ratios of 50:1, 25:1, and 12.5:1 (experimental wells). To determine the amount of ^{51}Cr that would be lost spontaneously, the target cells were incubated by themselves (minimum wells). To determine the maximum amount of ^{51}Cr that could be released, target cells were incubated in the presence of 1% SDS, which caused complete lysis of all target cells (maximum wells). All experiments were performed with duplicate wells. After the plates were prepared, they were centrifuged for five minutes to ensure maximal cell to

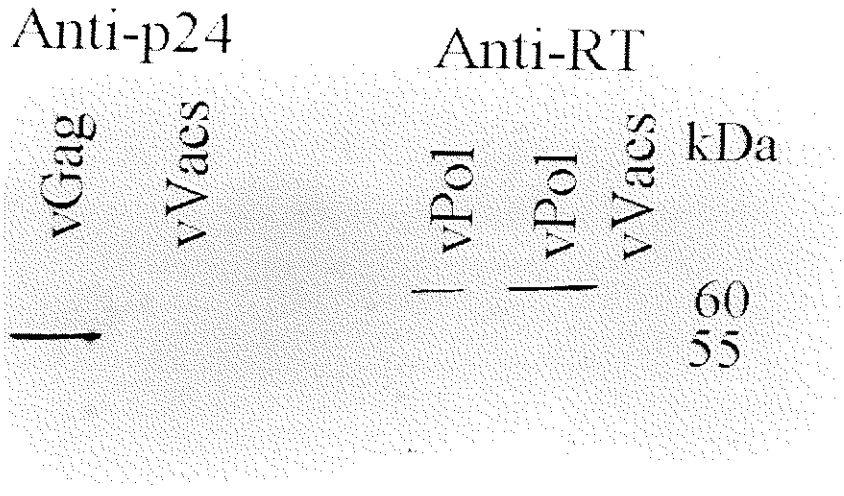


Figure 23: Immunoblots of Lysates from EBV Transformed B Cells Infected with Recombinant Vaccinia/HIV-1 Viruses.

Figure 23: Immunoblots of Lysates from EBV Transformed B Cells Infected with Recombinant Vaccinia/HIV-1 Viruses.

EBV transformed B cells were infected with the recombinant vaccinia/HIV-1 viruses vSC8 (wild type vaccinia with the ß-galactosidase gene), vDK1 (HIV-1 *gag* inserted into vSC8), vCF21 (HIV-1 *pol* inserted into vSC8) and vPE16 (HIV-1 *env* inserted into vSC8). After an overnight infection the cells were solubilized with detergent, the lysate resolved by PAGE (10μg total protein/well) and the proteins transferred to a support membrane. The antibodies used to detect the HIV-1 proteins are listed in Table II. The left side of the figure is the lysate from vDK1 and vSC8 infected cells (vGag and vVacs respectively) blotted with a HIV-1 p24 specific antibody. vDK1 infected cells produce an uncleaved *gag* protein that is 55 kilodaltons (kDa) and is recognized by the p24 antibody. The right side of the figure is the immunoblot of vCF21 (two lanes 5μg and 10μg per lane) and vSC8 infected cells (vPol and vVacs respectively). vCF21 infected cells produce viral reverse transcriptase (RT) which is 60 kDa and is recognized by the RT specific antibody. The HIV-1 gp160 specific antibody specifically recognized a 160 kDa protein produced from vPE16, *env*, infected cells (data not shown).

cell contact. The plates were incubated at 37°C for four hours and 100μl of supernatant was removed and added to 1ml scintillation cocktail and counted in the liquid scintillation counter. The per cent spontaneous release (%SR) of chromium from the cells and the per cent specific lysis (%SL) for each vaccinia vector were calculated using the following formulae:

$$\%SR = \frac{minimum}{maximum} x100 \quad \text{and} \quad \%SL = \frac{experimental - minimum}{maximum - minimum} x100$$

For each experiment to be considered valid, the per cent spontaneous release of chromium was less than 35%. For the HIV-1 specific lysis to be considered significant it had to be titratable and $>10\%$ the specific lysis generated by the vaccinia virus with no added HIV-1 gene. Thirteen individuals were tested for HIV-1 specific cytotoxic responses, 11 resistant prostitutes, one HIV-1 negative Kenyan mother and one Winnipeg laboratory worker. Three additional low risk seronegative controls were established but at the eleventh day of culture there were not enough effector cells to perform the assay. Five of the eleven resistant prostitutes tested positive with at least one of the recombinant vaccinia vectors. The HIV-1 specific cytotoxic data, summarized in Table XV, showed that ML 1376 recognized all of the HIV-1 vaccinia vectors, ML 857 recognized the vaccinia *pol* vector and MLs 887, 889 and 1070 recognized the vaccinia *env* vector. The low risk HIV-1 seronegative controls did not show any specific recognition of the HIV-1 proteins. In six of the resistant prostitutes HIV-1 specific cytotoxic activity was not detected. Examples of positive and negative cytotoxic responses are presented in Figure 24. To date we have been able to detect HIV-1 specific cell mediated cytotoxic responses in 45% (5/11) of the resistant prostitutes tested.

Table XV: Specific Lysis of Recombinant Vaccinia/HIV-1 Infected B cells by HIV-1 Stimulated PBMC.

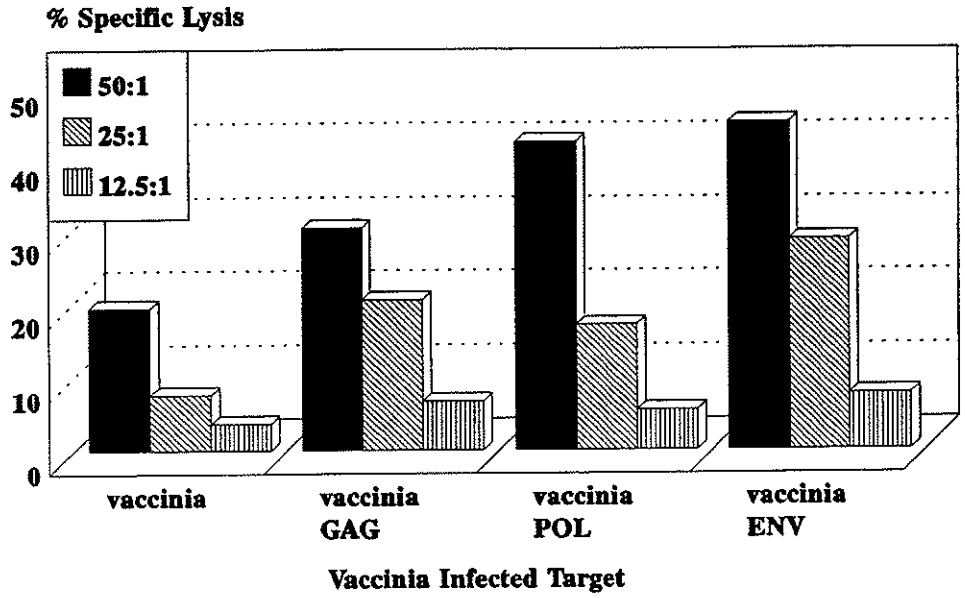
Study # and HIV Status	Recombinant Vaccinia/HIV Vectors	% Specific Lysis at Effector:Target Ratio		
		50:1	25:1	12.5:1
1376 RES	Vacs	19.3	7.5	3.6
	Gag	**30.1**	**20.3**	**6.6**
	Pol	**41.7**	**16.9**	**5.4**
	Env	**44.3**	**28.5**	**7.5**
889 RES	Vacs	19.1	7.0	2.9
	Gag	21.0	16.5	7.0
	Env	**34.8**	**17.5**	**15.4**
857 RES	Vacs	19.7	11.0	6.6
	Pol	**28.3**	**18.4**	**13.5**
	Env	6.8	2.8	0.5
1070 RES	Vacs	2.6	1.5	3.2
	Gag	11.2	5.7	3.0
	Env	**13.4**	**11.0**	**7.8**
887 RES	Vacs	7.0	3.5	4.3
	Env	**21.5**	**11.0**	**7.0**
KRF LR	Vacs	49.0	26.7	14.0
	Gag	37.7	21.9	14.1
	Pol	39.3	23.2	7.5
	Env	39.9	21.0	11.5
1:180 LR	Vacs	(32:1) 33.8	(16:1) 22.3	(8:1) 15.7
	Env	31.8	19.1	15.3

Note: RES = Resistant Prostitute
LR = Low Risk HIV-1 Seronegative Control
BOLD = Positive Cytotoxic Responses
Vacs = Targets infected with vaccinia vector vSC8
Gag Pol Env = Targets infected with recombinant vaccinia/HIV-1 viruses vDK1, vCF21 and vPE16, respectively

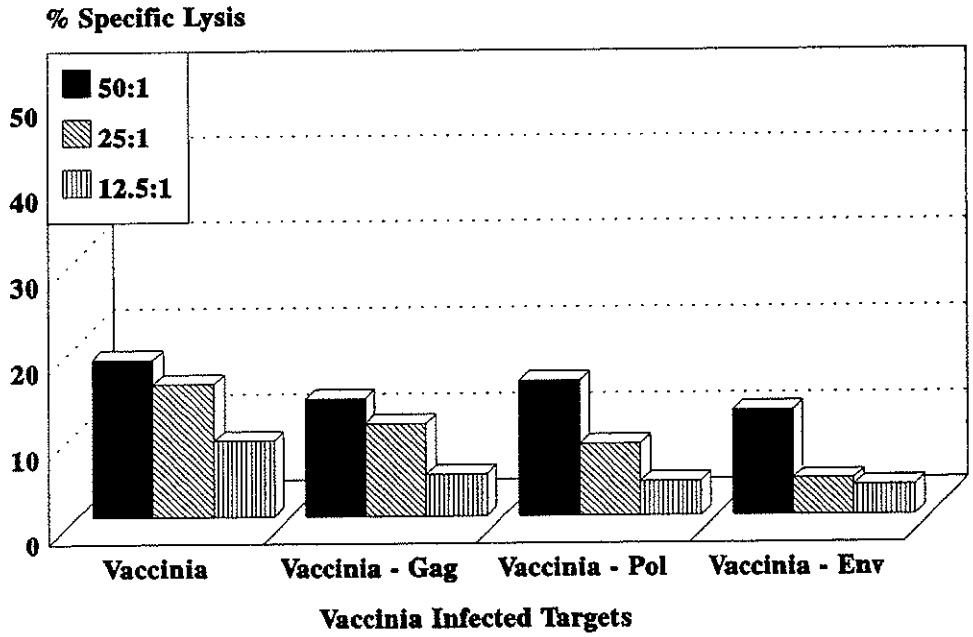
Table XV (con't)

Study Number	Recombinant Vaccinia/HIV Vectors	% Specific Lysis at Effector:Target Ratio		
		50:1	25:1	12.5:1
1327 RES	Vacs	26.5	20.9	15.1
	Gag	-15.8	-22.6	-35.5
	Pol	23.6	23.6	19.6
	Env	24.2	27.2	15.9
851 RES	Vacs	20.0	7.4	3.6
	Gag	25.0	12.2	-0.2
	Pol	21.1	4.6	-0.7
	Env	10.8	1.7	0.8
893 RES	Vacs	4.7	2.0	1.7
	Gag	4.9	1.6	1.6
	Pol	9.0	7.7	4.4
	Env	8.5	4.6	3.2
935 RES	Vacs	12.6	5.5	3.1
	Env	16.1	17.4	7.3
466 RES	Vacs	18.3	15.5	8.9
	Gag	13.7	10.8	4.9
	Pol	15.7	8.4	4.0
	Env	12.2	4.3	3.5
1286 RES	Vacs	-1.6	-1.0	ND
	Gag	0.9	1.4	ND
	Env	-1.4	-0.3	-4.4

Note: RES = Resistant Prostitute
LR = Low Risk HIV-1 Seronegative Control
BOLD = Positive Cytotoxic Responses
Vacs = Targets infected with vaccinia vector vSC8
Gag Pol Env = Targets infected with recombinant vaccinia/HIV-1 viruses vDK1, vCF21 and vPE16, respectively



ML 1376



ML 466

Figure 24: A Positive and Negative Example of HIV-1 Specific Cytotoxic Responses

Figure 24: A Positive and Negative Example of HIV-1 Specific Cytotoxic Responses.

^{51}Cr release assays were performed on HIV-1 stimulated PBMC from resistant prostitutes and negative controls using EBV transformed B cells, infected with recombinant vaccinia/HIV-1 viruses were, as targets. ML 1376, which shows HIV-1 specific lysis, and ML 466, which shows no lysis above vaccinia alone are given as examples. Three effector to target ratios were used; 50:1 (solid bar), 25:1 (diagonally striped bar) and 12.5:1 (vertically striped bar). The specific lysis is shown on the Y axis.

DISCUSSION:

I. DEMONSTRATING THAT THERE IS RESISTANCE TO HIV-1:

A. Humoral Immune Responses of the Prostitutes:

The presence of IgG antibodies specific for HIV-1 antigens is indicative of an HIV-1 infection. Despite continued sexual exposure to HIV-1, a small group of the women from the Nairobi prostitute cohort have remained persistently seronegative (PSN) for at least three years and some up to nine years. Blood was drawn from the women of the cohort biannually and screened for the presence of antibodies to HIV-1 (Table VI).

The possibility exists that these individuals have been infected by HIV-2 despite the extremely low prevalence of HIV-2 in East Africa (O'Brien TR, et al. 1992). There is however, no serological evidence to support this hypothesis since antibodies from HIV-2 are generally cross-reactive to HIV-1 *gag* and *pol* encoded proteins (Clavel F, et al. 1987) and would be detected by the western blot analyses performed. The current antibody screening and confirmatory assays (Behring and Cambridge Biotech) have been used since 1991 and were specifically designed to detect HIV-1 and HIV-2 antibodies. All PSN women have been screened with this assay and none have reacted suggesting there are no HIV-1 or HIV-2 specific antibodies in this population.

Evidence that the absence of HIV-1 specific antibodies in the PSN women resulted from the absence of HIV-1 infection rather than a dysfunctional humoral immune system has been presented. Antibodies to Rubella virus were detected at titres several fold above

the minimum cut off limit for protective humoral responses in all of the PSN women tested (Table VII). This suggests their humoral immune systems were capable of responding to a viral infection.

A theoretical concern was that due to a clerical error in the patient study codes or a labelling error of the blood samples, the PSN phenomenon was actually the a result of mixing of samples from two or more individuals and that no single individual had remained HIV-1 seronegative for greater than three years. A method was required that could demonstrate differences between individuals of the same species (allotypic marker) using sera or plasma as the source. Serum protein analysis has the ability to detect allotypic complement proteins (Chen FT, et al. 1994). Initially this technique was used on the Nairobi sera, however, due to technical problems, it was abandoned. The greatest degree of allelic variation in the human species is in the human leucocyte antigens (HLA) of the major histocompatibility complex (MHC) class I and II genes. At the time this study was initiated, there were several reports of genetic typing of class II genes which were aimed at replacing serotyping of Class II HLA proteins (Erlich HA, et al. 1986; Erlich HA, et al. 1990). These techniques were based on the polymerase chain reaction (PCR) and therefore required only small amounts of DNA template. It was reasoned that due to the natural half life of a cell and the rigorous process of centrifugation, serum or plasma would contain small amounts of DNA from lysed nucleated cells. If the cellular DNA could be isolated from sera or plasma it could be used as template for the HLA class II genotyping system. Since the Nairobi sera were used for many studies, a protocol that would use minimal amounts of sera was required. A protocol was

developed that used a silica gel matrix to isolate DNA from 50μl of serum or plasma. The isolated DNA was then targeted for amplification and subsequent genetic typing using the HLA DQA1 system. The HLA DQA1 system was chosen because it combined the power of allelic differentiation with a minimum number of allele specific oligonucleotides. The method of DNA isolation from sera and HLA DQA1 typing was established using sera from Winnipeg laboratory staff (Figure 1). The method was applied to paired serum or plasma samples collected from 13 PSN women in 1985/86 and 1990/91 (Figure 2). For each of the 13 PSN women tested the paired samples were shown to possess the same HLA DQA1 alleles (Table VIII). The HLA DQA1 system can detect eight alleles and because the human cell is diploid this generates a differentiation power of 2^8. There is a 1 in 256 (0.4%) chance that two samples with the same genetic type are from two separate individuals. The conclusion from the HLA DQA1 analysis was that it was highly probable that the paired samples originated from the same individual. These data are in press (Fowke KF, et al. 1995). This suggested that the phenomenon of persistent seronegativity was real and not artifactual.

Note that in Table VIII the paired samples of subject 768 were determined to have three HLA DQA1 alleles by the binding patterns of the oligonucleotides. This allele has been cloned and sequenced by Salman Qureshi and J. Neil Simonsen and was determined to be a previously described but rare allele 2 subtype (J. N. Simonsen personal communication). Since only 1 of 13 (7.7%) PSN women tested possessed this allele, there was no correlation with remaining persistently seronegative.

It can be concluded from the serological data that women of the Nairobi prostitute

cohort have remained persistently HIV-1 seronegative for 3-9 years, that their immune system is capable of mounting a humoral response to viral infection, and that the phenomenon of persistent seronegativity is real. But are these women truly uninfected?

B. Molecular Determination of Infection Status:

It is theoretically possible that persistently seronegative women are HIV-1 infected but do not produce a humoral response (a seronegative HIV-1 infection). Seronegative HIV-1 infections among long-term seronegatives have been described by one group (Imagawa DT, et al. 1989) but not reproduced by others (Pan L-Z, et al. 1991; Bruisten SM, et al 1992.; Lee T-H, et al. 1991; Yerly S, et al. 1991; Nielsen C, et al. 1991; Willerford DM, et al.1993; Coutlee F, et al. 1994). Amplification of proviral DNA by the polymerase chain reaction (PCR) is a highly sensitive and specific method of detecting HIV-1 (Ou C-Y, et al. 1988). Due to the lack of established amplification systems at the time this study was initiated (1989), it was decided to develop novel primer sets that had optimal homology to African isolates. The *vif*, *nef* and *rev* amplification systems were established (Figure 7). The *env* system, which was based on published sequences (Ou C-Y, et al. 1988), was also used so that the results from the new amplification systems could be compared to those in the literature. The limit of detection of the *vif* and *nef* PCR systems were comparable to the *env* system; detecting 3.8 viral copies in a background of DNA from 150,000 uninfected cells. This level of detection was similar to other reported PCR systems (Boni J, et al. 1993). The effect of sequence variation on these novel PCR systems was not known since, at the time of

this study, there had been no sequence information from East African HIV-1 isolates. A pilot study confirmed that the *vif*, *nef* and *env* PCR systems could detect Kenyan HIV-1 samples and therefore were suitable for this study (Table X). This pilot study also demonstrated that the *rev* system was not able to detect any of the Kenyan HIV-1 samples and its use was discontinued. These data have been published (Dawood MR, et al. 1992). The sensitivity and specificity of the combined amplification systems, determined in a second pilot study of Kenyan women, were 100% and 88% respectively (Table XI). The specificity may seem low, however, because PCR is a more sensitive assay than serology, the gold standard; PCR can detect an HIV-1 infection before the development of antibodies (Horsburgh CR Jr, et al. 1989).

The accuracy of the amplification systems were confirmed by the extremely low limit of detection, the ability to detect Kenyan HIV-1 samples and the demonstrated high level of sensitivity and specificity among the population to be tested. HIV-1 PCR was performed on the PBMC from the PSN women (Table XII). MLs 1358 and 1378 tested PCR positive although they were negative by serology (Table VI). These individuals had remained seronegative for the minimum three years required to be categorized as persistently seronegative. It is possible these individuals have been recently infected and have not developed HIV-1 specific IgG antibodies, however, only longitudinal observation can confirm this hypothesis.

The majority of the PSN women tested 20/22 (91%) were PCR negative. HIV-1 PCR is the most sensitive method of detecting an HIV-1 infection (Horsburgh CR Jr, et al. 1989) and therefore confirms that the PSN women tested were truly not infected by

HIV-1. The theoretical possibility exists of a cryptic HIV-1 infection of the lymph nodes. However, in our estimation and others (Pan L-Z, et al. 1991; Bruisten SM, et al. 1992; Lee T-H, et al. 1991; Yerly S, et al. 1991; Nielsen C, et al. 1991; Willerford DM, et al. 1993; Coutlee F, et al. 1994) it is highly unlikely that an infection would remain cryptic for nine years without eventually being detected in the peripheral circulation by an assay as sensitive as PCR. Therefore, it can be concluded that, within the limits of our detection, the majority of PSN women are not infected by HIV-1. This is an important observation as it suggests that serology and infection are highly correlated and that there are no apparent long-term seronegative HIV-1 infections.

C. Evidence that Persistent Seronegative Women are Resistant to HIV-1:

Serological and molecular data have demonstrated that a subgroup of exposed prostitutes have remained uninfected by HIV-1 for 3-9 years. This occurred despite estimates, based on epidemiological data, that suggested the annual number of sexual exposures to HIV-1 had increased from 24 in 1984 to 60 in 1993. If exposure were the only parameter for infection then this constantly increasing level of exposure to HIV-1 would predict that, given time, all of the prostitutes should become infected. However, when the length of time a woman remained seronegative after enrollment into the cohort (seronegative survival time) was plotted for the entire 424 initially seronegative women of the cohort, a change in the slope of the curve was observed around three years post enrollment (Figure 3). This change in the slope of the curve and eventual plateau effect suggested that, over time, the risk of infection declines. Figure 4 demonstrated this

decline in the risk of infection was statistically significant and did not simply represent chance ie. the tail end of a normal distribution curve. A survival model (the mixture model), which assumed a portion of the population were resistant to HIV-1 infection, was a better description of the seronegative survival data ($p < .00001$) than the exponential model, which assumed a normal distribution. These results are significant since they suggest that chance is not a sufficient explanation of the decline in risk seen among the PSN women. They also suggest that the degree of exposure to HIV-1 is not the only determinant for infection and that resistance, as predicted by the mixture model, may account for the declining risk of infection.

If this decline in the risk of HIV-1 infection were related to an immune mechanism the degree of protection should be related to extent of exposure. In other words, the more HIV-1 exposures a woman has survived the less likely the next exposure will cause an infection. For each year a prostitute remained uninfected there was an increased protective effect over women who started prostitution the following year (Figure 5). Due to the annual increase in the infection pressure, because of increasing annual exposures to HIV-1, the protective effect of the later years was higher than the earlier years. The cumulative protective effect of nine years of seronegative prostitution afford that individual a 100 fold reduction in the risk of infection over an individual starting prostitution in 1994.

Multivariate analyses by Cox proportional hazards modelling demonstrated that when age, number of sex partners, number of regular sex partners, condom use, gonococcal and chlamydial cervicitis and the frequency of genital ulcer disease were

included as independent variables there was still a significant association between duration of prostitution and risk of seroconversion. This demonstrated that the protective effect of duration of prostitution was not due to the selection of women with safer sexual behaviours or immunity to other sexually transmitted diseases which facilitate HIV-1 transmission.

Are these women who have remained uninfected for 3-9 years despite high level of exposure, resistant to HIV-1 infection? The only direct way to answer this question, a challenge experiment, is not possible so a deductive approach is necessary. These women are not remaining PSN because their immune systems are incapable of mounting a humoral response to a viral infection, nor is the persistently seronegative phenomenon the product of laboratory error. These PSN women do not have seronegative HIV-1 infections. Statistically these women should be infected, however, chance does not account for the lack of infection yet a survival model that assumes resistance to infection does fit the seronegative survival data best. The risk of infection does not remain constant rather it declines with increasing exposure (as measured by duration of prostitution). Finally, this highly significant association between duration of prostitution and declining risk can not be accounted for by safer sexual practices or STD co-factors that facilitate transmission of HIV-1. Having discounted all other reasonable possibilities it is concluded that the lack of infection in these PSN women is the result of resistance to HIV-1 infection. For the duration of this discussion the PSN women will be referred to as resistant.

II. MECHANISMS OF RESISTANCE:

A. Receptor Mediated Resistance to HIV-1 Infection:

There are two major categories of resistance to infections pathogens; innate or acquired. For this study, innate resistance was defined as the inability of HIV-1 to establish a productive infection in the peripheral blood mononuclear cells (PBMC) of an individual. As described in the introduction, the first step in the establishment of infection is the specific interaction between the viral gp120 and the major cellular receptor CD4. The gp120 binding region of CD4, amino acids 41-55, is homologous to the complementary determining region two (CDR2) of immunoglobin light chains (Arthos J, et al. 1989) and is located in extracellular domain one. This epitope is thought to be a conformational epitope (Ibegbu CC, et al. 1989) and likely explains why regions outside the direct gp120 contact points are known to decrease the affinity of CD4 to gp120 (Brodsky M, et al. 1990; Ashkenazi A, et al. 1990). A clear demonstration of the effects of regions distant to CDR2 of domain one on gp120 binding was given by Fleury S, et al. 1991. They constructed 30 mutant CD4 proteins that contained single and cluster amino acid changes throughout the four extracellular domains of CD4. They were able to demonstrate that particular alterations in the CD4 structure, in any of the four domains, significantly reduced binding to gp120. One mutation in the third domain completely abolished binding altogether.

CD4 is apparently non-polymorphic (Maddon PJ, et al. 1986). It remains possible that CD4 is a polymorphic molecule and that changes *in vivo* could mimic the *in vitro* situation and inhibit gp120 binding. It was hypothesized that changes in the structure of

the CD4 molecule of the resistant prostitutes decreased gp120 binding thus giving them a selective advantage which resulted in resistance to HIV-1 infection. To test this hypothesis, the entire CD4 cDNAs from six prostitutes were amplified (Figure 12), cloned (Figure 13), analyzed (Figure 14), and sequenced (Figure 15). At the time of analysis three of the prostitutes were defined as being resistant to HIV-1 while three were HIV-1 seropositive and therefore susceptible to infection. However, after the analysis was complete two of the resistant women seroconverted (emphasizing that resistance is relative and not absolute). As a result of the seroconversions, of the six women anlayzed five were HIV-1 infected and one was resistant. Sequence data revealed that, relative to the type strain pT4B, there were two non-synchronous changes and one synchronous change (Table XIII) in the prostitutes CD4 cDNAs. All six individuals possessed the same non-synchronous change at nucleotide position 351 while the second non-synchronous change was present only in the HIV-1 susceptible subject 069 (nucleotide 783). The synchronous change at nucleotide 868, which encoded an arginine to tryptophan change at amino acid 240, was observed in two susceptible subjects (one of whom seroconverted after the analysis). During this study Hodge TW, et al. 1991 published the identical observation. This amino acid change resulted in the loss of binding of the monoclonal antibody OKT4 and has been reported at a frequency of 8.3% in American blacks (Casey T, et al. 1986) but rarely in other racial groups (Aozasa M, et al. 1985; Tollerud DJ, et al. 1985; Hodge TW, et al. 1991). This epitope has been suggested to be associated with lupus and general haematologic disorders although the number of patients studied were too few to be convincing (Stohl W, et al. 1987;

Imashuku S, et al. 1987). This substitution of the basic arginine residue with the more hydrophobic tryptophan at position 240 is in the centre of ß sheet (Maddon PJ, et al. 1987) and could alter the tertiary structure of CD4. However, because this alteration was observed in the individual who susbequently seroconverted it was concluded that this alteration does not confer protection to HIV-1 infection. Similarly, the non-synchronous changes were not associated with resistance, therefore, they are not believed to be involved in protection. This study has demonstrated that the CD4 molecule is polymorphic and, although the numbers are small, the frequency of the amino acid polymorphism at position 240 may be significantly higher among the women of this cohort (33%) than that observed in Black American (8.3%). However, it was concluded that the alterations observed in the CD4 molecule are unlikely to confer resistance to HIV-1 infection.

B. Post Receptor Resistance Mechanisms:

Several models of innate resistance in the murine system were described in the introduction. The first example was resistance to *Mycobacterium sp.* in strains of mice that had a mutation in a membrane transport protein, encoded by the *Nramp* gene, in the susceptible host cell; the macrophage/monocyte. In the second example resistance to murine leukemia virus (MuLV) was associated with a polymorphic locus, *Fv-1*, which prevents integration of the provirus. Similar observations have been observed in HIV-1 infection in humans. Like the *Mycobacterium sp.* model, Williams LM, et al. have noted that PBMC from different individuals differ in their susceptibility to low MOIs of HIV-1.

Similar to the MMTV model the same group has observed that differential susceptibility to HIV-1 in human is at the level of the susceptible cell and segregates within a particular family, suggesting the inheritance of a protective polymorphic gene. In both animal models innate resistance was determined by the resistance of the susceptible cell to infection by the pathogen. To determine if a post receptor blockade in viral replication was occurring in the resistant prostitutes, PBMC from two populations, resistant and control, were exposed *in vitro* to HIV-1_{IIIB}. Bulk PBMC cultures were used because different CD4+ T lymphocyte clones from one individual were shown to have differential susceptibility to HIV-1 infection (Linette G, et al. 1989) and any variations observed between individuals would likely be dependent on the clones chosen. A productive infection, as determined by p24 production, was observed in both groups and there were no statistical differences in the mean p24 production of each group (Figure 16). This demonstrated that the PBMC of the resistant prostitutes were capable of supporting viral replication, thus excluding a post receptor blockade. However, data from Williams LM, et al. suggested that any differences in susceptibility to HIV-1 infection were only observed at low MOIs ie. dose dependent. However, when PBMC from resistant women were exposed to extremely low MOIs, all (n=3) (Figure 17) were susceptible to infection even at the lowest MOI (0.0003). Although the numbers were too small to draw firm conclusions, a trend seemed to be that the PBMC of the resistant women actually produced more p24 than the controls (n=2, Figure 18) at Day 6 of the lowest MOI. Due to the great degree of variation in the infectivity of different strains of HIV-1 (Cloyd MW, et al. 1990) these infection experiments should ideally be

reproduced with Kenyan isolates of HIV-1. However, our attempts to establish Kenyan HIV-1 isolates have failed to date. Yet the results from the infection experiments confirmed that the even at extremely low MOIs the PBMC of the resistant prostitutes were productively infected by HIV-1_{mn}. Taken together with the CD4 analysis it was concluded that the PBMC of the resistant prostitutes supported HIV-1 replication, therefore, the resistance mechanism was not innate.

C. Acquired Mechanisms of Resistance:

For this study acquired resistance was defined as that which occurred over time, ie. immune mediated. An individual may innately possess certain factors, eg. HLA genes, that are associated with resistance however, that individual is not born resistant to the infection rather the immune system must acquire resistance after initial exposure to the infectious agent.

An essential factor in the development of specific immunity is the presentation of HLA antigen associated peptide fragments of the pathogen to T lymphocytes via the T cell receptor (TCR). The nature of the peptide presented is dependent on the binding characteristics of each HLA antigen (Engelhard VH, 1994). This variability in antigen presentation is thought to explain the association of particular HLA antigens with certain autoimmune diseases (Lepage V, et al. 1993) and infectious diseases (Hill AVS, et al. 1991). Multivariate analyses by Cox proportional hazards modelling demonstrated independent and significant associations between HLA class I alleles A69 and B18 and a reduced risk of infection (protective alleles) and A30 and A23 with an increased risk

of infection (susceptible alleles). Kaplan-Meier plots of the time to seroconversion of the entire seronegative cohort demonstrated the significant survival value afforded by the protective antigens (Figure 19, Middle and Bottom). The current study reports several novel associations with particular class I alleles and resistance. HLA associations with resistance to HIV-1 infection have been reported by Just J, et al. (HLA DQA1) and Fabio G, et al. (HLA B52 and B44). The differences in the particular HLA antigens associated with resistance are expected since HLA frequencies and HIV-1 sub-types vary from one population to another. The HLA associations from the Nairobi prostitute cohort corroborates Just and Fabio's data that resistance to HIV-1 infection is associated with particular HLA antigens. The exact mechanism of the HLA associated resistance is not known but may involve certain HLA antigens presenting protective epitopes to the immune system. However, it is also possible that HLA antigens are acting as markers for the resistance factor that is held in linkage disequilibrium with the particular HLA gene.

The current study furthers strengthens the association between HLA and HIV-1 resistance by demonstrating that the rarity of the HLA haplotype is strongly associated with a decline in the risk of HIV-1 infection (Figure 20). The survival value of possessing a rare HLA haplotype was highly significant, $p < .005$ (Figure 21). The mechanism of this association is not known. However, it is known that the prostitutes are continuously exposed to foreign HLA antigens via their client's sperm. It may be hypothesized that the rarer the HLA haplotype of the prostitute, the greater the exposure to non-self HLA antigens, hence the greater the likelihood of developing a secondary

allo-immune response. Both cell associated and free HIV-1 virus contain HLA molecules (Arthur LO, et al. 1992; Meerloo T, et al. 1993) and could serve as targets for cellular or humoral allo-responses. This is analogous to transplant rejection occurring in persons receiving organs from HLA mismatched donors. The viral envelope may also act as a target for an allo response since cross reacting epitopes between gp120 and HLA molecules have been identified (Dorak MT, et al. 1994; Hounsell EF, et al. 1991; Clerici M, et al. 1993d). This study confirms previous reports that specific HLA antigens are associated with resistance and suggests that the rarity of the HLA haplotype is also associated with resistance, perhaps through an allo-immunization mechanism.

D. Cell Mediated Immune Responses to HIV-1:

Di. HIV-1 Specific CD4+ T cell responses:

One mechanism to explain the association between specific HLA antigens and resistance to HIV-1 infection was by the presentation of protective epitopes to T lymphocytes of the immune system. To determine if HIV-1 specific T cell responses existed in resistant women, PBMCs from control and resistant prostitutes were exposed to HIV-1 antigens (gp120 and *env* peptides (Berzofsky JA, et al. 1991)) *in vitro* and cellular activation was measured by IL-2 production (Table XIV). The level of IL-2 production (relative to media alone) in the low risk seronegative subjects was low (Figure 22). Due to reports that readily measurable immune responses to recall antigens, such as HIV-1 peptides, were common in asymptomatic HIV-1 infected individuals (Clerici M, et al. 1991), only asymptomatic HIV-1 seropositive prostitutes were chosen as

positive controls for this study. However, the levels of IL-2 production in the HIV-1 seropositive controls were surprising low. Shearer GM, et al. 1991 have reported that the loss of this recall response was predictive of CD4+ cell number decline, time to AIDS and death. This lack of response may be due to advanced T cell dysfunction among the seropositive prostitutes, in part accounting for the rapid progression to HIV-1 disease among the prostitutes of this cohort (Anzala AOA, et al. 1994). The lack of response is not due to an inability of the women from this cohort to recognize antigens based on North American HIV-1 isolates because the resistant prostitutes did respond to the antigens. Five of seven resistant prostitutes demonstrated immune recognition of the HIV antigens, as determined by IL-2 production. Despite significant variations in the data, preliminary experiments suggested that proliferative responses, as measured by ^{3}H-thymidine incorporation, to these same peptides demonstrated the same categorical pattern of responses; low risk and HIV-1 seropositive subjects did not respond while resistant subjects did respond to the HIV-1 antigens (data not shown). These results need to be confirmed in future experiments.

It is generally believed that the CD4+ T lymphocyte proliferates in response to exogenous antigens and peptides (Abbas AK, et al. 1991). This study suggests that the resistant women have CD4+ T cells that recognize HIV-1 specific antigens. This corroborates other studies that have observed CD4+ T cell responses among seronegative HIV-1 exposed individuals; homosexual men (Cleric M, et al. 1992), children born to HIV-1 positive women (Borkowsky W, et al. 1990; Clerici M, et al. 1993), health care workers with needle sticks (Clerici M, et al. 1994b) and partners of HIV-1 infected

persons (Kelker HC, et al. 1992). The current study has a significant advantage over the others in that the degree of exposure to HIV-1 is known. Although the other groups were also exposed to HIV-1, only the present study demonstrated statistically significant resistance to HIV-1 infection. Therefore, the association between the HIV-1 specific cellular immune response and resistance to HIV-1 infection assumes greater significance.

Dii. Cytotoxic Responses to HIV-1 Antigens:

This study has demonstrated the ability of CD4+ T cells to provide help (IL-2 production) in response to HIV-1 antigens. To determine if HIV-1 specific effector cellular responses could be detected in resistant women, chromium release assays were performed (Figure 24). Seven separate HIV-1 specific cytotoxic cellular responses representing *gag*, *pol*, and *env* activity were observed among 5 of the eleven resistant prostitutes (Table XV). HLA A28 (of which the protective allele A69 is a subtype) was observed in 4 of 5 women with HIV-1 specific cytotoxicity versus 1 of 6 women without detectable HIV-1 specific cytotoxicity (Fisher's exact test $p=.067$). Molecular analysis, which is more accurate than serological typing, of the A28 alleles of the resistant women is necessary to determine which allele is associated with resistance. Two low risk seronegative controls did not have any HIV-1 specific cytotoxicity. Additional negative controls were established, however, after the eleven day culture period there were insufficient effector cells to perform the assay suggesting a lack of specific (HIV-1 or otherwise) proliferation. The cell type responsible for the HIV-1 specific cytotoxicity was not determined although many reports have defined CD8+ T cell (reviewed by Levy

JA, et al. 1993), CD4+ T cell (Orentas RJ, et al. 1990; Curiel TJ, et al. 1993) and non-MHC restricted (McChesney M, et al. 1990) HIV-1 specific cytotoxic activity. These data corroborate previous reports of HIV-1 specific cytotoxic responses among seronegative partners of HIV-1 infected persons (Cheynier R, et al. 1992), a child born to an HIV-1 positive woman (Rowland-Jones, et al. 1993) and among Gambian prostitutes (Rowland-Jones, et al. 1995). The added significance of the results from the current study is an estimate of the degree of exposure to HIV-1. While the other studies have shown immune responses, the current study is the most convincing demonstration of resistance and that resistance is correlated with HIV-1 specific immune responses.

CD4+ T cell activation and cell-mediated cytotoxicity data from the current study strongly suggest that, among seronegative resistant prostitutes, HIV-1 specific cellular immune responses are dominant over humoral immune responses. The specific stimulation of IL-2 producing CD4+ T lymphocytes in the absence of a specific humoral response is characteristic of a type 1 T helper response (TH1). TH1 responses have been associated with protection to infection in *Leishmania major* (Bretscher PA, et al. 1992), *Chlamydia sp.* (Brunham RC, personal communication) and simian immunodeficiency virus (SIV) (Clerici M, et al. 1994). The evidence presented here supports the hypothesis that a TH1 dominant cellular immune response is protective and may account for resistance to HIV-1 infection.

Data from this study are not consistent with a single mechanism of resistance. The data suggest that a combination of HIV-1 specific and allotypic responses may be responsible for HIV-1 resistance.

E. Future Studies and Hypothesis Generation:

The present study has resulted in many potentially interesting lines of future investigation. These include: the use of Kenyan HIV-1 isolates in the determination of innate resistance; the identification and characterization of epitope specificity of HIV-1 specific cytotoxic cells; the determination of epitope binding motifs of resistance associated HLA molecules; the characterization of allo immune responses in resistant prostitutes and; further characterization of the cytokine profiles of HIV-1 specific CD4+ T cells. To test the hypothesis that these factors are associated with a reduced risk of infection they should be observed in a longitudinal study of resistant and newly enrolled seronegative prostitutes. Areas of preliminary data from this study that require confirmation are the proliferative responses to HIV-1 antigens and molecular determination of cytotoxic associated HLA alleles.

CONCLUSION

The objectives of the current study were to determine if a group of highly exposed persistently seronegative women from the Nairobi prostitute cohort were resistant to HIV-1 infection and, if so, by what mechanism. It was determined that the phenomenon of persistent seronegativity was real and even by sensitive molecular analyses the women were shown to lack any detectable HIV-1 infection. Statistical analyses suggested that these women should be infected by HIV-1 and the seronegative survival data from the entire cohort was best described by a model that assumed some of the individuals were resistant to infection. The association between duration of prostitution and reduced risk of infection could not be accounted for by differences in exposure to the virus or other factors associated with HIV-1 acquisition. Since all other reasonable explanations were eliminated, it was concluded that resistance did account for the lack of infection. The resistance was shown not to be at the level of the cellular receptor or at a post-receptor stage and, therefore, it was concluded that, using our cellular definition, resistance was not innate. An HLA association with resistance suggested acquired immune responses, possibly allo responses, may have been involved. HIV-1 specific CD4+ T cell and cellular cytotoxic responses (A28 associated) were demonstrated among the resistant prostitutes. The majority of the data presented suggested that the resistance to HIV-1 infection among the women of the Nairobi prostitute cohort was acquired. Although potentially protective acquired responses were demonstrated, the mechanism of resistance was likely multifactorial involving HIV-1 specific and allo responses.

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APPENDIX

I. ABBREVIATIONS:

AIDS	acquired immune deficiency syndrome
ADCC	antibody-dependent cellular cytotoxicity
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
cpm	counts per minute
CTL	cytotoxic T lymphocyte
DNA	deoxyribonucleic acid
FCS	fetal calf serum
gp	glycoprotein
HIV-1	Human Immunodeficiency Virus type 1
HLA	human leukocyte antigen
LM	lymphocyte medium
LN_2	liquid nitrogen
LTR	long terminal repeat
MCH	study code for antenatal subjects
MHC	major histocompatibility complex
ML	study code for Nairobi prostitute cohort
MOI	multiplicity of infection
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PSN	persistent seronegative
RNA	ribonucleic acid
SDS	sodium dodecylsulfate
Tm	melting temperature of DNA