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EVALUATION OF HIV TESTING STRATEGIES AND MONITORING OF IMMUNE RESPONSES IN HIV-VACCINATED INDIVIDUALS IN TANZANIA

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ABSTRACT

This thesis describes studies on the evaluation of human immunodeficiency virus (HIV) enzyme-linked immunosorbent assays (ELISAs) and simple rapid HIV assays for use in HIV testing strategies in resource-limited settings and studies of HIV vaccine-induced immune responses. Peripheral blood mononuclear cell (PBMC) preparation techniques were also studied in preparation for use in the HIV vaccine trials.

The performance of two antibody ELISAs (Vironostika Uni-Form II plus O and Enzygnost anti-HIV-1/2 Plus) and two new diagnostic HIV antigen/antibody combination ELISAs (Murex and Vironostika HIV Uni-Form II antigen/antibody) was evaluated using 1380 serum samples from Tanzanian individuals (paper I). The sensitivity at initial testing was 100% for all assays except Vironostika Uni-Form II plus O which showed one false negative sample at initial testing but 100% sensitivity after repeat testing. The initial specificity was 99.8% for Enzygnost, 98.9% for each of the antigen/antibody ELISAs and 97.0% for Vironostika Plus O ELISA. An alternative confirmatory HIV testing strategy based on initial testing on any of the two antigen/antibody assays followed by testing of reactive samples on the Enzygnost anti-HIV-1/2 Plus assay gave 100% specificity (95% CI; 99.7-100%).

The performance of five simple rapid HIV antibody assays was evaluated using 1433 whole blood samples (paper II). The sensitivity at initial testing of Determine, SD Bioline and Uni-Gold was 100% while First Response and Stat-Pak had a sensitivity of 99.5% and 97.7%, respectively, which increased to 100% on repeat testing. The initial specificity of the Uni-Gold assay was 100% while the specificities were 99.6%, 99.4%, 99.6% and 99.8% for Determine, SD Bioline, First Response and Stat-Pak assays, respectively. An alternative confirmatory HIV testing strategy based on initial testing on SD Bioline followed by testing of reactive samples on the Determine gave 100% sensitivity (95% CI; 99.1-100) and 100% specificity (95% CI; 96-99.1) with Uni-Gold as tiebreaker for discordant results and was adopted as a national algorithm in Tanzania.

Standard Ficoll-Paque gradient (FIP) centrifugation, BD vacutainer cell preparation tube (CPT) and Greiner Bio-One LeucoSep tube techniques for PBMC preparation were evaluated (paper III). No differences in mean recovery or mean viability of fresh PBMCs were observed between FIP centrifugation and CPT techniques used in Stockholm. In Dar es Salaam, recovery and viability of PBMCs isolated by FIP technique was higher compared to CPT purified cells. LeucoSep cell separation gave a higher yield and viability than FIP cell separation. The cells purified by the different techniques at the two sites performed equally well in interferon-gamma (IFN-γ) enzyme-linked immunospot (ELISpot) assays.

In a phase 1 HIV-1 DNA prime MVA boost vaccine trial in Sweden (HIVIS01/02), HIV-specific lymphoproliferative responses were tested by a [3H]-thymidine uptake assay and a flow-cytometric assay using whole blood (FASCIA-WB) (paper IV). A FASCIA using PBMC (FASCIA-PBMC) was also employed (n=14).Two weeks after the HIV-MVA boost 35 of 38 (92%) vaccinees were reactive by the thymidine uptake assay. Thirty-two of 38 (84%) vaccinees were reactive by the $CD4^+$ T-cell FASCIA-WB, and 7 of 38 (18%) also exhibited $CD8^+$ T-cell responses. There was strong correlation between the proliferative responses measured by the thymidine uptake assay and $CD4^+$ T-cell FASCIA-WB ($r=0.68$; $P < 0.01$). Fourteen vaccinees were analyzed using all three assays. Ten of 14 (71%) and 11/14 (79%) demonstrated $CD4^+$ T-cell responses in FASCIA-WB and FASCIA-PBMC, respectively. $CD8^+$ T-cell reactivity was observed in 3/14 (21%) and 7/14 (50%) using the FASCIA-WB and FASCIA-PBMC, respectively. All 14 were reactive by the thymidine uptake assay. A FASCIA-PBMC, which allows simultaneous phenotyping, may be an option to the [3H] thymidine uptake assay for assessment of vaccine-induced T-cell proliferation, especially in isotope-restricted settings.

In the HIVIS03 phase I/II HIV vaccine trial in Tanzania, sixty HIV-uninfected volunteers randomised to three groups of 20, received DNA plasmid vaccine 1 mg intradermally (id) or 3.8 mg intramuscularly (im) or placebo using a needle-free injection device (paper V). DNA plasmids vectoring HIV-1 genes gp160 subtypes A, B, C; rev B; p17/p24 gag A, B and Rtmut B were given at weeks 0, 4 and 12. Recombinant MVA (10^8 pfu) expressing HIV-1 Env, Gag, Pol of CRF01_AE or placebo was administered im at month 9 and 21. The vaccines were well tolerated. Two weeks after the first HIV-MVA boost 35/35 (100%) vaccinees had IFN-γ ELISpot responses; 35 (100%) to Gag and 31 (89%) to Env. Two to four weeks after the second HIV-MVA boost, 28/29 (97%) vaccinees had IFN-γ responses. The id -primed recipients had significantly higher responses to Env than im recipients after HIV-MVA boost. Intracellular cytokine staining for Gag-specific IFN-γ/IL-2 production showed both $CD8^+$ and $CD4^+$ T-cell responses. All vaccinees had HIV-specific lymphoproliferative responses. All vaccinees reacted in diagnostic HIV serological tests and 26/29 (90%) had antibodies against gp160 after the second HIV-MVA boost. A high neutralizing antibody response rate (31-83% depending on the clade B or AE virus tested) was demonstrated using a PBMC assay. In conclusion, this vaccine approach was safe and highly immunogenic.

LIST OF PUBLICATIONS

I. **Aboud S,** Urassa W, Lyamuya E, Mhalu F, Biberfeld G. Evaluation of HIV antibody and antigen/antibody combination ELISAs for use in an alternative confirmatory HIV testing strategy in Dar es Salaam, Tanzania. J Virol Methods 2006 August; 135(2):192 – 196.

II. Lyamuya EF, **Aboud S,** Urassa WK, Sufi J, Mbwana J, Ndugulile F, Massambu C. Evaluation of simple rapid HIV assays and development of national rapid HIV test algorithms in Dar es Salaam, Tanzania. BMC Infect Dis 2009 February; 9(1):19.

III. Nilsson C, **Aboud S,** Karlen K, Hejdeman B, Urassa W, Biberfeld G. Optimal blood mononuclear cell isolation procedures for gamma interferon enzyme-linked immunospot testing of healthy Swedish and Tanzanian subjects. Clin Vaccine Immunol 2008 April; 15(4): 585-589.

IV. **Aboud S,** Nilsson C, Karlen K, Marovich M, Wahren B, Sandstrom E, Gaines H, Biberfeld G, Godoy-Ramirez K. Strong HIV-specific $CD4^+$ and $CD8^+$ T lymphocyte proliferative responses in healthy individuals immunized with an HIV-1 DNA vaccine and boosted with HIV-1 recombinant modified vaccinia virus Ankara (MVA) expressing HIV-1 genes. Clin Vaccine Immunol 2010 July; 17(7):1124-1131.

V. Bakari M, **Aboud S,** Nilsson C, Francis J, Buma D, Moshiro C, Aris EA, Lyamuya EF, Janabi M, Earl P, Robb M, Marovich M, Wahren B, Pallangyo K, Biberfeld G, Mhalu F, Sandström E, for the HIVIS study group. Broad and potent immune responses to a low dose of intradermal HIV-1 DNA boosted with HIV-1 recombinant MVA among healthy adults in Tanzania. Vaccine, in press.

LIST OF ABBREVIATIONS

Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADC	Antibody-dependent cytotoxicity
ADCVI	Antibody-dependent cellular viral inhibition
Ad5	Adenovirus 5
Ag	Antigen
AIDS	Acquired Immune Deficiency Syndrome
APOBEC	Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like
ART	Antiretroviral therapy
ARV	Antiretroviral
AT-2	Aldrithiol-2
AZT	Azidothymidine
CAF	Cell antiviral factor
CCR5	Chemokine receptor 5
CRF	Circulating recombinant form
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
cDNA	Complementary negative strand DNA
CEF	Cytomegalovirus, Epstein-Barr and influenza virus
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CMV	Cytomegalovirus
CPT	Cell preparation tube
CTC	Care and treatment center
CTL	Cytotoxic T-lymphocytes
CXCR4	Chemokine receptor 4
DBS	Dried blood spot
DC	Dendritic cells
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EC	Elite controllers
ELISA	Enzyme-linked immunosorbent assay

ELISpot	Enzyme-linked immunospot
Env	Envelope
FASCIA	Flourescent activated cell sorting for cell-mediated assay
FC-LPA	Flow-cytometry lymphoproliferation assay
FDA	US Food and Drugs Authority
Gag	Group-specific antigen
GM-CSF	Granulocyte macrophage-colony stimulating factor
Gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HESN	Highly exposed seronegative
HIV	Human immunodeficiency virus
HIVIS	HIV vaccine immunogenicity study
HLA	Human leukocyte antigen
HVTN	HIV vaccine trials network
IAVI	International AIDS vaccine initiative
IFNs	Interferons
IFN-γ	Interferon-gamma
ICS	Intracellular cytokine staining
id	Intradermal
IL	Interleukin
im	Intramuscular
IMC	Infectious molecular clone
LIA	Line immune assay
LPA	Lymphoproliferation assay
LPS	lipopolysaccharide
LTNP	Long-term non-progressors
LTRs	Long terminal repeats
Mabs	Monoclonal antibodies
MBL	Mannose-binding lectin
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory proteins
MPER	Membrane proximal external region
MSM	Men who have sex with men
MTCT	Mother to child transmission

MVA	Modified Vaccinia Virus Ankara
MUHAS	Muhimbili University of Health and Allied Sciences
NAAT	Nucleic acid amplification tests
Nabs	Neutralizing antibodies
Nef	Negative regulatory factor
NIH	National Institutes of Health
NIMR	National Institute for Medical Research
NK	Natural killer
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitors
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEP	Post-exposure prophylaxis
PFU	Plaque forming unit
PHA	Phytohaemaglutinin
PMTCT	Prevention of mother to child transmission
PPD	Purified protein derivative
PrEP	Pre-exposure prophylaxis
PRR	Pattern recognition receptors
RANTES	Regulated upon activation, normal T-cell expressed and secreted
Rev	Regulator of virion
RNA	Ribonucleic acid
RT	Reverse transcriptase
SEAB	Staphylococcal enterotoxin A and B
SHIV	Simian/human immunodeficiency virus
SIV	Simian immunodeficiency virus
SMI	Swedish Institute for Communicable Disease Control
STIs	Sexually transmitted infections
Tat	Transactivating factor
TB	Tuberculosis
TFDA	Tanzania Food and Drugs Authority
Th	T-helper
TLR	Toll-like receptor

TNF-α	Tumor necrosis factor-alpha
TRIM5-α	Tripartite motif 5-alpha
tRNA	Transfer RNA
WHO	World Health Organization
UNAIDS	Joint United Nations Program on HIV and AIDS
VCT	Voluntary counseling and testing
Vif	Viral inhibition factor
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X
VRC	Virus Research Center
WB	Western blot
WRAIR	Walter Reed Army Institute for Research

CONTENTS

1 GENERAL BACKGROUND

1.1 Introduction

Acquired immunodeficiency syndrome (AIDS), which is characterized by a cellular immunodeficiency leading to life threatening opportunistic infections and/or Kaposi's sarcoma and malignant lymphoma was first described in the early 1980s [1-2]. AIDS is caused by two retroviruses, human immunodeficiency virus types 1 (HIV-1) and 2 (HIV-2) [3-6]. HIV-1 infection is found worldwide while HIV-2 infection has its epicenter in West Africa. HIV infection has spread extensively in most parts of the world but the highest prevalence and incidence of HIV infection are found in sub-Saharan Africa. HIV can be transmitted through sexual intercourse, blood and blood products including contamination during intravenous drug use and vertically from mother to child [7]. The primary target for HIV is the $CD4^+$ T-lymphocytes, which are crucial to the normal function of the human immune system. Laboratory diagnosis of HIV infection can be done by detection of HIV antibodies, HIV antigens and viral nucleic acids, and by virus isolation [8]. HIV infection can be treated by antiretroviral (ARV) drugs to prolong and improve the quality of life of HIV-infected individuals. There are several ways to prevent the spread of HIV infection including health education on HIV, condom use, HIV screening of blood and blood products, voluntary counseling and testing, diagnosis and treatment of sexually transmitted infections (STIs), pre (PrEP) and post exposure ARV prophylaxis (PEP), prevention of mother to child transmission (PMTCT) of HIV and male circumcision. A safe, successful and affordable vaccine would be the most effective means to prevent the spread of HIV infection especially in vulnerable and highly at risk populations throughout the world and in particular those living in sub-Saharan Africa where nearly 70% of people living with HIV are found.

1.2 The epidemiology of HIV infection

1.2.1 Global situation

WHO/UNAIDS estimated that at the end of 2009 globally there were 33.3 million people living with HIV including 30.8 million adults, 15.9 million women and 2.5 million children under 15 years [9]. The total number of people living with HIV in 2009 was more than 14% higher than the 28.6 million in 2001, and the prevalence in 15-49 year-adults was similar (0.8%). HIV prevalence in young women (15-24 yr.) was double (0.6%) compared to young men (0.3%). It is estimated that during the year

2009, 2.6 million people became infected with HIV which was 21% fewer than the 3.2 million at the epidemic's peak 12 years earlier in 1997. The incidence rate in adults was <0.1 in 2009. An estimated 1.8 million people died of AIDS-related illnesses worldwide which decreased from 2.1 million in 2004 [9] due to increased availability of antiretroviral therapy (ART), care and support to people living with HIV and AIDS and decreasing incidence. However, the number of orphans due to AIDS has increased from 10 million in 2001 to 16.6 million in 2009. At the end of 2009, 5.25 million people were reported to be receiving ART in low- and middle-income countries following revised guidelines on $CD4^+$ T-cell count for initiating treatment in adults and recommendations on ART for infants and children, adults and adolescents including pregnant women [10]. This represents an increase of over 1.2 million people from December 2008 [10]. Based on the new criterion for ART initiation ($CD4^+$ T-cell count $\leq$350 cells/uL), ART coverage for eligible patients increased from 28% at the end of 2008 to 36% at the end of 2009 [10].

1.2.2 HIV infection in sub-Saharan Africa

Sixty-seven percent of people living with HIV worldwide are found in sub-Saharan Africa and at the end of 2009, there were 22.5 million people living with HIV [9]. Approximately 54% of the people living with HIV infection (12.1 million) in the region were women in 2009 compared to 10.9 million women in 2001 [9]. The prevalence of HIV infection in 15-49 year-adults was 5% in 2009 compared to 5.9% in 2001. HIV prevalence in young women (15-24 yr.) was more than double (3.4%) compared to that in young men (1.4%). Sub-Saharan Africa remains the most heavily affected region globally accounting for 69% of all new HIV infections in 2009 with an estimated 1.8 million people. The HIV incidence has fallen by more than 25% between 2001 and 2009 in 33 countries in the world of which 22 are in sub-Saharan Africa. The HIV incidence rate in adults was <0.41 in 2009 compared to 0.61 in 2001 [9]. Seventy-two percent of the total global AIDS deaths occurred in the sub-Saharan region in 2009 with an estimated 1.3 million people. The estimated number of orphans due to AIDS in the region was 14.8 million [9]. Sub-Saharan Africa had the greatest increase in the number of people receiving ARV treatment in 2009, from 2,950,000 at the end of 2008 to 3,911,000 a year later [10]. Eight low- and middle-income countries including Botswana had already achieved universal access to ART at the end of 2009 [10]. At 39%, ART coverage in the region was higher among women compared with 31% among men. In 2009, the average retention rate at 12 months across low- and middle-income countries was 82% and was approximately the same among men and women [10].

1.2.3 HIV and AIDS in Tanzania

In Tanzania, cases of AIDS were first observed in the Kagera region at the end of 1983 [11]. Early sero-epidemiological studies showed that the prevalence and incidence of HIV-1 infection differed considerably in various parts of the country and in various population groups [11-24]. A population-based study conducted in the Kagera region in 1987 showed that the HIV-1 seroprevalence among adults was 24.2% in the Bukoba urban zone, 10% in the neighbouring Bukoba rural and Muleba area and only 0.6% in more remote rural areas [13]. A subsequent study in the Kagera region showed a fall in HIV seroprevalence among adults in Bukoba urban to 18.2% in 1993 and down to 13.3% in 1996 and the decline was most significant in young women [25]. Furthermore, the high incidence of 47.5 per 1000 person years in 1989 in the Bukoba urban area declined to 9.1 per 1000 person years in 1996 [25]. In the Kagera region, age-adjusted prevalence of HIV infection among antenatal clinic attendees decreased from 22.4% in 1990 and down to 13.7% in 1996 [26].

In Dar es Salaam, the prevalence of HIV infection among pregnant women increased from 1.3% in 1984-1985 [27] and 3.6% in 1986 [11] up to 15.2% in 1993 [19] and then declined to 13.7% in 1996 [23], 11% in 2001-2003 [28] and 11.1% in 2004-2006 [29]. A national surveillance of HIV infection conducted among antenatal clinic attendees in 2003-2004 showed that the Mbeya region had the highest prevalence of HIV infection (15.7%) followed by Dar es Salaam (10.8%) and Tanga (9.2%) regions while the Kagera region (4.7%) had the lowest HIV prevalence [30]. The prevalence among blood donors in Dar es Salaam rose from 2% in 1984-1985 to 10% in 1988 [27] and then declined to 8.7% in 1999 [31] and 3.8% in 2004-2005 [32]. The overall prevalence of HIV among voluntary blood donors also decreased from 4.0% in 2006 to 2.7% in 2008 [33].

UNAIDS estimates that at the end of 2009 there were around 1.4 million people living with HIV including 1.2 million adults and 730,000 women and 160,000 children under 15 years in a total population of 41 million [9]. The overall HIV prevalence in adults was 5.6% in 2009 compared to 7.1% in 2001. HIV prevalence in young women between 15 and 24 years was more than double (3.9%) compared to

1.7% in young men. An estimated 100,000 people (88,000 adults and 12,000 children) became newly infected in 2009 [9]. The incidence rate in adults was 0.45 in 2009 compared to 0.64 in 2001. An estimated 86,000 people died of AIDS-related illnesses in 2009 compared to 110,000 people in 2001. The number of orphans due to AIDS increased from 840,000 in 2001 to 1.3 million in 2006 [9].

In 2008, the Tanzanian Ministry of Health and Social Welfare reported that about 85% of HIV transmission occurred through heterosexual sex followed by mother to child transmission (MTCT) (6%) and less than 1% through blood transfusion [33]. During the same year, the overall prevalence of HIV infection among voluntary counseling and testing (VCT) attendees was 11.4%, ranging from 3% in Tanga to 24.6% in Iringa regions [33]. The number of patients on ART by the end of March 2009 was 235,092 representing 55.6% of the 422,632 estimated numbers of ART eligible and 51.4% of 457,314 patients enrolled into HIV care and treatment center (CTC) services.

1.3 Virology and replication cycle of HIV

HIV belongs to the subfamily lentivirinae and family retroviridae. There is 50% homology in the genome between HIV-1 and HIV-2. HIV-1 was reported to have been transferred from chimpanzees to humans at least three times to form the HIV-1 M, N and O groups [34]. Similarly, the origin of HIV-2 has been related to a transfer from sooty mangabey into human beings on multiple occasions [35].

HIV is a spherical enveloped RNA virus with a diameter of 80 to 120 nm. The envelope is a lipid bilayer containing viral glycoprotein and is acquired by budding from the host cell membrane. The envelope spikes are the glycoprotein gp120 which interacts with the $CD4^+$ molecule on the surface of T-lymphocytes and gp41 which mediates fusion of the HIV with the cell membrane of the $CD4^+$ T-lymphocyte. The envelope surrounds a capsid that contains two identical copies of positive strand single stranded RNA genome inside the core part of the virus (Figure 1). The virion which resembles a truncated cone also contains the reverse transcriptase and integrase enzymes. The HIV genome consists of three major genes that encode polyproteins for enzymatic and structural proteins of the virus including *gag* for gag-specific antigen, capsid, matrix and nucleic acid-binding proteins; *pol* for polymerase, protease and integrase; and *env* for envelope glycoproteins. The genome also includes six accessory genes. The genome is flanked at its 5´and 3´ end by long terminal repeats (LTRs) [36].

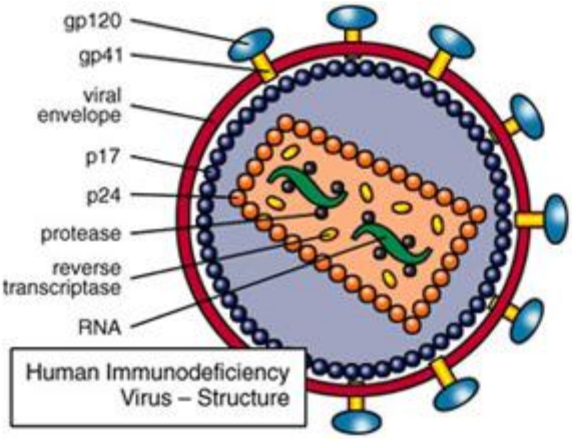


Figure 1.

(Source http://www.avert.org/media-gallery/image-115-the-structure-of-hiv)

HIV targets cells expressing the $CD4^+$ receptor, including T-helper lymphocytes, monocytes/macrophages and dendritic cells. Replication of HIV starts with binding of the viral glycoprotein spikes, the trimer of gp120 and gp41 molecules to the primary receptor, the $CD4^+$ protein and subsequently to one of the main chemokine co-receptors (Figure 2) either CCR5 (R5 viruses) or CXCR4 (X4 viruses). R5 viruses (also known as macrophage-tropic) predominate during early infection while X4 viruses (also known as T-cell-tropic) are more frequent during the advanced stages of infection [37]. A small percentage of people are resistant to infection because they have mutations in the CCR5 receptor gene [38]. HIV can also bind to a cellular adhesion molecule, integrin $\alpha4\beta7$, present on gut-associated lymphoid tissue. The transmembrane gp41 mediates fusion of the viral and cellular membranes which leads to the release of the viral core into the host cell. Once the genome is released into the cytoplasm after uncoating, the early phase of replication begins. The reverse transcriptase transcribes viral RNA to DNA which is transported to the cell nucleus and is integrated into the host cell chromosomal DNA. HIV reverse transcriptase is very prone to errors due to lack of proof reading ability and cause point mutations during transcription to proviral DNA [39-42]. Integration requires cell growth but the complementary negative strand DNA (cDNA) of HIV can remain in the nucleus and cytoplasm in a non-integrated circular DNA form until the cell is activated. Once integrated, the late phase begins and proviral DNA is transcribed as a cellular gene by the host RNA polymerase. Transcription of the genome produces a full-length RNA which is processed to produce several mRNAs that contain the *gag*, *gag-pol,* or *env* gene sequences. The proteins translated from the gag, gag-pol and env mRNAs are synthesized as polyproteins and are subsequently cleaved to functional proteins. The envelopment and release of mature HIV virions occur at the cell surface. The HIV envelope picks up cellular proteins including major histocompatibility complex (MHC) molecules upon budding. HIV replication is regulated by six accessory gene products which are important in the life cycle of HIV. The Tat protein is a transactivator of transcription of viral and cellular genes while Rev protein regulates and promotes transport of viral mRNA into the cytoplasm. The Nef protein reduces cell surface expression of CD4$^+$ and MHC class I molecules, alters T-cell signaling pathways, regulates the cytotoxicity of the HIV and is required to maintain high viral loads. The Vif protein helps in virion assembly and promotes viral infectivity by mediating degradation of the intracellular antiviral apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like (APOBEC-3G) factor [43]. Viral protein u (Vpu) reduces cell surface CD4$^+$ expression and enhances the release of virion. Vpr is important for transport of cDNA into the nucleus and for arresting of cell growth [36].

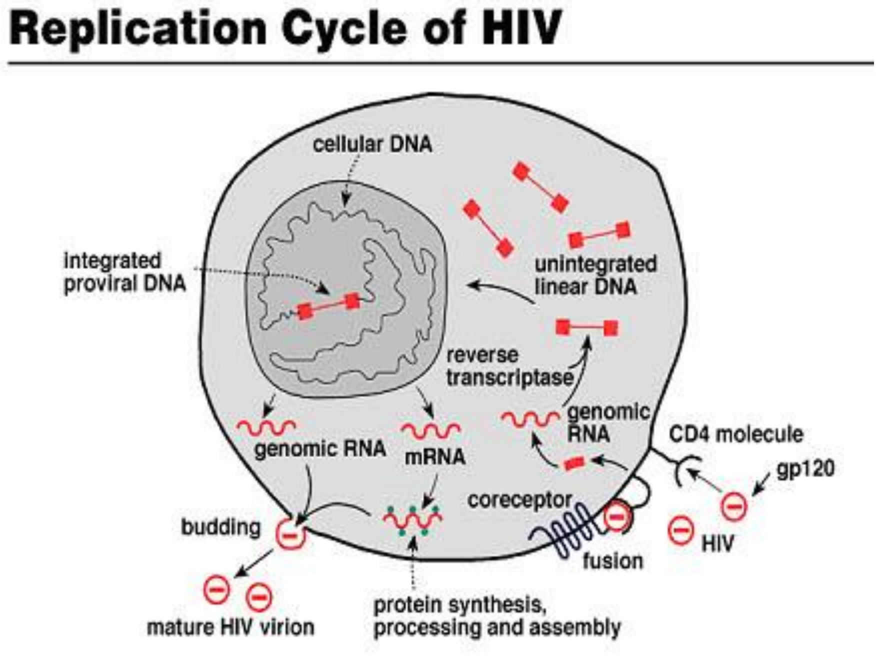


Figure 2.

(Source http://www.avert.org/media-gallery/image-875-replication-cycle-of-hiv)

1.4 HIV subtypes and genetic diversity

HIV-1 consists of 3 groups called M (major), O (outlier) and N (non M or O) [44]. Eight HIV-2 groups have been described so far [45]. Nine HIV-1 clades (or subtypes) have been described within the M group [46-47] and are designated A to D, F to H, J and K. There are also circulating recombinant forms (CRFs). Globally, clades A through D and the CRF-01AE and CRF-01AG recombinants account for more than 90% of infections worldwide [48]. Close to 75% of the new infections occurring in the world are caused by subtypes A, C, and CRF-02AG. Clade C is the most prevalent in the world spreading through Central Africa down to South Africa. Clade C is also becoming dominant in parts of China, India and Ethiopia and may present up to 50% of all HIV infections worldwide. In the group M, clade A is found primarily in Central Africa, clade B in North Africa, North and South America and Europe, clade C in South Africa and India, and clade D in Central Africa. Subtype F has been isolated from Brazil [49]. In Tanzania, the prevalent HIV-1 subtypes are A, C, D and CRFs [50-54] and no HIV-2 infections have been reported to date. Other subtypes of group M include viruses from Russia (G) [55], Africa in Zaire (clade J) [56] and Cameroon (clade K) [57]. There are 16 recognized CRFs derived from the group M HIV isolates [46-47]. Subtype E (A and E) which is prevalent in Thailand has been renamed CRF-01AE [58]. CRF-02AG dominates the HIV epidemic in some parts of Africa [59]. In addition to the M group, other isolates initially found in Cameroon [60] are considered outliers and form the O group which has also been found at low frequency in other African countries [60]. The N group of HIV-1 has been found in a few HIV-infected individuals in Cameroon [46, 61]. The enormous HIV genetic diversity may have implications for possible differential rates of HIV disease progression, response to ART, emergence of resistance to ARV drugs and development of vaccine [62].

1.5 Modes of transmission of HIV infection

AIDS was initially described in homosexual and bisexual men and intravenous drug abusers [2, 63-67]. HIV can be transmitted through sexual intercourse, exposure to infected blood and blood products, intravenous drug use [7, 68-69] and vertically from mother to child [70-74]. HIV can also be transmitted through use of infected needles and surgical instruments and accidental needle-stick injury [75]. HIV is primarily transmitted through heterosexual intercourse in sub-Saharan Africa including Tanzania [76]. High viral load [77], high-risk sexual behavior including having multiple sex partners and the presence of STIs increase the risk for HIV transmission [21, 78-79].

The number of infectious viruses and infected cells are highest during acute and symptomatic infection, especially AIDS [80-85]. Presence of cell free infectious virus and/or virus infected cells has been demonstrated in the seminal and vaginal fluids in 10 to 30% of specimens tested from HIV-infected individuals [83, 86-92]. It has been reported that clade C virus infection was associated with increased HIV-1 vaginal shedding [93]. Another study showed that subtype C was preferentially transmitted in utero compared to subtype A and D [94]. MTCT of HIV is associated with high viral loads in blood and breast milk, a larger number of infected breast milk cells and mastitis in HIV-infected breastfeeding mothers [95-96].

1.6 Immunopathogenesis of HIV infection

$CD4^+$ T-lymphocyte depletion and chronic immune activation are central immunopathogenic features of HIV infection. Possible mechanisms of $CD4^+$ T-cell destruction include a direct cytopatic effect of HIV and its proteins, apoptosis induced by immune activation, $CD8^+$ T-cell cytotoxicity and ADCC activity [37]. The decrease of $CD4^+$ T-cell counts and the level of HIV-1 viral load in plasma correlate with disease progression [97]. Immune activation changes include polyclonal B cell activation, increased CD8 and $CD4^+$ T-cell expression of activation markers such as CD38 and HLA-DR, increased T-cell turnover and elevated serum levels of proinflammatory cytokines and chemokines [98]. Immune activation measured as elevated expression of CD38 on $CD8^+$ and $CD4^+$ T-cells has been reported to be a better predictor of disease progression than plasma viral load [99]. A recent study of chronically HIV-1-infected individuals in Uganda showed that levels of $CD4^+$ T-cell activation measured as expression of CD38, HL-DR and the programmed death (PD-1) receptor correlated directly to viral load and inversely to $CD4^+$ T-cell count and that the levels of these cells also correlated to plasma levels of soluble CD14 and IL-6 which are markers of innate immune activation [100]. High-level chronic immune activation is found in pathogenic simian immunodeficiency virus (SIV) infection in macaques but not in non-pathogenic SIV infection in natural non-human primate hosts [98].

Following sexual transmission, $CD4^+$ T-cells and Langerhans cells are the first targets of HIV [101-102]. There is evidence that a single CCR5 virus usually is responsible for initial sexual infection. Studies of early infection in the SIV macaque model have shown that following vaginal SIV inoculation, central memory $CD4^+$ T-cells expressing high levels of the $\alpha4\beta7$ integrin receptors are the predominant early target cells [103].

These cells were also shown to include Th17 cells which are important in the defence against bacterial infections. These cells are abundant in the gut-associated lymphoid tissue. After initial propagation of virus in the mucosa for a few days at the mucosal portal of entry, infection is spread to draining lymph nodes. Dendritic cells (DC) can bind virus particles and contribute to the spread of virus to the lymph nodes where activated $CD4^+$ T-cells are targets for further infection. Subsequent dissemination of infection to gut-associated and other lymphatic tissues results in a massive depletion of memory $CD4^+$ T-cells, especially in the gastrointestinal tract [101-102]. Damage to the mucosal barrier in the gastrointestinal tract results in translocation of microbial products, e.g. lipopolysaccharide (LPS), into the systemic circulation [98]. Studies in chronically HIV-infected individuals and in SIV-infected macaques have shown that circulating microbial products are a cause of systemic immune activation [104].

During the acute HIV infection the viral replication is very high. Viral RNA is usually first detectable in plasma one to two weeks after initial infection. After the initial peak the plasma viral RNA declines and reaches a viral set point two to six months after the initial infection. The initial decline of viremia coincides in time with the appearance of HIV-specific $CD8^+$ T-cells [105-106]. The viral load increases during the advanced stages of HIV disease (Figure 3). The rapid decrease of $CD4^+$ T-cells during the acute HIV infection is followed by an increase of the $CD4^+$ T-cell count after the resolution of the primary infection. However, subsequently there is a gradual decline of $CD4^+$ T-cells during the course of infection (Figure 3). An impairment of HIV-specific $CD4^+$ T-cell function occurs early in infection which is then followed by defects in $CD4^+$ T-cell responses to other recall antigens and to novel antigens [107].

1.7 Natural history of HIV-1 infection

HIV disease progresses from acute primary infection to a chronic asymptomatic phase followed by a symptomatic phase leading to full-blown AIDS [108-109]. Studies on the natural history of HIV-1 infection conducted in resource-rich countries have shown that in ART-naive HIV-infected individuals the time from seroconversion to development of AIDS is about 10 years [110-112]. The natural history of HIV infection has also been documented from studies conducted in Africa [113-119]. Studies in Uganda and Tanzania [116, 119] showed that the rates of HIV-1-associated disease progression and $CD4^+$ T-lymphocyte decline were similar to those reported in Europe and the US. In contrast, in a study of female sex workers in Kenya a rapid clinical progression from

HIV-1 seroconversion to AIDS was reported [113]. HIV-2 infection is characterized by lower viral load and slower progression to AIDS than HIV-1 infection [120].

The initial symptoms following acute phase of HIV infection may resemble those of influenza or infectious mononucleosis. As in mononucleosis, the symptoms arise from immune response triggered by a widespread infection of lymphoid tissue. These symptoms subside spontaneously after 2 to 3 weeks and are followed by a period of asymptomatic infection or a persistent generalized lymphadenopathy that may last for several years. During this clinical latency period, the virus continues to replicate mainly in the lymphoid tissue. In intermediate immunodeficiency, HIV replication is very high and $CD4^+$ T-cell turnover is rapid. Deterioration of the immune response is indicated by increased susceptibility to opportunistic pathogens.

Full-blown AIDS usually occurs when the $CD4^+$ T-cell counts are less than 200/µL and involves the onset of more significant diseases including HIV wasting syndrome and occurrence of indicator diseases such as malignancy or opportunistic infections. Opportunistic pulmonary infections including tuberculosis (TB) and *Pneumocystis jirovecii* pneumonia are the major causes of morbidity and mortality in AIDS patients [121]. Oral candidiasis, cerebral toxoplasmosis, cryptococcal meningitis, pneumococcal infections, bacterial enteritis and prolonged and severe viral infections caused by cytomegalovirus (CMV), herpes simplex virus types 1 and 2 and varicella-zoster virus also occur [122]. The most notable malignancy to develop in patients with AIDS is the human herpesvirus 8-associated Kaposi’s sarcoma, a rare and otherwise benign skin cancer that disseminates to involve visceral organs in immunodeficient patients [123]. Non-Hodgkin lymphoma and Epstein-Barr virus (EBV)-related lymphomas are also prevalent [124]. AIDS-related dementia may result from opportunistic infection or HIV infection of the macrophages and microglial cells of the brain. Studies in Africa showed that infection with HIV-1 subtype D is associated with faster disease progression compared to infection with other subtypes [125-128].

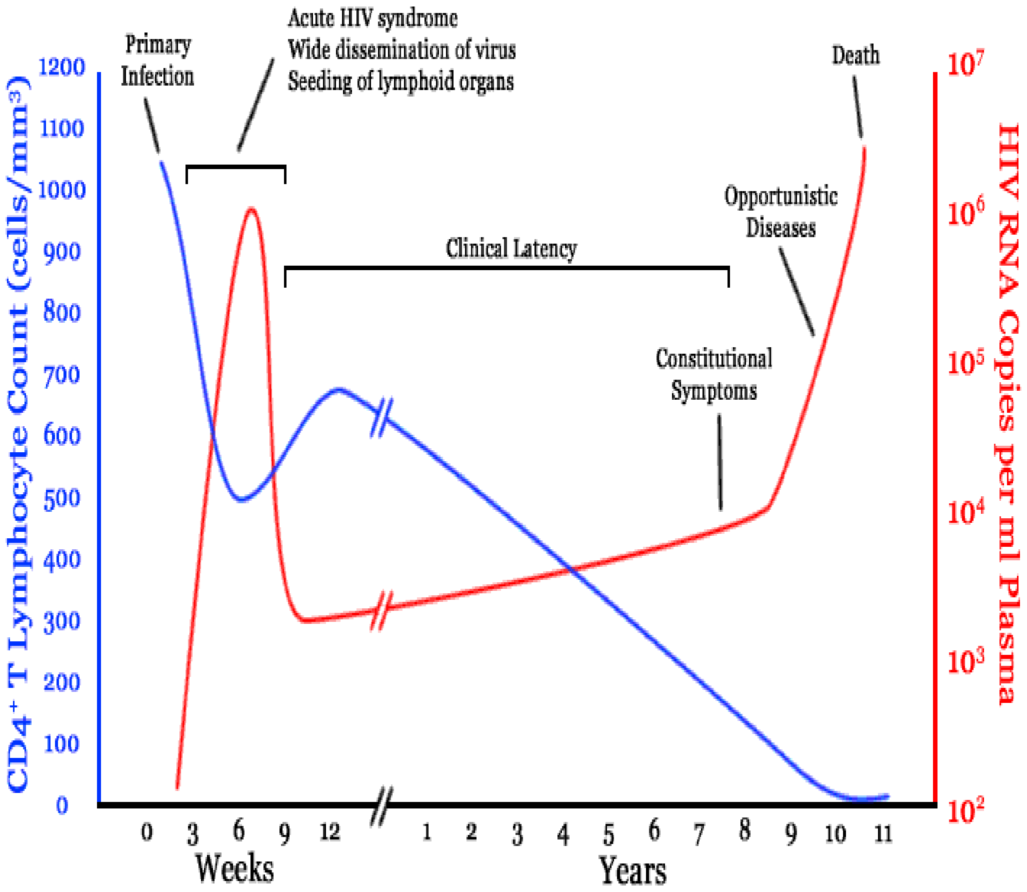


Figure 3. HIV-1 disease progression (Source http://en.wikipedia.org/wiki/Hiv)

1.8 Innate immunity

Innate immunity is the first line defense mechanism against HIV infection that can respond in minutes to a few days. It is characterized by its presence since birth, non-utilization of MHC molecules, non-specificity, lack of memory and its intensity does not increase with intensity of HIV exposure. Intact skin and mucosa serve as physical barriers to the HIV entry in the body. Chemical barriers such as low pH acts to create unfavorable environment for invading pathogens. Secretions in the mucosa such as defensins and type I interferons (IFNs) can inactivate HIV and prevent the entry through mucosa. Mannose-binding lectin (MBL) is another soluble factor with anti-HIV activity [129]. The innate immune system is comprised of several cell types, including DC, macrophages, neutrophils, natural killer (NK) cells, NK T-cells and γδ T-cells. Cells of the innate immune system recognize pathogen-associated molecular patterns of various pathogens, e.g. viral RNA and bacterial LPS, via pattern recognition receptors (PRR), such as Toll-like receptors (TLR) [130]. Activation of cells of the innate immune system results in the production of various cytokines, such as type I

IFNs, and β-chemokines, such as Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES), Macrophage inflammatory proteins (MIP)-1α and MIP-1β which can inhibit HIV replication [129, 131]. The β-chemokines can prevent HIV infection by blocking the CCR5 co-receptors. IFN-α activates NK cells which can kill virus-infected cells and produce IFN-γ and other cytokines which help cytotoxic function of $CD8^+$ T-cells [132]. Furthermore, IFN-α up regulates the expression of the intracellular antiviral factors APOBEC3G and TRIM5-α [131]. Complement proteins can control HIV infection through several mechanisms, including lysis of virions in association with antibodies [133-134], binding to virions and activation of the alternative pathway [135], binding to gp120 and activation of the classical complement pathway [136] and increased HIV binding via immune complexes [137-138]. $CD8^+$ T-cell non-cytotoxic antiviral activity mediated by a soluble factor (CAF) is another component of the immune system with anti-HIV activity [129]. The activation of the innate immune system also contributes to the induction of adaptive immune responses.

Studies of possible correlates of protection in various cohorts of HIV-1 highly exposed seronegative (HESN) individuals, including female sex workers, partners of HIV-infected individuals and intravenous drug users, have demonstrated an association not only with HIV-specific cellular immune responses and mucosal HIV antibody responses but also with innate immune responses [139]. Increased NK cell activity including cytolytic activity and production of cytokines, e.g. IFN-γ has been associated with resistance to HIV in HESN individuals. Protective NK receptor alleles have also been shown to be more frequent in HESN individuals than in HIV-infected individuals. Furthermore, increased DC responses and production of antiviral soluble factors including β-chemokines and defensins have been associated with reduced risk of HIV infection in HESN individuals [139].

1.9 Adaptive immunity

Adaptive immunity is characterized by its acquisition after exposure to HIV, specificity, keeping of memory, antigen recognition by either MHC class I or II, and by the increasing intensity of immunity with increasing intensity of exposure. Adaptive immunity consists of cell and antibody mediated immune responses.

1.9.1 HIV-specific cellular immune responses

$CD4^+$ and $CD8^+$ T-lymphocytes play major roles to fight HIV infection. $CD4^+$ T-lymphocytes, also called T-helper (Th) cells, recognize antigens in association with MHC class II. $CD4^+$ T-lymphocytes secrete cytokines which activate other cells of the immune system. $CD4^+$ T-cells can also have cytolytic antiviral activity [140]. There are several subsets of $CD4^+$ T-lymphocytes including Th1, Th2, Th17 and regulatory T-cells. Th1 cells produce IFN-γ and tumor necrosis factor (TNF) which have a significant role in the control of HIV infection. Th1 cells also produce IL-2 which causes activation and differentiation of $CD8^+$ T-cells. Th2 cells produce IL-4, IL-5, IL-6 and IL-13 which facilitate priming of humoral immune response and clearance of extracellular pathogens. HIV-specific $CD4^+$ T-cell proliferative responses resulting in the production of IFN-γ and β-chemokines have been shown to be associated with control of HIV replication and prevention of HIV disease progression [141].

$CD8^+$ T-lymphocytes, also called cytotoxic T-lymphocytes (CTL), recognize HIV-infected cells in association with MHC class I. Activated $CD8^+$ T-cells release perforin and granzymes A and B which target HIV-infected cells and induce apoptosis [142-144]. $CD8^+$ T-cells can also induce apoptosis of target cells by ligation of Fas. Furthermore activated $CD8^+$ T-cells produce antiviral cytokines such as IFN-γ and TNF-α. HIV-specific $CD8^+$ CTL activity has been shown to be associated with the initial control of viremia in acute HIV-1 infection [105-106]. HIV-specific CTL activity declines with disease progression [145]. In the macaque SIV infection model, depletion of $CD8^+$ T-cells led to a marked increase of viremia [146-147]. Data from a large cohort study among 578 treatment naïve HIV-infected individuals from KwaZulu-Natal in South Africa and a smaller study in Tanzania among 56 female bar workers showed that $CD8^+$ T-cell responses to Gag were associated with low viral loads [148-149]. Furthermore, HIV-infected female bar workers with HLA class I alleles B5801, B8101 and B0702 had lower viral loads compared to other alleles [149]. Certain HLA types including HLA-B27 and HLA-B57 have been reported to be associated with slow HIV disease progression [37].

HIV-1 specific $CD4^+$ and $CD8^+$ T-cell responses have been observed in HIV-exposed uninfected individuals [150-151]. Recently, HIV-specific lymphoproliferative responses have been associated with reduced acquisition of HIV in commercial sex workers in Kenya [152]. Long-term non-progressors (LTNP) have been reported to show a stable $CD4^+$ T-cell count and variable but low viral load [153]. Elite controllers (EC) demonstrate a stable $CD4^+$ T-cell count and viral load of <50 copies/mL of plasma [154]. LTNP and EC show a higher HIV-specific CTL activity with increased levels of functional granzyme B and perforin compared to that seen in progressors [155]. Furthermore, LTNP show broader and more polyfunctional HIV-specific immune responses compared to progressors [156]. High CAF activity has also been demonstrated in LTNP [37].

1.9.2 HIV-specific antibody responses

HIV antibodies are usually detectable 3 to 4 weeks after HIV infection [8]. HIV antibodies circulate in the blood and are also found in mucosal surfaces. HIV-1 specific binding antibodies are detected earlier after initial infection than neutralizing antibodies (Nabs). Nabs are directed against HIV gp120 and gp41 [48, 157-158]. The Nabs develop too late to influence the course of the acute HIV infection. The earliest Nab response is usually specific for the early autologous virus. However, viral mutants develop which are resistant to the Nabs and the Nabs in chronically infected subjects can usually neutralize early virus isolates but not concurrent autologous virus variants. It has been reported that approximately 20% of chronically HIV-infected individuals develop Nabs that can neutralize several heterologous primary virus isolates but only 2% have high titers of broadly cross-reacting Nabs against most HIV-1 strains [159]. Rare broadly neutralizing monoclonal antibodies (Mabs) directed against different epitopes of gp120 or against the membrane proximal external region (MPER) of gp41 have been identified [159-160]. Recently two new broadly neutralizing Mabs called PG9 and PG16 with reactivity to conserved regions of variable loops of gp120 have been generated from a clade A-infected African donor [161]. Passive immunization experiments in macaques using broadly neutralizing Mabs or polyclonal IgG showed that these antibodies could protect against simian/human immunodeficiency virus (SHIV) infection [160]. Nabs to HIV-1 have been demonstrated in the cervical fluid in HIV HESN [151]. Furthermore, genital neutralizing IgA has been reported to be associated with reduced acquisition of HIV infection in Kenyan female sex workers [152].

Antibodies to HIV Env have also been shown to mediate antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cell-mediated virus inhibition (ADCVI) through binding to Fc receptors on effector cells, such as NK cells and monocytes [162-166]. A study of the Multicenter AIDS Cohort in the US showed that rapid progressors had significantly lower ADCC antibody titers as compared to nonrapid progressors [167]. A study of HIV-infected individuals with undetectable viral replication showed that these individuals had higher ADCC antibody titers than viremic individuals [168]. It has been reported that macaques vaccinated with replicating recombinant adenovirus 5 (Ad5)-SIV followed by SIV gp120 developed ADCC antibody activity which correlated with reduced acute viremia after intrarectal SIV challenge [169].

1.10 Laboratory diagnosis of HIV infection

1.10.1 Detection of HIV antibodies

HIV-specific antibody detection is the most commonly used approach for the diagnosis of HIV infection. However, antibodies usually appear about 3-4 weeks after initial HIV infection [8]. Several types of assays for HIV antibody detection have been developed and promoted for HIV screening and diagnosis [8]. Enzyme-linked immunosorbent assay (ELISA) is the most commonly used technique for screening purposes in developed countries, followed by confirmatory testing most commonly by using conventional Western blot (WB). There are many different commercially available ELISAs for detection of antibodies to HIV. In 1985, first-generation indirect ELISAs employed whole virus antigens obtained from cell cultures which were bound to the solid phase on the bottom of the wells of microtitre plate [170]. The first generation ELISAs were sensitive but less specific with capacity to detect early HIV antibodies slightly more than 40 days after infection [170]. The second-generation ELISAs used an indirect format, HIV recombinant antigens and peptides bound in solid phase [171]. The assays had increased specificity and good sensitivity that reduced the window period to detect antibodies as early as 33-35 days after infection [171]. In 1990s, due to diverse HIV variability, ELISAs were introduced which also included antigens from HIV-2 and new antigens from viruses of the HIV-1 groups M, N and O [UNAIDS [172-173]. Third generation ELISAs which used antigen sandwich technique and included recombinant HIV-1 and HIV-2 proteins and/or peptides bound on a solid phase either in the bottom of microplate or a bead were introduced in 1994 [171]. These ELISAs had higher sensitivity and specificity and reduced the window period to about 22 days after infection [171]. Fourth-generation ELISAs that can detect both HIV p24 antigens and antibodies have been introduced recently. These assays offer advantages of early detection of acute HIV infection by reducing the window period to almost the levels of the detection of HIV RNA [171, 174]. Fourth generation ELISAs have been used in developed countries [175-177] and introduced in resource-limited settings in recent years.

There are several simple rapid HIV assays that are used for the diagnosis of HIV infection. The assay principles are based on particle agglutination, immunodot, immunofiltration and immunochromatography [178-183]. The assays offer several advantages including utilization of whole blood or capillary blood obtained from a finger prick, lack of requirements for laboratory facility, affordability, expansion of access to HIV testing and giving results within 15-30 minutes on the same day. There are a number of simple rapid assays that do not require refrigeration [178-183]. Many rapid tests contain a built-in internal control such as a control band indicating whether the samples and reagents have been added correctly to ensure accuracy and reliability of results. Presently, many rapid tests include antigens from both HIV-1 and HIV-2. There are two commercially available fourth generation rapid HIV tests.

The most commonly used confirmatory antibody assays are WB and line immune assays (LIA). The WB consists of HIV denatured proteins, separated by electrophoresis according to size and blotted on strips of a nitrocellulose membrane which are then incubated with patient serum [8]. HIV-1 proteins detectable by WB include the Env (envelope) glycoproteins (gp41, gp120, gp160), the Gag (p17, p24/p25, p55) and the Pol (p34, p40, p52, p68). Most of the commercially available Western blots include also a protein from HIV-2 in order to detect both HIV-1 and HIV-2 infections. The consortium for retrovirus serology standardization recommends the presence of at least one of the gp120 or gp160 proteins and one of p24 or p32 proteins for a positive WB [184]. CDC considers a positive WB if at least two of the p24, gp41, and gp120/160 proteins are present [185-186]. WHO recommends a positive WB if only two Env bands are found [186]. WB is limited by the high costs, unavoidable subjectivity when reading and interpreting results and frequent occurrence of indeterminate results that can delay the diagnosis and increase costs. LIA such as Inno-Lia assay are based on recombinant proteins and/or synthetic peptides capable of detecting antibodies to specific HIV-1 and/or HIV-2 proteins. The assays produce fewer indeterminate results as compared to WB but are equally expensive.

Due to high costs, WB is not used routinely as a confirmatory antibody assay in resource-limited countries but applied to resolve discrepancy between two ELISAs or in rapid HIV testing algorithms where ELISA could not resolve the discrepancy. Several studies in resource-limited countries have shown that a combination of antibody ELISAs based on different test principles and/or different antigens can be used in alternative confirmatory testing strategies [187-194]. Combinations of various simple rapid HIV assays have also been evaluated for use in alternative confirmatory HIV testing strategies and when carefully selected can perform similar to more conventional ELISA and WB combinations [192, 195-202]. Simple rapid assays are commonly used for the diagnosis of HIV infection in VCT, PMTCT and CTC facilities in resource-limited settings [197-199].

1.10.2 Detection of HIV antigens

The p24 antigen can be detected by an ELISA in which the solid phase consists of antibodies to p24 antigen of HIV. The assay detects the viral protein p24 in the blood of HIV-infected individuals where it exists either as unbound or bound to anti-p24 antibodies. Several studies have been conducted to evaluate the performance of p24 antigen assay for the diagnosis and monitoring of HIV infection in infants [203-214]. The sensitivity of the assay has increased with modifications introduced to dissociate p24 antigen from anti-p24 antibodies [215]. Ultrasensitive p24 antigen assay performed on plasma samples for the diagnosis of HIV infection showed a sensitivity of 97% to 100% within the first 6 months of life [207, 209, 212, 216]. The assay has been used much less frequently than HIV-1 DNA or RNA amplifications tests because of the relative poor sensitivity of p24 antigen assay, absence of readily available FDA-approved reagents and high costs in resource limited settings. Recently, a study conducted in South Africa has reported on the development of a p24 antigen rapid test for the diagnosis of acute HIV infection in infants with an overall sensitivity of 95% and specificity of 99% [217].

1.10.3 Detection of viral nucleic acid

Nucleic acid amplification tests (NAAT) can detect acute HIV infection by detecting HIV-1 RNA as early as 9 days before seroconversion [218]. Very sensitive methods for detecting plasma HIV RNA include target nucleic acid sequence-based amplification, reverse transcriptase-polymerase chain reaction (PCR) and signal branched-chain DNA amplification [219]. HIV-1 RNA PCR is commonly used to diagnose HIV-1 infection in infants in high-income countries [219]. Detection of viral RNA to levels of ~50 copies/mL or lower can be achieved [220]. More recent viral RNA assays can even detect virus levels as low as 2 RNA molecules/mL [221-222]. The half-life of HIV is so short that it is estimated that half the entire plasma virus population is replaced in <30 minutes [223]. Use of HIV-1 RNA assays for the diagnosis of HIV-1 infection in infants has been reported in several studies [224-236] with reported sensitivity ranging from 25% to 50% within the first few days of life to 100% by 6 to 12 weeks of age [226, 228]. HIV-1 RNA assays are used commonly for monitoring response to ART and as a prognostic marker for HIV disease progression where affordable in resource-limited settings.

HIV-1 DNA PCR test using peripheral blood mononuclear cells (PBMC) has been used in low resource settings for early infant diagnosis of HIV infection in children less than 18 months [50, 237]. The use of venous blood sample has limitations including lack of expertise needed for venipuncture of small infants, transportation and storage at 2-25 °C, and processing within 4 days of specimen collection. Various studies in several settings have demonstrated excellent results using dried blood spot (DBS) specimen, which has the advantages of requiring only a few drops of blood (20-50 µL) obtained from a heel prick and applied to the filter paper. Once dried, a filter paper can be stored at room temperature eliminating the need to store and transport whole blood at 2-25 °C [238-241]. Use of filter papers also provides fewer chances for mislabelling though it can occur, because there are fewer transfer steps once the blood is applied to the paper. The DNA in the filter paper also remains stable for a longer time. Recently, usefulness of DBS specimens has been emphasized as a means for ensuring greater accessibility to HIV testing for the paediatric population [242]. DBS specimens have also been used for viral loads to detect HIV infection in resource-limited settings.

1.10.4 Virus isolation

The use of virus culture for the laboratory diagnosis of HIV-1 infections in infants and young children has been reported previously [203, 224, 243-246]. Virus isolation has remained as a research method, however, its use is limited by the fact that it is labor intensive, time consuming, costly, requires biosafety level 3 facility and well trained laboratory personnel, and poses a biohazard risk. The availability of viral culture facilities for routine use is limited in resource-constrained settings.

1.11 Treatment of HIV-infected individuals

The primary goals of ART are suppression of viral load to undetectable level, restoration and/or preservation of immunologic function, improvement of quality of life, and reduction of AIDS-related morbidity and mortality. There are six groups of licensed antiretroviral drugs that are available for treatment including binding inhibitors, fusion-penetration inhibitors [Maraviroc, Enfuvirtide], nucleoside reverse transcriptase inhibitors (NRTI) [abacavir, emtricitabine (FTC), zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), lamivudine (3TC), tenofovir (disoproxil fumarate), and stavudine (D4T)], non-nucleoside reverse transcriptase inhibitors (NNRTI) [nevirapine, efavirenz (EFV)], integrase or protease inhibitors [247-248]. AZT was the first successful antiretroviral drug which came to use in 1987.

ART is currently given as a cocktail of several ARV drugs called highly active antiretroviral therapy (HAART). The use of triple therapy with different mechanisms of action has less potential to lead to resistance to ARV drugs. Multidrug therapy can reduce plasma viral load to undetectable levels and the widespread use of HAART has dramatically reduced morbidity and mortality due to AIDS [249-250]. There are several different regimens but each regimen depends on several factors. In resource-rich countries first line HAART usually includes a protease inhibitor which is an expensive regimen. In many developing countries including Tanzania, two NRTI drugs and one NNRTI drug are given to HIV-infected individuals when initiated on ART [251]. Some HAART are combined in a single pill to enhance compliance to ART. The use of ARV drugs is associated with problems including poor adherence, development of side effects and emergence of HIV resistance to the ARV drugs. Significant side effects include for instance anemia and neutropenia due to bone marrow suppression by AZT and liver toxicity by nevirapine. Customization of the HAART for each patient can minimize the ARV drug side effects, ease the pill-taking regimen and allow the patient to return to nearly normal health and lifestyle.

According to the current HIV care and treatment strategy in Tanzania, ART should be initiated for individuals showing symptoms of AIDS, AIDS-defining illness or if CD4 T-cells drop below 200 cells/µL [251]. However, WHO has recently revised the criterion for ART initiation to be CD4$^+$ T-cell count $\leq$350 cells/µL [10]. In Tanzania, the first pilot HIV CTC in Dar es Salaam with availability of ART was set up in June 2004. The clinic was part of the National HIV care program that was started countrywide to provide care and treatment including provision of free ARV drugs [252]. A recent study conducted in treatment-naïve HIV-infected individuals in Dar es Salaam showed resistant mutations that were associated with drugs currently used in first-line therapy and in the PMTCT of HIV which can result in treatment failure and the spread of ARV-resistant strains [54].

1.12 Prevention of HIV infection

There are several ways that can be used to prevent and control the spread of HIV infection. The principal way HIV infection can be controlled is by educating the population about the modes of transmission and measures that may curtail spread of HIV including monogamous relationship, safe sex practice, the use of condoms to reduce the possibility of HIV exposure and voluntary counselling and testing. A successful anti-HIV education campaign in Uganda has been cited as more effective than ARV drugs for saving lives of people [253]. In 2007, the president of Tanzania, Jakaya Kikwete, led the national campaign on voluntary HIV counselling and testing (himself and his wife underwent the test in public) to motivate Tanzanians to know their HIV status and take appropriate control measures thereafter. The national campaign led to voluntary testing of more than 3 million individuals in six months in the whole country. When male condom is used properly, it is thought to reduce HIV transmission by as much as 70% [254]. A female condom is also available and is an effective barrier to HIV and other STIs though its use is limited by high costs and low acceptance rates [255].

The proof-of-concept, double-blinded, placebo-controlled trial conducted by the Centre for the AIDS Programme of Research in South Africa (CAPRISA) showed that a vaginal microbicide candidate consisting of 1% tenofovir gel reduced the HIV incidence by 39% in South African women [256]. Male circumcision has been reported to reduce transmission of HIV-1 by 50%-60% in Kisumu, Kenya and Rakai, Uganda [257-258]. Contaminated needles are a major source of HIV infection in intravenous drug abusers and people must be educated that needles must not be shared. The reuse of contaminated needles in clinics was the source of outbreaks of AIDS in the former Soviet bloc and other countries [259-260]. In some high-income countries, efforts have been launched to provide sterile equipment to intravenous drug abusers [261-263].

Potential blood and organ donors are screened for HIV and other blood born infections before they donate blood, tissue and blood products. People testing positive for HIV must not donate blood. People who anticipate a future need for blood such as those awaiting elective surgery, should consider donating blood beforehand. The Tanzanian government introduced nation-wide blood donation screening for HIV in 1989. Blood safety remains an issue of major concern in transfusion medicine in Tanzania where national blood transfusion services and policies, appropriate infrastructure, trained personnel and financial resources are yet to satisfy increased demands. National blood transfusion services were established in 2004 with aims to ensure availability of safe blood and blood products for transfusion to health facilities [33]. The strategy of blood donation is focused on low risk of HIV, voluntary non-enumerated blood donors and this has gradually discouraged replacement/family blood donors due to high-risk of transfusion transmissible infections [264].

Screening for STIs and providing early treatment prevent the transmission of HIV-AIDS [265]. In resource-limited settings, a syndromic approach has been adopted for the management of STIs [266-267]. PrEP has been shown to reduce the risk of HIV infection [268]. PEP which includes administration of ART within 72 hours after HIV exposure prevents the risk of infection [269]. Although AZT monotherapy may be effective in PEP, most PEP protocols specify dual or triple therapy because it is likely to be more effective than monotherapy [270].

Without interventions, the risk of MTCT of HIV varies from 14% to 48% and is highest in breastfeeding women [70]. The rate of MTCT of HIV has been reduced to less than 1% in resource-rich countries by the use of prophylactic HAART to the mother combined with caesarean section and avoidance of breastfeeding [271]. However, in many resource-limited countries the majority of HIV-infected women breastfeed since they do not have acceptable, affordable, sustainable and safe infant feeding options [272]. Short-course prophylactic ART around delivery which is used in many resource-limited settings significantly reduces MTCT of HIV but does not prevent postnatal HIV transmission through breastfeeding [273-274]. Several recent studies in sub-Saharan Africa [271, 275], including the Mitra [28] and Mitra Plus [29] studies in Tanzania have shown that prophylactic ART of HIV-infected mothers or their infants during breastfeeding prevents postnatal HIV transmission. Infant or maternal ARV prophylaxis for 6 months during breastfeeding in the Mitra and Mitra

Plus study, respectively, resulted in a similar low risk of acquisition of infection between 6 weeks and 6 months (1%) and a similar low cumulative infant HIV infection rate at 6 months of age (5%) [28-29].

1.13 Prevention of HIV infection by immunization

HIV immunization is potentially the most effective and affordable strategy to reduce and to prevent the spread of HIV infection in the population worldwide. There are two main types of immunization, namely prophylactic and therapeutic. This thesis includes only studies of prophylactic immunization and therapeutic immunization will not be discussed. Several different types of HIV vaccines to prevent HIV infection have been evaluated in different phases of clinical trials [276-280].

1.13.1 Challenges associated with development of an HIV-1 vaccine

It is generally believed that an effective HIV vaccine should be able to elicit durable immunity including broadly Nabs systemically as well as mucosally and potent polyfunctional cellular immune responses, especially CTL responses [280]. However, there are several scientific challenges associated with the development of an HIV vaccine [281]. The greatest problem is the extremely high variability of HIV. The viral diversity is highest in sub-Saharan Africa, including Tanzania, where infections with different subtypes of HIV-1 are prevalent. Another major problem is that HIV infects cells of the immune system, mainly CD4$^+$ T-lymphocytes which have a central role in the induction of immune responses. Furthermore, HIV DNA integrates in the host cell genome and establishes a latent reservoir of infected cells. Infection may also be transmitted by infected cells. HIV has the ability to escape immune elimination through viral mutations. In addition, the envelope glycoprotein is heavily glycosylated which protects neutralization epitopes. Most successful vaccines against viral diseases are live attenuated or whole-killed virus vaccines. However, live attenuated vaccines are not being considered for use in humans because of the risk for reversion of an attenuated strain to a pathogenic strain. A way of mimicking the effect of live attenuated vaccines is to use live recombinant vectored vaccines. Infection of macaques with SIV or chimeric SIV-HIV are useful animal models for HIV vaccine studies but there is no appropriate animal model that allows the replication of HIV-1 and development of HIV disease similar to that seen in humans.

1.13.2 Possible correlates of protection against HIV infection

The correlates of vaccine-induced protection against HIV infection in humans have not yet been identified. Only one HIV efficacy trial has shown modest vaccine-induced protection against infection [282]. Possible correlates of protection against HIV infection include broadly Nabs and other inhibitory antibodies such as ADCC antibodies, $CD8^+$ T-cell activity, especially cytotoxicity, $CD4^+$ T-cell activity and NK cell activity and other innate immune responses as described in the sections on cellular and humoral immune responses (1.9) and innate immune responses (1.8). Vaccine-induced antibodies should ideally be able to neutralize the incoming virus at the site of entry and prevent the spread to other parts of the body. However, it has so far proven difficult to induce broadly Nabs by immunization. If infection occurs after HIV exposure, vaccine-induced cellular immune responses will be needed to control virus replication and thereby prevent development of disease. Potential correlates of $CD8^+$ T-cell immunity against HIV including cytotoxicity, cytokine production, phenotypic markers and other markers, have been discussed recently in a review article [283]. A recent study of immune and genetic correlates of SIV vaccine-induced protection in a large number of macaques showed that a SIV-DNA prime/recombinant Ad5 boost vaccine regimen induced complete protection against mucosal infection with heterologous SIVsmE660 in 50% of the vaccinated monkeys. Protection was associated with low levels of Nabs and an envelope-specific $CD4^+$ T-cell response. Furthermore, monkeys that expressed two TRIM5 alleles were more often protected than those that expressed one TRIM5 allele. Vaccinated, SIV-infected monkeys which expressed the major histocompatibility class I allele Mamu-A*01 had a lower peak plasma virus RNA level than control monkeys [284].

1.13.3 Prophylactic HIV vaccine trials

In June 2011, more than 200 trials of HIV-1 vaccine candidates were listed in the International AIDS Vaccine Initiative (IAVI) clinical trials database [285]. More than 40 different HIV-1 vaccine candidates have been tested alone or in combination in clinical trials [280]. The most frequently tested HIV immunogens include Env subunits, DNA vaccines and live recombinant virus vaccines. Many HIV vaccine trials have been based on the heterologous prime boost approach which usually implies using HIV DNA plasmids or recombinant Env glycoprotein in combination with a nonreplicating viral vector based vaccine. The most commonly used viral vectors are poxvirus, including canarypox, MVA and NYVAC, and adenovirus [278, 286]. The earliest vaccine trials focused on the use of Env proteins with the purpose of inducing neutralizing antibodies. The first phase I trial of a human candidate HIV vaccine was conducted in 1987 in the United States among 72 HIV-negative healthy adults using a recombinant envelope gp120 or gp160 given with alum adjuvant [287-288]. Studies in the chimpanzee HIV-1 infection model and the macaque SHIV infection model demonstrated that immunization with gp 120 could induce protection against challenge with the homologous virus strain but not against challenge with distant heterologous virus strains [280]. Several phase I clinical trials showed that rgp120 and rgp160 elicited antibodies which neutralized laboratory-adapted HIV-1 isolates but not primary HIV isolates [289]. Immunization with recombinant gp120 in two phase III efficacy trials failed to induce protection against HIV infection or delay HIV disease progression [290-292].

The first phase I vaccine trial in an African country started in 1999 in Uganda and included 40 HIV-seronegative volunteers who received a recombinant attenuated canary pox vaccine encoding Env, Gag and protease based on subtype B [293-294]. Subsequently more than 20 prophylactic vaccine trials have been conducted in Africa testing various HIV vaccines based on subtypes which are prevalent in that continent [277].

Canarypox vector based HIV vaccine constructs, called ALVAC, in combination with recombinant envelope glycoprotein vaccines have been evaluated in several phase I and phase II trials and in one phase III trial [277, 282, 295-296]. The first phase II trial with an ALVAC canarypox virus vector vCP205 expressing subtype B gp120, p55, and protease was conducted in the United States with 435 volunteers receiving or not receiving an HIV-1 SF-2 recombinant gp120 boost. The majority of volunteers (94%) given the canarypox vaccine plus gp120 had Nabs to the MN strain as compared to 56% of the volunteers given the canarypox vaccine alone. About one-third of the volunteers had anti-HIV cytotoxic T-lymphocytes whether they received gp120 or not [295]. Another phase I/II trial (RV135) with an ALVAC expressing CRF01_AE HIV-1 gp120 linked to a portion of subtype B gp41 (vCP1521) and also expressing HIV-1 gag and protease was conducted in Thailand among 133 HIV-negative adults who also were boosted with a high or low dose of a bivalent HIV gp120 vaccine containing a B envelope from strain MN and a CRF01_AE envelope from strain A244 (AIDSVAX B/E) [296]. Lymphoproliferative responses to gp120 E were shown in 63% of vaccinees and HIV-specific CD8$^+$ CTLs in 24% of the vaccinees [296]. Nabs against HIV-1 subtype E were detected in 71% of the vaccinees. Furthermore, ADCC activity was demonstrated in 84% of vaccinees to CRF01-AE gp120 [297]. The vaccine combination used in the RV 135 trial was subsequently used in the RV144 phase III efficacy trial in Thailand [282].

HIV vaccines based on plasmid DNA and/or live recombinant virus vectors mainly stimulate cellular immune responses. T-cell vaccines are usually not expected to prevent acquisition of infection but to protect against development of disease by reducing the viral load. Several studies in the SIV and SHIV macaque models have shown that vaccines which predominantly elicit T-cell responses can control virus replication and delay or prevent CD4$^+$ T-cell decrease [298-300]. In a recent vaccine study in macaques using recombinant replication competent rhesus CMV expressing SIV Gag, Rev, Nef, Tat and Env, effector memory T-cell responses were elicited in the absence of Nabs and 4 of 12 vaccinated animals controlled rectal mucosal SIV infection without progressive systemic dissemination [301].

HIV DNA vaccines are usually poor immunogens when used alone in humans but they can efficiently prime immune responses when used in prime boost regimens with a live recombinant HIV vaccine [278]. The immunogenicity of HIV DNA vaccines can be enhanced by improving the delivery of the vaccine and by the use of adjuvants, such as IL-12, IL-15 and granulocyte macrophage colony-stimulating factor (GM-CSF) [302]. Use of needle-free injection devices, such as the Biojector, to deliver DNA vaccine has been shown to improve immunogenicity [303-304]. Electroporation has been shown to enhance DNA uptake and to increase the breadth of immune responses after im administration of a multigene vaccine in rhesus macaques [305].

Phase I and II HIV vaccine trials of HIV-1 DNA and MVA encoding HIV-1 clade A p24/p17 sequences and a string of CD8$^+$ T-cell epitopes have been conducted in the UK and East Africa. The frequency of IFN-γ enzyme-linked immunospot (ELISpot) responses was found to be less than 15% to Gag [276]. However, in a subsequent small trial that administered higher doses of these vaccine constructs, HIV-specific IFN-γ ELISpot responses were demonstrated in 50% of the vaccinees [306]. The HIVIS01/02 phase I trial in Sweden evaluated priming with DNA expressing *gp160* of HIV-1 subtypes A, B and C; rev B; *p17/p24 gag* A and B, and *RTmut* B, given with the

Biojector on days 0, 30 and 90 and boosting 6 months after the last HIV-DNA immunization with heterologous MVA-CMDR expressing *env, gag* and *pol* of CRF01A_E. The HIV-specific IFN-γ ELISpot response rate was 92% (34/37), 86% to Gag and 65% to Env. A low dose of HIV-1 DNA administered id was as effective as a higher dose im in priming for the MVA boosting vaccination. The use of recombinant GM-CSF as an HIV-DNA adjuvant did not enhance the immune responses [304].

A recent DNA prime MVA boost phase I HIV vaccine trial was conducted among healthy individuals in the United States (HVTN 065) who were randomized to receive either placebo, 2 doses of DNA followed by 2 doses of rMVA (DDMM), or one dose of DNA followed by 2 doses of rMVA (DMM) or 3 doses of rMVA (MMM) [307]. The DNA and rMVA vaccines encoded Gag, Protease, RT and the native membrane-bound trimeric form of Env that produced noninfectious virus-like particles. Immune responses for CD4$^+$ (77% vs. 43%) and CD8$^+$ (42% vs. 17%) T-cells assessed by intracellular cytokine staining (ICS) were found to be highest in the DDMM group compared to the lowest in the MMM group [307]. Furthermore, the response rates for Env binding and Nabs were observed to be the highest in the MMM group [307].

A phase I trial which compared HIV DNA-C prime NYVAC-C boost to NYVAC-C vaccination alone was conducted among volunteers in Lausanne, Switzerland and London, UK (EV02). Volunteers were randomized to receive two doses of recombinant DNA and poxvirus vector NYVAC (DNA-C group; n=20) or two doses of NYVAC-C alone (NYVAC-C group; n=20). Both vaccines expressed HIV-1 clade C *env, gag, pol* and *nef* genes. HIV-specific IFN-γ ELISpot responses were demonstrated in 90% (18/20) of vaccinees who received DNA-C and NYVAC-C compared to 33% (5/15) of vaccinees who received NYVAC-C alone [308-309]. The vaccine-induced T-cell responses were most frequently directed against Env [308-309]. Testing by ICS showed that both CD4$^+$ and CD8$^+$ T-cell responses were polyfunctional. However, CD4$^+$ T-cell responses were more frequent. T-cell responses were still demonstrable in 70% of vaccinees one year after the last immunization. Binding antibodies to Env gp140 were demonstrated in 75% of vaccinees who received DNA-C plus NYVAC-C. Tests for Nabs were negative [308-309].

A phase I/II HIV DNA prime rAd5 boost vaccine trial was conducted between May and October 2006 among 324 individuals (RV 172) from 3 East African countries

(Kenya, Tanzania and Uganda). The volunteers were randomized to receive placebo, a single dose of rAd5 at 10^{10} or 10^{11} particle units, or priming with 3 injections of multiclade HIV-1 DNA at 0, 1 and 2 months followed by the boost of a single dose of rAd5 at 10^{10} or 10^{11} particle units at 6 months [310]. The DNA vaccine consisted of HIV-1 *env* subtypes A, B and C and subtype B *gag*, *pol* and *nef* genes while the rAd5 expressed identical genes with the exception of *nef*. This was the first phase HIV vaccine trial to be conducted in Tanzania. The vaccine regimen was reported to be safe and well tolerated. HIV-specific T-cell responses assessed by IFN-γ ELISpot were detected in 63% of vaccinees. Pre-existing Ad5 Nab titers influenced significantly the response rates in individuals who received rAd5 alone [310].

Four HIV vaccine efficacy trials have been conducted and one is ongoing [277]. The first two phase III efficacy trials used bivalent subtype B and subtype B/E envelope glycoprotein. One trial was conducted during 1998 and 1999 in the USA, Canada and the Netherlands among 5403 HIV-seronegative men who have sex with men (MSM) (VAX004) and the other trial included 2527 HIV-uninfected injection drug users in Bangkok, Thailand (VAX003). These trials did not show protection against HIV infection [290-292].

A test-of-concept trial in the United States (STEP), including about 3000 individuals, evaluated a recombinant adenovirus 5 (MRKAd5) vector vaccine expressing clade B Gag, Pol and Nef in a three-dose regimen [311-312]. In 2007, the trial was discontinued because the vaccine did not protect vaccinees from HIV infection and there was increased risk of HIV acquisition in vaccinated men who had pre-existing Ad5 antibody titres and were uncircumcised [311, 313]. A similar trial that was conducted in South Africa (Phambili) including heterosexual women and men was also stopped [314]. Another phase IIb trial (HVTN 505) is being conducted through VRC at the NIH in which a DNA vaccine consisting of clade B Gag, Pol, Nef, and Env plus clade A and C Env DNA is given as a prime followed by a boost with rAd5 vector that expresses a clade B Gag/Pol fusion protein and the clade A, B and C envelope glycoproteins to Ad5 antibody negative circumcised MSM [315].

The hope for getting an efficacious HIV vaccine was revived recently. A phase III vaccine trial that was conducted in Thailand (RV 144) using a recombinant canary pox vector (ALVAC) expressing clade E antigens of HIV-1 gp120 linked to the transmembrane-anchoring portion of clade B gp41 and HIV-1 clade B *gag* and protease boosted with the AIDSVAX gp120 B/E (VaxGen) bivalent HIV-1 gp120 envelope showed a moderate efficacy of 31% in heterosexual Thais with a low risk for HIV infection [282, 316]. HIV-specific IFN-γ ELISpot responses were demonstrated in 19.7 % of vaccinees. The lymphoproliferation assay (LPA) response rate to gp120 was 87.3% and to gp120A244 90.1%. Binding antibodies to Env gp 120 developed in 98.6 % of the vaccinees. An immunologic correlate of protection against HIV infection has not yet been reported.

2 RATIONALE OF THE STUDY

Paper I and II: It is important to evaluate new and better assays as they become available in the markets in the context in which they will be used before adopting them in order to improve the diagnosis of HIV infection in resource-limited countries.

Paper III: High levels of recovery, viability and functionality of PBMCs are essential for reliable assessment of cell-mediated immune responses. The cell preparation technique best suited for use in two clinical trial sites: Stockholm, Sweden and Dar es Salaam, Tanzania was studied in preparation for the conduct of phase I/II HIV vaccine trials.

Paper IV: Analyses of cell-mediated immune responses are vital in the evaluation of HIV vaccine efficacy. Here we wanted to further define the HIV-1-specific lymphoproliferative responses in vaccinees in the HIVIS01/02 HIV-1 DNA prime-MVA boost vaccine trial in Stockholm by applying a flow cytometry-based assay employing either whole blood (FASCIA-WB) or PBMC (FASCIA-PBMC) to assess vaccine-induced $CD4^+$ and $CD8^+$ T-cell proliferation. We also explored the use of FASCIA especially suitable in isotope-restricted settings as an alternative to the conventional [^{3}H]-thymidine uptake LPA.

Paper V: An effective and safe prophylactic HIV vaccine is urgently needed especially in many African countries where the incidence of HIV is high. A phase I/II HIV vaccine trial (HIVIS03) using an HIV-1 DNA prime-MVA boost regimen was conducted among healthy adults in Dar es Salaam, Tanzania with the aim of comparing id and im delivery of the DNA vaccine and to build capacity for clinical trials and establish methods for assessment of vaccine-induced immune responses on site.

3 OBJECTIVES

3.1 Broad objective

To improve methodologies for laboratory diagnosis of HIV infection suitable for use in developing countries and to monitor immune responses in a phase I and a phase I/II HIV-1 vaccine trial in Sweden and in Tanzania, respectively.

3.2 Specific objectives

Paper I: To evaluate the performance of two antibody ELISAs (Vironostika Uni-Form II plus O and Enzygnost anti-HIV-1/2 Plus) and two new diagnostic HIV antigen/antibody combination ELISAs (Murex and Vironostika HIV Uni-Form II antigen/antibody) for use in an alternative confirmatory HIV diagnostic testing strategy in Dar es Salaam, Tanzania.

Paper II: To evaluate the performance of five simple rapid HIV assays and to formulate an alternative confirmatory strategy based on rapid HIV testing algorithms suitable for use in Tanzania.

Paper III: To study techniques for blood mononuclear cell isolation best suited for use in two HIV vaccine trial sites: Stockholm, Sweden and Dar es Salaam, Tanzania.

Paper IV: To compare different assays for the assessment of HIV-specific lymphoproliferative responses in a phase I trial in Sweden of healthy volunteers primed with HIV-1 DNA and boosted with recombinant HIV-1 MVA (HIVIS01/02).

Paper V: To evaluate the safety and immunogenicity of a low dose id priming compared to a higher dose im priming with the HIVIS multigene, multiclade HIV-1 plasmid DNA vaccine followed by heterologous HIV-MVA boosting in a phase I/II clinical trial (HIVIS03) among healthy HIV negative volunteers in Dar es Salaam, Tanzania.

4 METHODS

4.1 Paper I and Paper II

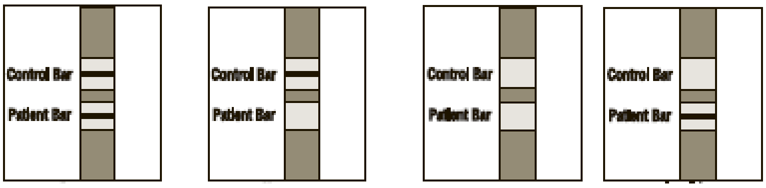
In the study reported in paper I, we evaluated the performance of two HIV antibody ELISAs (Vironostika Uni-Form II plus O and Enzygnost anti-HIV-1/2 Plus) and two new diagnostic HIV antigen/antibody combination ELISAs (Murex and Vironostika HIV Uni-Form II antigen/antibody) for use in an alternative confirmatory HIV diagnostic testing strategy in Tanzania. The Enzygnost anti-HIV-1/2 Plus assay is made of recombinant HIV-1, HIV-2, and HIV-1 subtype O proteins while the Vironostika HIV Uni-Form II plus O assay consists of a mixture of HIV-1 p24, HIV-1 gp160, HIV-1 ANT70 peptide, and HIV-2 env peptide containing amino acids 592-603. The Murex and Vironostika antigen/antibody assays use anti-HIV-1 p24 monoclonal antibodies for both solid phase capture and probe conjugation for detection. We included a total of 1380 serum samples; 508 from blood donors, 511 from pregnant women and 361 from hospital patients between July 2003 and March 2004. The samples were left over after HIV testing/screening and were to be discarded. All ELISA reactive samples were tested on a confirmatory antibody assay, the Inno-Lia immunoblot assay (Innogenetics, Belgium).

In the Inno-Lia immunoblot assay, rrecombinant proteins and synthetic peptides from HIV-1 and HIV-2 and also a synthetic peptide from HIV-1 group O are coated as discrete lines on a plastic backed nylon strip. Five HIV-1 antigens are coated, namely sgp120 and gp41 which are specific for HIV-1, and p31, p24 and p17 which may also cross react with antibodies to HIV-2. Peptides from HIV-1 group O are present in the HIV-1 sgp120 line. The antigens gp36 and sgp105 are coated to detect specific antibodies to HIV-2. In addition four control lines are also coated on each strip: an anti-streptavidin line, a +/- cut-off line (human IgG), a 1+ positive control line (human IgG) and a 3+ strong positive control line which is also the sample addition control (anti-human Ig).

In the study reported in paper II, we evaluated five rapid HIV assays: Determine HIV-1/2 (Inverness Medical), SD Bioline HIV 1/2 3.0 (Standard Diagnostics Inc.), First Response HIV Card 1-2.0 (PMC Medical India Pvt Ltd), HIV1/2 Stat-Pak Dipstick (Chembio Diagnostic System, Inc) and Uni-Gold HIV-1/2 (Trinity Biotech) between

June and September 2006 using 1433 whole blood samples from hospital patients, pregnant women, voluntary counseling and testing attendees and blood donors. All samples that were reactive on all or any of the five rapid assays and 10% of non-reactive samples were tested on a confirmatory Inno-Lia HIV I/II immunoblot assay (Innogenetics, Belgium).

Determine HIV-1/2 is a rapid immunochromatographic test for the qualitative detection of antibodies to HIV-1 and HIV-2. The sample is added to the sample pad. As the sample migrates through the conjugate pad, it reconstitutes and mixes with the selenium colloid-antigen conjugate. This mixture continues to migrate through the solid phase to the immobilized recombinant antigens and synthetic peptides at the patient window site. If antibodies to HIV-1 and/or HIV-2 are present in the sample, the antibodies bind to the antigen-selenium colloid and to the antigen at the patient window, forming a red line at the patient window site. If antibodies to HIV-1 and/or HIV-2 are absent, the antigen-selenium colloid flow past the patient window and no red line is formed at the patient window site. To insure assay validity, a procedural control bar is incorporated in the assay device.

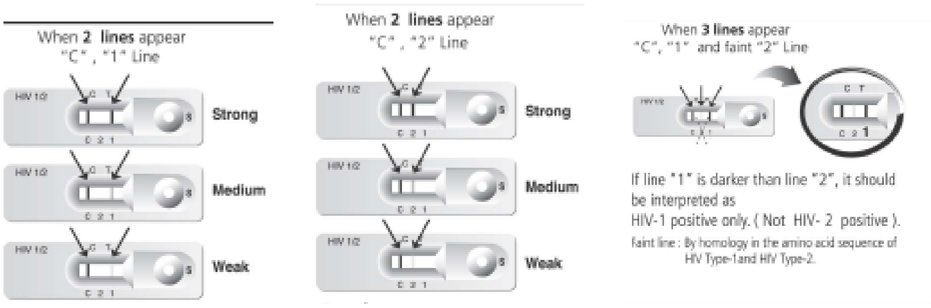


A B C D

Figure 4. Determine HIV-1/2 test device. A shows control and patient bands; B shows control band only; C shows no band in control or patient bar and D shows patient band and no control band and is invalid

(Source www. determinetest.com/about_hiv.aspx)

The SD BIOLINE HIV-1/2 3.0 is an immunochromatographic test for the qualitative detection of antibodies of all isotypes (IgG, IgM, IgA) specific to HIV-1 and HIV-2 in human serum, plasma or whole blood. The SD BIOLINE HIV-1/2 3.0 rapid test contains a membrane strip, which is precoated with recombinant HIV-1 capture antigen (gp41, p24) on test band 1 region and with recombinant HIV-1/2 capture antigen (gp36) on test band 2 region, respectively. The recombinant HIV-1/2 antigen (gp41, p24 and gp36) - colloid gold conjugate and the specimen sample move along the membrane chromatographically to the test region (T) and form a visible line as the antigen-antibody-antigen gold particle complex forms with high degree of sensitivity and specificity. This test device has a letter of 1, 2 and C as Test Line 1 (HIV-1), Test Line 2 (HIV-2) and Control Line on the surface of the device. Both the Test Lines and Control Line in the result window are not visible before applying the sample. The Control Line is used for procedural control. The control Line should always appear if the test procedure is performed properly and the test reagents of Control Line are working.



If line "1" is darker than line "2", it should be interpreted as HIV-1 positive only. (Not HIV- 2 positive).

Faint line : By homology in the amino acid sequence of HIV Type-1and HIV Type-2.

Figure 5. SD BIOLINE HIV-1/2 3.0 test device

(Source www. standardia.com)

First Response HIV Card 1-2.0 is a rapid lateral-flow immunochromatographic test that can be performed on whole blood, serum, or plasma. The test requires only 10 uL of serum or 20 uL of whole blood as sample and one drop of developer solution. The results are obtained in 5 minutes. HIV1/2 Stat-Pak Dipstick assay is a single use, immunochromatographic screening test which uses a cocktail of antigens to detect antibodies to HIV-1 and 2 in serum, plasma or whole blood. The assay employs a combination of antibody binding protein, which is conjugated to colloidal gold dye particles, and antigens to HIV-1/2 which are bound to the membrane solid phase.

The Chembio HIV 1/2 STAT-PAK DIPSTICK is a single use, immunochromatographic screening assay which utilizes a cocktail of antigens to detect HIV-1 and HIV-2 antibodies in serum, plasma and whole blood. The assay employs a combination of antibody binding protein, which is conjugated to colloidal gold dye particles, and antigens to HIV1/2, which are bound to the membrane solid phase. The sample being tested and running buffer are applied to the sample pad. The running buffer facilitates the lateral flow of the specimen through the membrane and promotes the binding of antibodies to the antigens. The antibodies if present bind to the gold conjugated antibody binding protein. In a reactive sample, the dye conjugated-immune complex migrates on the nitrocellulose membrane and is captured by the antigens immobilized in the test area producing a pink/purple line. In the absence of HIV-1/ HIV-2 antibodies, there is no pink/purple line in the test area. The sample continues to migrate along the membrane and produces a pink/purple line in the control area containing immunoglobulin G antigens. The procedural control serves to show that specimens and reagents have been applied properly and have migrated through the test device.

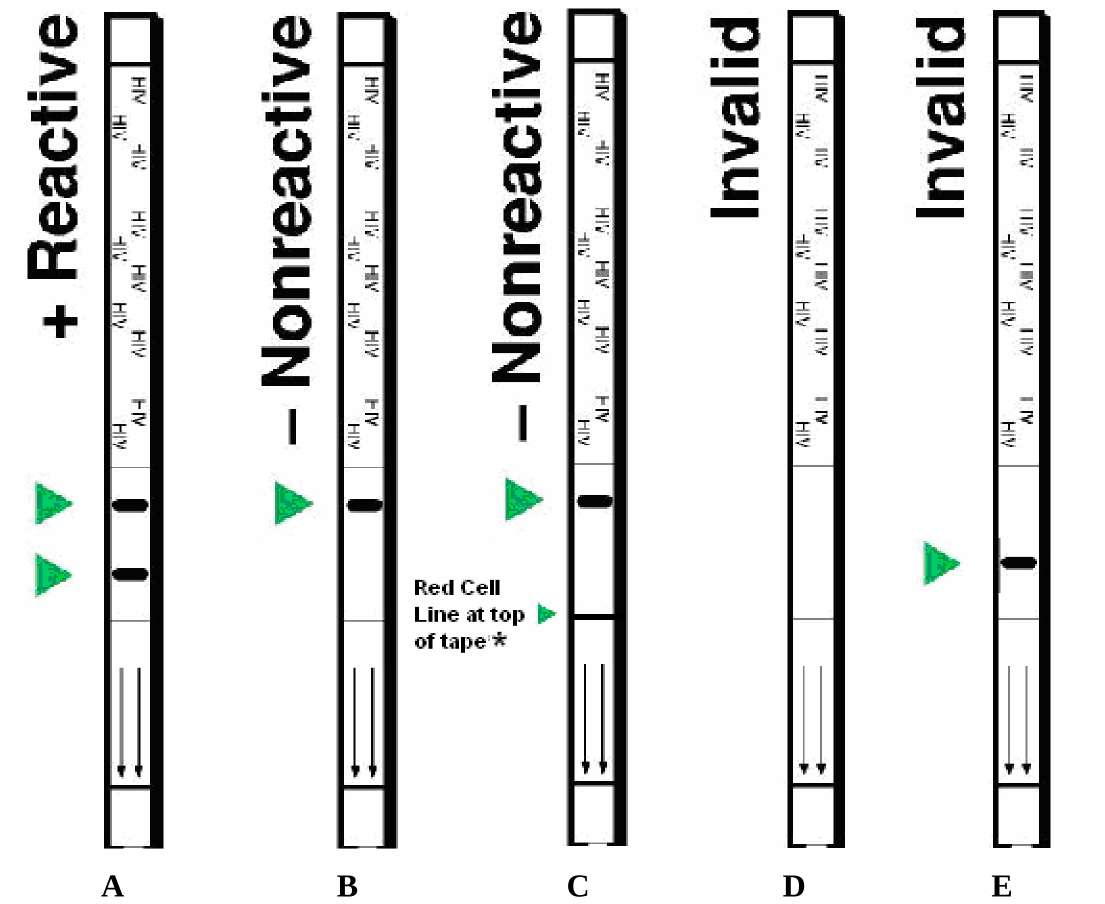


Figure 6. Chembio HIV 1/2 STAT-PAK DIPSTICK test device. A shows control and patient bands; B and C show control band only; D shows no band in control or patient bar and is invalid; and E shows patient band and no control band and is invalid (Source www. chembio.com/humantest2.html)

The Uni-Gold™ HIV test was designed as a rapid immunoassay based on the immunochromatographic sandwich principle and is intended to detect antibodies to HIV-1 and HIV-2 in human serum, plasma and whole blood. The Uni-Gold™ HIV Test employs genetically engineered recombinant proteins representing the immunodominant regions of the envelope proteins of HIV-1 and HIV-2, glycoprotein gp41, gp120 (HIV-1), and glycoprotein gp36 (HIV-2). The recombinant proteins are immobilized at the test region of the nitrocellulose strip. These proteins are also linked to colloidal gold and impregnated below the test region of the device. A narrow band of the nitrocellulose membrane is also sensitized as a control region. During testing two drops of serum, plasma or whole blood is applied to the sample port, followed by two drops of wash buffer and allowed to react. Antibodies of any immunoglobulin class, specific to the recombinant HIV-1 or HIV-2 proteins will react with the colloidal gold linked antigens. The antibody protein-colloidal gold complex moves chromatographically along the membrane to the test and control regions of the test device. A positive result is visualized by a pink/red band in the test region of the device. A negative reaction occurs in the absence of detectable levels of human immunoglobulin antibodies to HIV-1 in the specimen; consequently no visible band develops in the test region of the device. Excess conjugate forms a second pink/red band in the control region of the device. The appearance of this band indicates proper performance of the reagents in the kit.

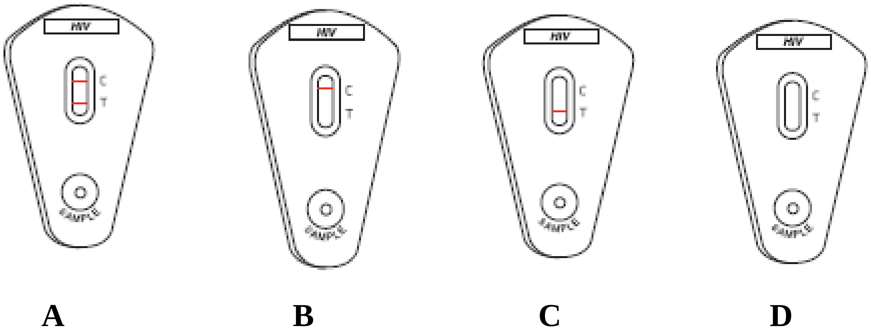


Figure 7. Uni-Gold™ HIV test device. A shows control and patient bands; B shows control band only; C shows no band in control but patient bar and is invalid; and D shows neither control band nor patient band and is invalid

(Source www.trinityusa.com)

4.2 Paper III

Three techniques for the isolation of PBMC from heparinised blood were evaluated including standard Ficoll-Paque gradient centrifugation, the use of BD vacutainer cell preparation tubes (CPT) and and the use of Greiner Bio-One LeucoSep tube techniques. Cell yield and viability were determined using a NucleoCounter (ChemoMetec A/S, Allerod, Denmark). The functionality of the purified PBMC was determined by IFN-γ ELISpot testing using as antigen a pool of peptides from cytomegalovirus, Epstein-Barr virus and influenza virus (CEF).

4.3 Paper IV

HIV-specific lymphoproliferative responses were determined by different assays (Table 1) in the HIVIS01/02 phase I trial of an HIV-DNA prime MVA boost vaccine regimen conducted in Stockholm. The HIV DNA plasmids contained gp160 of HIV-1 subtypes A, B, and C, *rev* B, P17/p24 *gag* A and B and *RTmut* B. The MVA contained *env*, *gag* and *pol* of CRF01A_E. The HIV-1 DNA vaccine was produced in Sweden by Professor Britta Wahren and collaborators at the Swedish Institute for Communicable Disease Control (SMI) and Karolinska Institute and the HIV-1 MVA vaccine was produced at the Walter Reed Army Institute for Research (WRAIR), Rockville, USA. The phase I trial of the vaccines was performed in Sweden in 40 volunteers. The trial took place at Venhälsan, Södersjukhuset Stockholm. The volunteers were randomized to 4 groups of 10 individuals each. Different modes of administration of the DNA vaccine were compared. The HIV-DNA was given with a needle-free injection device (biojector) id (1 mg) or im (3.8 mg) with or without GM-CSF at weeks 0, 4 and 12. The HIV-MVA boost was given id (10^7 pfu) or im (10^8 pfu) at month 9. Blood samples for immunological studies were collected prior to the first injection, on the day of injection, 2 weeks after the second and third HIV-DNA immunizations and 2 weeks after the HIV-MVA injection.

Lymphoproliferative responses to aldrithiol-2 (AT-2)-inactivated-HIV-1 antigen were tested by a [^{3}H]- thymidine uptake assay and a flow-cytometric assay of specific cell-mediated immune response in activated whole blood (FASCIA-WB) 2 weeks after the HIV-MVA boost. A FASCIA using PBMC (FASCIA-PBMC) was also employed (n=14). The methods and analysis used in the three LPAs are summarized in Tables 1 and 2.

Table 1: Methods used in three HIV lymphoproliferation assays

	[^{3}H]-**thymidine uptake assay (n=38)**	***FASCIA-WB (n=38)**	***FASCIA-PBMC (n=14)**
Cell material	200 000 cells/well	Diluted whole blood in tubes (1/10)	200 000 cells/well
Stimuli	Aldrithiol-2 (AT-2) treated HIV-1_{MN}	Aldrithiol-2 (AT-2) treated HIV-1_{MN}	Aldrithiol-2 (AT-2) treated HIV-1_{MN}
Stimulation time	6 days	7 days	7 days
Processing	6 hour [^{3}H]-thymidine incorporation	Stained with anti-CD3 FITC and anti-CD4 PerCP	Stained with anti-CD3 FITC and anti-CD4 PerCP
Readout/Instrument	cpm/ Microbeta counter	Flow cytometric/ FACSCalibur or FACSCan	Flow cytometric/ FACSCalibur or FACSCan

*Flow-cytometric Assay of Specific Cell-mediated Immune response in Activated whole blood [317].

Table 2: Analysis used in three HIV lymphoproliferation assays

	[^{3}H]-**thymidine uptake assay (n=38)**	***FASCIA-WB (n=38)**	***FASCIA-PBMC (n=14)**
Analysis	Stimulation index (SI) = mean [^{3}H]-thymidine incorporation in antigen stimulated wells/mean incorporation in medium wells.	Mean percentage stimulation (%S): [100xtest-negative control/positive control-negative control]	Mean percentage stimulation (%S): [100xtest-negative control/positive control-negative control]
Cut-off determination	27 normal healthy controls	38 baseline values	17 normal healthy controls
	SI >8	CD4$^+$ T-cells: %S >1.0 CD8$^+$ T-cells: %S >1.1	CD4$^+$ T-cells: %S >2.4 CD8$^+$ T-cells: %S >2.8

*Flow-cytometric Assay of Specific Cell-mediated Immune response in Activated whole blood.

4.4 Paper V

In the HIVIS03 phase I/II trial conducted in Dar es Salam the HIV-DNA and MVA vaccine constructs were the same as in the HIVIS01/02 trial. Sixty volunteers were recruited and randomized into three groups. Groups 1 and 2 were given id and im immunization modalities while group 3 which served as a control received placebo. Volunteers received three HIV-DNA/placebo vaccinations at months 0, 1 and 3 followed by two HIV-MVA/placebo boost vaccinations at month 9 and 21. HIV-1 specific cellular immune responses were determined by the IFN-γ ELISpot assay using pools of overlapping HIV peptides representing Env, Gag and Pol proteins. LPA by [^{3}H]-thymidine uptake was performed using four AT-2 treated HIV-1 antigens of four different clades. Four-colour ICS for assessment of Gag-specific IFN-γ/IL-2 production was performed 2-4 weeks after the second HIV-MVA boost. Fresh PBMC were used for all cellular immunological assays. Serum binding antibody determination was performed using Advanced Biotechnologies native gp160 in an in house ELISA, Abbott Murex and Dade Behring Enzygnost Plus ELISAs and Inno-Lia immune blot assay. Nabs were measured using pseudoviruses and a luciferase based assay in TZM-bl cells as previously described [318]. The assay measures the reduction in luciferase reporter gene expression in TZM-bl cells with a single round of pseudovirus infection. A result ≥ 50% is considered to be a positive response. A PBMC assay employing an infectious molecular clone (IMC) that carries a LucR gene as a reporter was also used [319]. The percent neutralization by post-vaccination serum was calculated based on the level of virus growth in the presence of the same dilution of pre-vaccination serum and a result ≥ 50% was considered a positive response.

4.4.1 Quality monitoring of PBMC purification technique and assays for the assessment of HIV-specific vaccine-induced immune responses in Tanzania

Quality assurance program was strictly implemented to ensure accuracy and reliability of laboratory results for HIV-specific vaccine-induced immune responses in the HIVIS03 trial. All laboratory personnel were trained on the PBMC processing, cell counting, cryopreservation, thawing, IFN-γ ELISpot assay, 4-colour ICS and [^{3}H]-thymidine LPA assay at the SMI. They were validated and certified to perform the assays after proficiency assessment. Laboratory personnel who performed IFN-γ ELISpot testing of cryopreserved PBMC from three donors in three consecutive runs with a mean coefficient of variation (CV) of <20% was deemed validated. Internal quality control procedures were performed including regular preventive maintenance of the lab equipments/instruments, use of pre-coated IFN-γ ELISpot plates and pretested reagents such as RPMI, fetal calf serum and HIV-1 specific peptide pools and inclusion of known controls. In every IFN-γ ELISpot assay run, phyto-haemagglutinin (PHA) (positive control), a peptide pool composed of cytomegalovirus (CMV), Epstein-Barr virus and influenza virus (CEF), a peptide pool of the pp65 protein of human CMV, normal human Tanzanian donor cells and RPMI medium only (negative control) were included. Staphylococcal enterotoxin A and B (SEAB), CEF and CMV peptide pools (positive controls) and RPMI medium only (negative control) were included in every 4-colour ICS assay run. In every [^{3}H]-thymidine LPA assay run, PHA (positive control), purified protein derivative (PPD), SUPTI and Jurkat Tat CCR5 microvesicles (control antigens) were also included. The Muhimbili University of Health and Allied Sciences (MUHAS) cellular immunology laboratory also performed IFN-γ ELISpot proficiency testing every other month using HIV-1 infected donor cells with known reactivity patterns provided by SMI.

4.5 Ethical considerations

No ethical approval was required for Paper I because left over blood samples after routine HIV screening and removals of identifiers were used. Ethical approval was waived for paper II because left over blood samples after routine screening and without patient/client identifiers were used. Ethical approvals for Paper III, IV and V were obtained before the implementation of the clinical trials both in Sweden and in Tanzania. For paper III and V, MUHAS Senate Research and Publication committee, National Institute for Medical Research (NIMR) and Tanzania Food and Drug Authorities (TFDA) approved the studies. Written informed consents were obtained prior to recruitment of study volunteers.

5 RESULTS AND DISCUSSION

5.1 Evaluation of HIV antibody and antigen/antibody testing strategies for the diagnosis of HIV infection (Paper I and II)

Evaluation of various HIV-1 antibody detection assays using panels of American and European sera have shown that many of these tests have a high sensitivity and specificity [320]. However, early studies showed that some HIV antibody assays did not have a similar test performance when used for testing of African sera [321]. It is generally recommended to evaluate HIV-1 assays in the context in which they will be used before adopting them for wide-scale use [172]. After extensive evaluation of various HIV antibody ELISAs at MUHAS in Dar es Salaam in the early 1990s, an HIV testing strategy was adopted whereby serum samples that were reactive by an initial ELISA were tested by a second ELISA based on a different test principle and/or different antigens [187-188]. One of the HIV antibody tests which had been used in the testing strategy at MUHAS for more than 10 years, the Wellcozyme HIV-1 recombinant competitive ELISA was withdrawn from the market in 2004 which made it necessary to evaluate new HIV antibody ELISAs. Furthermore, new combined HIV antigen and antibody test kits had become available in the market including Vironostika Organon and Abbott Murex HIV test kits.

The evaluation of two HIV antibody ELISAs and two HIV antigen/antibody ELISAs reported in paper I showed that the sensitivity at initial testing was 100% (95% CI; 98.8-100%) for the Murex and Vironostika HIV Uni-Form II antigen/antibody and Enzygnost anti-HIV-1/2 Plus assays whereas Vironostika Uni-Form II plus O antibody ELISA showed one false negative sample at initial testing (99.7%; 95% CI; 98.2-99.9%) but 100% sensitivity after repeat testing. The specificity at initial testing was 99.8% (95% CI; 99.3-99.9%) for Enzygnost anti-HIV-1/2 Plus, 98.9% (95% CI; 98.1-99.4%) for each of the antigen/antibody combination ELISAs and 97.0% (95% CI; 95.8-97.8%) for Vironostika Plus O ELISA. The final specificity after repeat testing was 100% (95% CI; 99.7-100%) for Enzygnost anti-HIV-1/2 Plus, 99.4% (95% CI; 98.8-99.8%) for each of the antigen/antibody combination ELISAs and 97.9% (95% CI; 96.8-98.6%) for Vironostika Plus O ELISA. A combination of the two antigen/antibody ELISAs was not suitable for use in an alternative confirmatory HIV testing strategy since the serum samples which showed false positive reactions by these two ELISAs were the same both at initial and repeat testing. Following this evaluation, we adopted at MUHAS an alternative confirmatory HIV testing strategy based on initial testing on Abbott Murex HIV antigen/ antibody ELISA followed by testing of reactive samples on the Enzygnost anti-HIV-1/2 Plus ELISA which gave 100% sensitivity and specificity. The discordant results between the two ELISAs were resolved by the Inno-Lia immunoblot assay. In Tanzania, ELISAs are used for laboratory diagnosis of HIV infection in some regional hospital and zonal laboratories, in the national blood transfusion services and at the Muhimbili National Hospital laboratory. The Vironostika HIV Uni-Form II antigen/antibody assay and the Enzygnost anti-HIV-1/2 Plus ELISA were adopted for the screening of blood and blood products in the national and zonal blood transfusion service centres in Tanzania. Recently, the Enzygnost anti-HIV-1/2 Plus ELISA has been phased out from the market and the fourth-generation Enzygnost HIV Integral II ELISA (Siemens Healthcare Diagnostics Products GmbH, Germany) has been introduced. An evaluation of the Enzygnost HIV Integral II ELISA using Tanzanian serum samples from different populations is warranted.

In a recently reported study from Japan, initial screening of serum samples from 6461 pregnant women by the Enzygnost HIV Integral antigen/antibody ELISA showed a specificity of 99.6%. Sequential testing of the samples reactive on this ELISA by another fourth generation ELISA, the VIDAS HIV DUO Quick ELISA resulted in 100% specificity [322]. In the evaluation reported in paper I, we did not detect any sample that was HIV antigen positive but HIV antibody negative. We instead used one seroconversion panel (AU PRB945) to determine the ability to detect acute HIV infection. Abbott Murex HIV antigen/antibody ELISA detected acute HIV infection 13 days after the first bleed and Vironostika HIV antigen/antibody ELISA 15 days after the first bleed. The study conducted in pregnant women in Japan revealed that HIV infection was detected with the VIDAS HIV DUO Quick ELISA earlier than with the Enzygnost HIV Integral ELISA in eight out of ten HIV-1 seroconversion panels with an average interval of 4.5 days [322]. It was reported further that VIDAS HIV DUO Quick ELISA was 16-32 times more sensitive for antigen detection than the Enzygnost HIV Integral ELISA when using serial two-fold dilutions of three HIV-1 antigen samples [322]. In a recent study, the analytical sensitivity of four HIV antigen/antibody assays (ARCHITECT HIV Ag/Ab Combo, AxSYM HIV Ag/Ab Combo, VIDAS HIV

DUO Quick and VIDAS HIV DUO Ultra) and one p24 assay, most commonly used in France was evaluated using the p24 WHO standard [323]. Four of the five assays had a lower limit of detection below 2 IU/ml (1.24 IU/ml for ARCHITECT HIV Ag/Ab Combo, 0.66 IU/ml for VIDAS HIV DUO Ultra, 0.43 IU/ml for VIDAS HIV DUO Quick and 0.73-1.15 IU/ml for VIDAS p24) while that of AxSYM was close to 2 IU/ml [323] showing that VIDAS HIV DUO Quick ELISA performed best to detect acute HIV infection compared to the other assays. It is important to note that the international (WHO standard) is based on a subtype B isolate and there might be subtype variations in antigen detection.

Following an evaluation of simple rapid HIV antibody assays at MUHAS several years ago, a rapid HIV testing algorithm was adopted which consisted of initial screening with the Capillus assay followed by confirmatory testing of reactive samples with the Determine assay [198]. However, the Capillus assay required cold storage making it unsuitable for use in peripheral areas where electricity is not readily available or in settings where power outages are frequent. Dependency for the cold chain by Capillus, need to scale up access to HIV screening, diagnosis and treatment together with availability of newer HIV rapid tests in the market made it necessary to embark on new evaluations of rapid HIV assays aiming at developing alternative HIV testing algorithms for use in Tanzania.

Our recent evaluation of five rapid HIV assays included 390 confirmed HIV-1 antibody positive samples, and 1043 HIV seronegative samples (Paper II). We found that the sensitivity at initial testing of Determine, SD Bioline and Uni-Gold was 100% (95% CI; 99.1-100) while First Response and Stat-Pak had a sensitivity of 99.5% (95% CI; 98.2-99.9) and 97.7% (95% CI; 95.7-98.9), respectively, which increased to 100% (95% CI; 99.1-100) on repeat testing. The initial specificity of the Uni-Gold assay was 100% (95% CI; 99.6-100) while specificities were 99.6% (95% CI; 99-99.9), 99.4% (95% CI; 98.8-99.7), 99.6% (95% CI; 99-99.9) and 99.8% (95% CI; 99.3-99.9) for Determine, SD Bioline, First Response and Stat-Pak assays, respectively. There was no sample which gave concordantly false positive results in Uni-Gold, Determine and SD Bioline assays. The Tanzanian Ministry of Health and Social Welfare has adopted a rapid HIV testing algorithm based on our study results which includes initial testing on SD Bioline assay followed by testing of reactive samples on the Determine assay which had a sensitivity of 100% and specificity of 100% with Uni-Gold as tiebreaker for discordant results. This rapid HIV testing algorithm is currently being used for diagnosis of HIV infection in the VCT, PMTCT and HIV CTC clinics.

Problems with the HIV diagnostic accuracy when using rapid HIV tests in non-laboratory settings have been encountered in African studies. In a screening study of 1517 males for trials of circumcision for HIV prevention in rural Uganda, an algorithm using initial testing with the Determine test followed by testing of reactive samples with the Stat-Pak assay and further testing with Uni-Gold to resolve discordant results showed a sensitivity of 97.7% and a low specificity of 94.1%. Exclusion of results with weak positive bands increased the specificity to 99.6% [180]. In our evaluation, the rapid testing algorithm that included initial testing on Determine followed by testing of reactive samples on Stat-Pak Dipstick assay showed a 100% sensitivity and specificity with no concordant false positive results (Paper II). Another study of rapid assays including Determine, Uni-Gold and Capillus used for voluntary counseling and testing of more than 6000 individuals in Uganda and Kenya showed that the sensitivity and specificity of rapid assays varied significantly across sites with a high rate of false positives in Uganda (positive predictive values ranging from 45.7% to 86.62%) [181]. A recent study of rapid HIV assays among pregnant women in a clinical setting in South Africa showed that First Response, Standard Diagnostic and Pareekshak rapid HIV tests which performed well under laboratory conditions showed poor sensitivity (94.5%, 87.5% and 90.2%, respectively) when used in the clinical setting [182].

Two fourth-generation rapid HIV assays based on the detection of both HIV antigen and antibody have been developed and introduced in the market. The Immunocomb Trispot (Orgenics, Yavne, Israel) was first introduced but to our knowledge no independent evaluation reports have been published. The more recently introduced Determine HIV-1/2 antigen/antibody Combo is an immunochromatographic test for the qualitative detection of p24 antigen and antibodies to HIV-1 and HIV-2. A recent evaluation of Determine HIV-1/2 Combo assay using serial serum panels including among others an HIV seroconversion panel and primary HIV infection samples showed 100% antibody sensitivity and 100% antibody specificity [324]. The antigen sensitivity of the assay was found to be 86.6% compared to a reference single antigen ELISA. However, the assay could not detect antigen in one group O, one subtype F and two subtype H cell supernatant isolates and none of the HIV-2 antigen could be detected

[324]. The assay is proposed as an alternative for antigen detection in the diagnosis of HIV infection in high-risk populations and blood donor screening in resource-limited settings.

5.2 Processing of blood mononuclear cells for use in HIV vaccine trials (Paper III)

The HIVIS01/02 HIV-1 DNA prime MVA boost phase I vaccine trial was conducted in Stockholm in 2005 and 2006 [304] and the phase I/II HIVIS03 trial using the same vaccine constructs was conducted in Dar es Salaam from 2007 to 2010 (Paper V). The primary immunogenicity endpoint in these trials was the determination of HIV-specific cell-mediated immune responses by the IFN-γ ELISpot assay. Before the start of these vaccine trials, procedures for the isolation of PBMC were tested at each of the two trial sites to find the techniques best suited for use at the respective sites (Paper III). In our evaluation of cell separation procedures, we found no differences in mean recovery or mean viability of fresh PBMC purified by Ficoll-Paque gradient centrifugation and CPT techniques used in Stockholm. In Dar es Salaam, recovery of PBMC isolated by Ficoll-Paque gradient technique was higher compared to CPT (1.58±0.6 vs.1.34±0.4 million cells/mL blood, $p=0.0469$) and the viability of PBMC processed by Ficoll-Paque gradient was higher compared to CPT purified cells (95.8±2.3 vs. 92.6±4.8 %, $p=0.0081$). Furthermore, LeucoSep cell separation gave a higher yield (1.10±0.3 vs. 0.92±0.3 million cells/mL blood, $p=0.0022$) and viability (95.7±2.0 vs. 93.4±3.2%, $p=0.0012$) than Ficoll-Paque cell separation. The studies in Stockholm as well as in Dar es Salaam of the functionality of purified PBMC showed no difference in the rates of responses to a CEF peptide pool in the IFN-γ ELISpot assay for the pair-wise comparisons of the different cell separation techniques. Following these evaluations, the CPT technique was adopted for use at the SMI while the LeucoSep cell separation technique is being used at MUHAS.

A recent study in Uganda of PBMC separation by LeucoSep processing of blood samples from a large number of HIV-uninfected individuals showed a yield of 1.3×10^6 cells per mL of whole blood and 97% viability which is similar to our findings in Tanzanian individuals [325]. It has been recommended that PBMC separation should yield $1\text{-}2 \times 10^6$ cells/mL of whole blood in which approximately 60%-70% of mononuclear cells are lymphocytes with >95% viability [326]. In most HIV vaccine trials cryopreserved cells have been used for monitoring of vaccine-induced cellular immune responses. In the HIV-1 DNA prime MVA boost vaccine trials in Sweden and in Tanzania, we have primarily used fresh PBMC for assessment of vaccine-induced cellular immune responses. However, PBMCs from the volunteers in these trials have also been cryopreserved for subsequent additional cellular immunological studies.

5.3 Assessment of HIV vaccine-induced lymphoproliferative responses (Paper IV)

In the HIVIS01/02 phase I HIV vaccine trial that was conducted in Stockholm, Sweden, vaccine-induced cell-mediated immune responses were monitored by the IFN-γ and the IL-2 ELISpot assays and by LPA. After receipt of three HIV-DNA immunizations and one HIV-MVA boosting immunization, 34 of 37 (92%) vaccinees had HIV-specific IFN-γ ELISpot responses and 25 (68%) had positive IL-2 responses. Thirty-five of 38 (92%) vaccinees were reactive by the conventional [^{3}H]-thymidine uptake assay [304]. In the study reported in paper IV, the HIV-specific lymphoproliferative responses in these vaccinees were further assessed by a flow cytometry LPA with simultaneous CD4$^+$ and CD8$^+$ T-cell immunophenotyping using either whole blood (FASCIA-WB) or PBMC (FASCIA-PBMC). Thirty-two of 38 (84%) vaccinees were reactive by the CD4$^+$ T-cell FASCIA-WB, and 7 of 38 (18%) also exhibited CD8$^+$ T-cell responses. There was a strong correlation between the proliferative responses measured by the [^{3}H]-thymidine uptake assay and CD4$^+$ T-cell FASCIA-WB ($r=0.68$; $P < 0.01$). Fourteen vaccinees were analyzed using all three assays. Ten of 14 (71%) and 11/14 (79%) demonstrated CD4$^+$ T-cell responses in FASCIA-WB and FASCIA-PBMC, respectively. CD8$^+$ T-cell reactivity was observed in 3/14 (21%) and 7/14 (50%) using the FASCIA-WB and FASCIA-PBMC, respectively. All 14 were reactive by the [^{3}H]-thymidine uptake assay. It was concluded that FASCIA-PBMC may be an alternative to the [^{3}H]-thymidine uptake assay for assessment of vaccine-induced T-lymphocyte proliferation especially in radioactive-restricted settings.

Another flow cytometric assay based on the use of carboxyfluorescein diacetate succinimidyl ester (CFSE) to monitor lymphocyte division has been reported to be an effective method to measure T-lymphocyte proliferation [306, 327-329]. CFSE is a fluorescein-based dye compatible with a wide range of fluorochromes making its application possible in multi-color flow cytometry. Recently, a comparison of three LPAs including [^{3}H]-thymidine uptake, FASCIA PBMC which has been renamed flow cytometry LPA (FC-LPA) and CFSE was performed to monitor the HIV-1-specific vaccine-induced T-cell responses in 24 vaccinees in the HIVIS05 trial [330]. In this trial, 24 vaccinees from the former HIVIS01/02 trial were recruited to receive a second HIV-MVA boost three years after the first HIV-MVA boost. Using the FC-LPA, $CD4^+$ T-cell responses were detected in 100% of the vaccinees and $CD8^+$ T-cell responses in 82% two weeks after the second HIV-MVA. The CFSE also revealed both $CD4^+$ and $CD8^+$ HIV-specific T-cell proliferation. However, the FC-LPA detected more $CD4^+$ T-cell responses than the CFSE assay (100% vs. 71%). There was a good correlation between the proliferative responses assessed by the ^{3}H-thymidine uptake and FC-LPA-CD4 ($r=0.66$; $p<0.01$), and by ^{3}H-thymidine uptake and the CFSE-CD4 ($r=0.53$; $p<0.05$). There was also a significant correlation between FC-LPA-CD4 and CFSE-CD4) ($r=0.52$; $p<0.01$) [330]. There are plans to use FC-LPA for assessment of vaccine-induced T-lymphocyte proliferation in the HIV-DNA-MVA vaccine trials at the National Health Institute in Maputo, Mozambique where the use of isotopes is not permitted.

5.4 Monitoring of immune responses in healthy individuals immunized with HIV-1 DNA and boosted with recombinant MVA (HIVIS03) (Paper V)

Preparations have been made for HIV-1 vaccine trials in humans in Tanzania since 1994. These preparations have included studies of a potential cohort for vaccine trials, consisting of police officers in Dar es Salaam [331], determination of prevalent HIV-1 subtypes in Dar es Salaam (subtypes A, C and D) [50], training of laboratory and clinical personnel, and transfer of virological and immunological methods to the MUHAS laboratories in Dar es Salaam from the collaborating laboratories in Stockholm. The HIVIS 01/02 trial in Stockholm, Sweden informed the design of the subsequent phase I/II HIV vaccine trial (HIVIS03) in Dar es Salaam, Tanzania. In the HIVIS03 trial, HIV-1 DNA vaccinations were given id or im without GM-CSF, the HIV-1 MVA boosting vaccinations were administered im at 10^8 pfu and volunteers recruited were younger than 40 years of age. The main aim of the HIVIS03 trial was to explore if priming with a low id dose of HIV-DNA was superior to a higher dose of HIV-DNA given im for eliciting strong and broad HIV-specific cellular immune responses after HIV-MVA boosting.

In the HIVIS03 trial, two weeks after the third HIV-DNA injection, 22/38 (58%) vaccinees had IFN-γ ELISpot responses to Gag (Paper V). Two weeks after the first HIV-MVA boost, all of 35 (100%) vaccinees responded to the Gag and 31 (89%) to Env. Two to four weeks after the second HIV-MVA boost, 28/29 (97%) vaccinees had IFN-γ responses, 27 (93%) to Gag and 23 (79%) to Env. After the first HIV-MVA boost all 35 (100%) vaccinees responded to Gag WR peptide pool while among 29 vaccinees after the second HIV-MVA boost, 23 (79%) had positive responses to Gag WR and 3 had positive responses to Gag II and one to Gag I. IFN-γ ELISpot responses to Gag WR were significantly higher after the first than after the second HIV-MVA boost. The id-primed recipients had significantly higher responses to Env but not to Gag than im recipients. There were more volunteers with responses to multiple peptide pools in the id group than in the im group. Four weeks after the second HIV-MVA boost, ICS for Gag-specific IFN-γ/IL-2 production showed both $CD8^+$ and $CD4^+$ T-cell responses. Two weeks after the first and the second HIV-MVA boost, all of 32 and all of 25 vaccinees had HIV-specific lymphoproliferative responses, respectively. It was concluded that the HIV-1 DNA prime/MVA boost was safe and highly immunogenic among healthy Tanzanian volunteers. Furthermore, the low dose id multigene multiclade HIV-DNA elicited higher and broader cellular immune responses to Env compared to a higher dose administered im after boosting with HIV-MVA.

The 100% HIV-specific IFN-γ ELISpot response rate found in the HIVIS03 trial is higher than that reported in other trials of HIV-DNA prime and poxvirus or rAd5 boost regimens [309-310]. The magnitude of the IFN-γ ELISpot responses was also higher in the HIVIS03 responders compared to that in HIV-DNA prime poxvirus or rAd5 boost trials [309-310]. In the HIVIS03 trial, the response rate and magnitude of the IFN-γ ELISpot responses were higher to Gag than to Env but the frequency of Env responses was also high (89%). In other trials of HIV-DNA prime and poxvirus or rAd5 boost regimens, IFN-γ ELISpot responses to Env predominated [309-310]. However, a recent trial of Geovac HIV-DNA and MVA vaccines (HVTN 065) showed that $CD4^+$ T-cell responses measured by ICS were evenly distributed between Gag and Env after two HIV-DNA immunizations followed by two HIV-MVA boosts [307]. The $CD8^+$ T-cell response rate to Gag and Env was also similar. Gag-specific cellular immune responses may be important for vaccine-induced protection since these responses have been shown to be associated with low viral loads in HIV-infected individuals [148-149].

In the HIVIS03 trial, lymphoproliferative responses to HIV-1 antigens of various clades including B, A, C and /A_E were also tested [332]. Two weeks after the first HIV-MVA boost, all of 32 (100%) vaccinees had strong positive lymphoproliferative responses to HIV-1 antigens from all four clades. Two weeks after the second HIV-MVA boost, all of 25 (100%) vaccinees showed positive lymphoproliferative responses to each of the AT-2-treated HIV-1 antigens of clades B, A, A_E and all except one vaccinee showed reactivity to the clade C antigen. Six months after the second HIV-MVA boost, 16 out of 18 (88.9%) vaccinees still had positive lymphoproliferative responses to HIV-1 antigens of clades B and A_E, while 14 (77.8%) and 13 (72.2%) vaccinees had positive lymphoproliferative responses to HIV-1 antigens of clades A and C, respectively [332]. Thus the HIV DNA-MVA vaccine approach induced strong and durable HIV-specific lymphoproliferative responses with a high degree of cross-clade reactivity.

In the HIVIS03, IFN-γ ELISpot and 4-colour ICS assays and the LPA were performed to monitor the HIV-specific vaccine-induced immune responses using fresh cells. Additional studies of vaccine-induced immune responses in the HIVIS03 trial will be performed using cryopreserved cells including epitope mapping of IFN-γ ELISpot responses and ICS for assessment of polyfunctional cytokine production by $CD4^+$ and $CD8^+$ T-cells and the expression of cytolytic markers in these cells. In the HIVIS05 trial, HIV-Gag specific immune responses have been assessed by 8-colour ICS for expression of cytokines (CD3/CD4/CD8/IFN-γ/IL-2/TNF-α/MIP1-β/VIVID). Polyfunctional $CD4^+$ and $CD8^+$ T-cell Gag-specific responses were detected in vaccinees who had IFN-γ-ELISpot Gag reactivity >175 SFC/million PBMC (unpublished data).

In the HIVIS03 trial, none of the vaccinees or placebo recipients was positive in the diagnostic HIV serological assays after three HIV-DNA immunizations or after the first HIV-MVA boost. However, four weeks after the second HIV-MVA boost, all 30 vaccinees (100%) were positive in the diagnostic HIV antigen/antibody (Abbott Murex, UK) and the Enzygnost anti-HIV-1/2 Plus (Dade Behring, Germany) ELISAs and in the Inno-Lia immunoblot assay. Seven out of 33 (21%) and 26 of 29 (90%) vaccinees had antibodies against gp160 in an in house ELISA after the first and second HIV-MVA boost, respectively. A recent follow up of HIVIS03 trial volunteers showed that

27 out of 27 (100%) and 26 of 26 (100%) vaccinees were still reactive in the Murex (Abbott Murex, UK) and Integral (Siemens, Germany) HIV antigen/antibody ELISAs, respectively, 17 to 22 months after the second HIV-MVA boost. Furthermore, on the Inno-Lia immunoblot (Inno-genetics, Belgium) assay, 19 out of 27 (70%) vaccinees fulfilled the diagnostic criteria for seropositivity and 8 were indeterminate. All of 27 (100%) vaccinees reacted against Gag and 19 out of 27 (70%) reacted against Env (gp120 or gp41) on Inno-Lia. Testing by the Roche HIV-1 DNA PCR assay excluded HIV-1 infection in all these volunteers.

Testing of sera from 29 vaccinees in the HIVIS03 trial 4 weeks after the second HIV-MVA boost for HIV Nabs was performed in collaboration with WRAIR using both PBMC and TZM-bl based assays. There was no demonstrable neutralizing activity in the TZM-bl pseudovirus assay using CM235 clade CRF01_AE, GS015 clade C and BaL clade B pseudoviruses. In contrast, a high antibody response rate was demonstrated using the PBMC assay. The response rates were higher against the CM235 clade CRF01_AE virus (overall 24/29, 83%) and the SF162 clade B virus (21/29, 72%) as compared to the BaL clade B virus (9/29, 31%). The response rate was not significantly different between id versus im HIV-DNA primed vaccinees ($p=0.43$). The observation that HIV antibodies can be inhibitory using a PBMC target cell assay, but non-functional in a cell line based pseudovirus assay has been reported in previous studies [48, 333-335], but has not been reported previously using human vaccine sera. The mechanism for the inhibitory activity in the PBMC assay employed in our study is currently under investigation.

The HVTN 065 trial showed that the frequency of Env binding and Nabs to HIV-1 isolates was higher after three HIV-rMVA immunizations than after two HIV-DNA immunizations followed by two HIV-rMVA boosts [307]. These findings suggest that further boosting with HIV-MVA of HIVIS03 vaccinees could improve HIV-specific humoral immune responses.

Based on the HIVIS03 findings, a phase II trial, Tanzania and Mozambique HIV Vaccine Programme (TaMoVac I) started in May 2010 to explore further the optimal HIV-1 DNA vaccine delivery method. The primary objectives of the TaMoVac I are to determine the safety and immunogenicity of three immunizations with HIVIS-DNA at a dose of 600 µg or 1000 µg administered id followed by two MVA-CMDR boosting immunizations im. Two clinical trial sites, Dar es Salaam and Mbeya in Tanzania participate in the TaMoVac I trial, each with enrolment of 60 volunteers with low risk for HIV acquisition. It is also planned in collaboration with the AfreVac group to further boost vaccinees with gp140 after two HIV-MVA boosts to enhance humoral immune responses. Furthermore, a new trial, TaMoVac II is planned to start in 2012 in Tanzania and Mozambique with the objectives to document further the immunogenicity and safety of the HIVIS DNA/MVA immunogens and to introduce novel delivery technologies of the HIVIS DNA vaccine such as electroporation to try to induce long term memory and enhance antibody production.

6 CONCLUSIONS

We have evaluated alternative serological HIV testing strategies based on the testing by HIV ELISAs and rapid HIV assays for the laboratory diagnosis of HIV infection in resource-limited settings. An alternative confirmatory HIV ELISA testing strategy has been adopted at MUHAS in Dar es Salaam and in the national blood transfusion services in Tanzania. The evaluation of various combinations of rapid HIV assays has resulted in formulation of a national confirmatory rapid HIV testing algorithm which has been adopted for use in VCT, PMTCT and HIV CTC clinics throughout Tanzania.

Testing of various procedures for optimal preparation of PBMC for use in HIV vaccine trials in Sweden and Tanzania, respectively, led to the adoption of different cell separation techniques in the two clinical trial sites.

Following investigation of three lymphoproliferative assays for monitoring of vaccine-induced immune responses in a phase I HIV-DNA prime and MVA boost trial in Stockholm, we concluded that a flow-cytometric assay using PBMC could be useful as an alternative to the [^{3}H]-thymidine uptake assay for assessment of HIV vaccine-induced T-cell proliferation, especially in isotope-restricted settings. Furthermore, the flow-cytometric assay has the advantage of allowing CD4$^+$ and CD8$^+$ T-cell immunophenotyping of the proliferating cells.

In a phase I/II HIV vaccine trial (HIVIS03) in Dar es Salaam, Tanzania, we found that the HIV-DNA prime MVA boost vaccine approach was safe and highly immunogenic eliciting HIV-specific cellular immune responses and binding antibody responses in 100% of the vaccinees. Furthermore, a high neutralizing antibody response rate was demonstrated using a PBMC assay. Capacity has been built by training of human resources, acquisition of laboratory equipments/instruments, establishment of assays for monitoring of vaccine-induced immune responses and experience of conducting an HIV vaccine trial. Capacity built through the HIVIS03 trial paved the way for the funding of the follow-up phase II HIV vaccine trials, TaMoVac I and II in Tanzania and Mozambique with aims to explore further the HIV-DNA prime MVA boost vaccine regimen for possible phase IIb or III HIV vaccine trials in the future.

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