THE MUCOSAL IMMUNITY IN HIV-1 EXPOSED SERONEGATIVE INDIVIDUALS AND EXPRESSION OF HIV-1 gp160 PROTEIN IN TRANSGENIC PLANTS

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DECLARATION

The results in thesis and its composition are solely the work of the author, except where otherwise indicated and all work of other authors is acknowledged.
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## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADCC</td>
<td>antibody-directed cellular cytotoxicity</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
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<tr>
<td>APC</td>
<td>antigen presenting cells</td>
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<tr>
<td>ALVAC</td>
<td>Albany vaccine</td>
</tr>
<tr>
<td>ARV</td>
<td>AIDS-associated retroviruses</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAF</td>
<td>CD8+ antiviral factor</td>
</tr>
<tr>
<td>CaMV</td>
<td>cauliflower mosaic virus</td>
</tr>
<tr>
<td>CCR</td>
<td>CC chemokine receptor</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-(3-cholamidopropyl)-dimethylammonio-1-propane sulfonate</td>
</tr>
<tr>
<td>CMV</td>
<td>human cytomegalovirus</td>
</tr>
<tr>
<td>CTB</td>
<td>Cholera toxin B subunit</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC chemokine receptor</td>
</tr>
<tr>
<td>2,4-D</td>
<td>2,4-dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3-diamonobenzidine</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>DC-specific ICAM-3 grabbing nonintegrin</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>3D-MPL</td>
<td>3-deacetylated monophosphoryl lipid</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked-immunosorbent assay</td>
</tr>
<tr>
<td>env</td>
<td>envelope</td>
</tr>
<tr>
<td>ESN</td>
<td>exposed seronegative individuals</td>
</tr>
<tr>
<td>EU</td>
<td>exposed uninfected individuals</td>
</tr>
<tr>
<td>gp</td>
<td>glycoprotein</td>
</tr>
<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
</tr>
<tr>
<td>HEPS</td>
<td>highly exposed, persistently seronegative</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HTLV</td>
<td>human T-cell leukaemia virus</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IN</td>
<td>integrase</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thio-galactopyranoside</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>LAS</td>
<td>lymphadenopathy syndrome</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>LAV</td>
<td>lymphadenopathy-associated virus</td>
</tr>
<tr>
<td>LCs</td>
<td>Langerhans cells</td>
</tr>
<tr>
<td>LT-B</td>
<td>heat-labile enterotoxin binding subunit (B) of <em>E. coli</em></td>
</tr>
<tr>
<td>LTNP</td>
<td>long term non-progressor</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeats</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MALT</td>
<td>mucosal-associated lymphoid tissue</td>
</tr>
<tr>
<td>MBN</td>
<td>mung bean nuclease</td>
</tr>
<tr>
<td>MCS</td>
<td>multiple cloning sites</td>
</tr>
<tr>
<td>MG-1</td>
<td>large molecular weight glycoprotein1</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>M-tropic</td>
<td>macrophage tropic</td>
</tr>
<tr>
<td>NC</td>
<td>nucleocapsid protein</td>
</tr>
<tr>
<td>nef</td>
<td>negative effector function</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>nptll</td>
<td>neomycin phosphotransferase</td>
</tr>
<tr>
<td>NSI</td>
<td>non-syncytia-inducing</td>
</tr>
<tr>
<td>NV</td>
<td>Norwalk virus</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCP</td>
<td><em>pneumocystis carinii</em> pneumonia</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohaemagglutinin</td>
</tr>
<tr>
<td>PND</td>
<td>principal neutralising domain</td>
</tr>
<tr>
<td>pol</td>
<td>polymerase</td>
</tr>
<tr>
<td>QS</td>
<td><em>Quilaja saponaria</em></td>
</tr>
<tr>
<td>RANTES</td>
<td>reduced upon activation normal T cell expressed and secreted</td>
</tr>
<tr>
<td>rev</td>
<td>regulator of expression of virion proteins</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Rose Park Memorial Institute</td>
</tr>
<tr>
<td>RRE</td>
<td>Rev-responsive element</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>SC</td>
<td>secretory component</td>
</tr>
<tr>
<td>SDF</td>
<td>stromal cell derived factor</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SI</td>
<td>syncytia-inducing</td>
</tr>
<tr>
<td>slgA</td>
<td>secretory IgA</td>
</tr>
<tr>
<td>SIV</td>
<td>simian immunodeficiency virus</td>
</tr>
<tr>
<td>TAR</td>
<td>trans-activation-responsive element</td>
</tr>
<tr>
<td>tat</td>
<td>trans-activator of transcription</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% tissue culture infectious dose</td>
</tr>
<tr>
<td>TCLA</td>
<td>T cell line adapted</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>T-DNA</td>
<td>transferred DNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TEV</td>
<td>tobacco etch virus</td>
</tr>
<tr>
<td>Ti</td>
<td>tumour-inducing</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane protein</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethylbenzidine</td>
</tr>
<tr>
<td>T-tropic</td>
<td>T cell tropic</td>
</tr>
<tr>
<td>uida</td>
<td>β-glucuronidase</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>vif</td>
<td>viral infectivity factor</td>
</tr>
<tr>
<td>vpr</td>
<td>virion protein R</td>
</tr>
<tr>
<td>vpu</td>
<td>virion protein U</td>
</tr>
<tr>
<td>vspB</td>
<td>vegetative storage protein gene</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside</td>
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ABSTRACT

Human immunodeficiency virus (HIV), which is the etiologic agent of the acquired immunodeficiency syndrome (AIDS), is mainly spread by heterosexual transmission. Therefore, initial exposure to HIV occurs most frequently at the genital mucosa and thus the induction of mucosal immune response may be important in protection against HIV infection. The finding that some individuals are highly exposed to HIV infection, but remain persistently seronegative (HEPS) suggests that there may be a natural immune protection mechanism possibly involving mucosal immunity. The aim of this thesis was firstly to investigate the possibility of local antibody in urine samples from HEPS individuals to evaluate the theory that such individuals may develop a protective local anti-HIV-1 antibody response, detectable in urine. Since elicitation of mucosal immunity is important in preventing viral infection by sexual transmission, development of a vaccine which is effective for the induction of mucosal immune responses may provide protection to mucosal surfaces against HIV-1 transmission. Transgenic plants expressing foreign protein have been demonstrated to be an inexpensive and easily delivered system for the production of animal and human vaccines which can effectively induce a mucosal immune response. The second aim of this work was to develop a transgenic plant system expressing HIV-1 gp160 protein to exploit the feasibility of HIV-1 oral vaccine.

The specificity of IgG, IgA and sIgA anti-HIV antibodies to different HIV-1 viral proteins present in paired urine and serum samples from 3 HIV-1 seropositive and 9 HEPS individuals were profiled using Western blot immunoassay. Specific sIgA antibodies to HIV-1 gp160, p65, gp41, p31 and p24 were detected in urine or the corresponding vaginal swab samples from HIV-1 seropositive individuals, which suggested that a local mucosal immune response was induced by HIV-1 infection. For samples from HEPS individuals, specific IgG antibodies to HIV-1 gp160, gp120 and p51, and IgA antibody to HIV-1 p51 were detected in 1 of 9 urine but not the corresponding serum samples, respectively. These results suggested that local mucosal immune responses may be elicited in those HEPS individuals. Furthermore, IgG are the predominant isotype of anti-HIV antibodies present in the genitourinary
tract of those HEPS subjects and may be associated with a natural protection mechanism in some individuals.

In order to express HIV-1 gp160 protein in transgenic plants, recombinant plant expression vectors containing HIV-1 env160 and leaf specific cauliflower mosaic virus (CaMV) 35S promoter or potato tuber specific expression promoter patatin were constructed. This first required amplifying the full-length of HIV-1 env160 and cloning this into plant plasmids pIBT210.1, behind plant leaf specific promoter 35S, and pIBT240.1, behind potato tuber specific promoter patatin. The final recombinant expression vectors were obtained by cloning DNA fragments containing env160 gene-35S promoter or env160 gene-patatin promoter into the plant binary vector pGPTV-KAN which contained the plant selectable marker gene nptII, which confers kanamycin resistance to plants.

The required DNA segment containing HIV-1 env160 associated with the appropriate promoter was introduced into tobacco (Nicotiana tabacum "sumsun"), potato (Solanum tuberosum, variety "Frito-Lay (FL) 1607") and tobacco NT (Nicotiana tabacum)-1 cell line by Agrobacterium-mediated plant transformation system. The integration of HIV-1 env160 into tobacco "sumsun" transformants was verified by PCR using 3 paired HIV-1 env primers, respectively. 29 out of 30 tested transformants showed the correctly sized PCR products, which indicated the target gene had been successfully introduced into the host genome. To investigate the transcription of integrated env160 gene in tobacco transformants, the presence of mRNA was analysed by RT-PCR. 14 out of 17 transformants showed specific transcription of env160 genes, which indicated the presence and proper processing of mRNA of gp160 gene in the leaves of tobacco plantlets. The expression of HIV-1 gp160 protein in transformed tobacco plantlet leaves, potato microtubers and NT-1 cells was detected by gp160/120 ELISA using monoclonal antibody to HIV-1 gp120. Results showed that HIV-1 gp160 protein was expressed in 84 out of 96 kanamycin-resistant tobacco plantlets with the expression level ranging from 0.03 to 15.88 ng/mg total soluble protein, corresponding to gp160 protein accumulated in the leaves of tobacco transformants from 0.000003 to 0.002% of the total soluble
leaf protein. The presence of HIV-1 gp160 protein was also detected in 8 out of 32 tested potato microtubers and 6 out of 32 tested NT-1 cell transformants, with the expression level up to 14.92 ng/mg total soluble protein in potato microtubers and 2.76 ng/mg total soluble protein in NT-1 cell transformants, corresponding to about 0.002% and 0.0003% gp160 of the total soluble protein, respectively. These results demonstrate the feasibility of expressing the HIV-1 gp160 protein in transgenic tobacco, potato and tobacco NT-1 cell systems. Such transgenic plants may provide a valuable tool for the development of edible oral vaccines.
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1.1 Human immunodeficiency virus

1.1.1 Discovery

The first cases of what later became known as acquired immunodeficiency syndrome (AIDS), manifested as *Pneumocystis carinii* pneumonia (PCP) were reported in June 1981 in the United States (Gottlieb *et al.*, 1981). The other immunodeficiency-associated conditions such as Kaposi's sarcoma, mucosal candidiasis, disseminated cytomegalovirus infection, and chronic perianal herpes simplex virus ulcers were described in next several months. All the affected patients had the common features of T-lymphocyte dysfunction. By the end of 1982, the outbreak of this immunodeficiency syndrome was reported in over 30 states and different populations hemophiliacs, transfusion recipients, sex partners of risk-group people, and children born to mothers at risk. This all pointed to a transmissible agent which spread through genital secretions and blood.

Several infectious agents were suggested as possible etiologic agents for this newly described immunodeficiency syndrome such as human cytomegalovirus (CMV), hepatitis B (HBV) and human T-cell leukaemia virus type 1 (HTLV-1). However, all three viruses were excluded as there was limited evidence of infection with these viruses in the AIDS affected patients, particularly children. Barre-Sinoussi *et al.* (1983) recovered a reverse transcriptase-containing virus, suggestive of a retrovirus, from the lymph node of a man with persistent lymphadenopathy syndrome (LAS). This first isolation of a retrovirus, later called lymphadenopathy-associated virus (LAV), was initially believed to be a member of known human retrovirus groups such as human T-cell leukemia virus (HTLV), until Montagnier *et al.* (1984) found that this human retrovirus had some quite distinct properties from HTLV. They found that it grew substantially in peripheral blood mononuclear cells (PBMC), killing CD4⁺ lymphocytes, not immortalising them. In 1984, Gallo *et al.* and Levy *et al.* reported another two human retrovirus HTLV-III and AIDS-associated retroviruses (ARV) respectively. Both of them confirmed that these two viruses had the same properties as LAV and greatly expanded the evidence linking these three retroviruses (LAV, HTLV-III, ARV) to the
immunodeficiency syndrome.

In 1986, the three prototype viruses (LAV, HTLV-III and ARV) were recognised as members of the same group of retroviruses, and the International Committee on Taxonomy of Viruses recommended giving the AIDS virus a separate name, the human immunodeficiency virus (HIV).

Subsequently a second human immunodeficiency virus (HIV-2) was isolated in West Africa and North America. It appeared that individuals infected with HIV-2 also develop AIDS, but with a longer clinical latency period and lower morbidity (Clavel et al., 1986).

1.1.2 Transmission

According to new estimates from the Joint United Nations Program on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) (HIVInSite 1999), 33.6 million people were living with HIV/AIDS by the end of 1999. By the end of 1999, it is estimated that total number of AIDS deaths was 16.3 million since the summer of 1991. Approximately, 22% of these deaths occurred in children and 38% in women. In 1999, 2.6 million people died from HIV/AIDS, a figure that is higher than for any year since the beginning of the epidemic. With the HIV-positive population expanding, the annual number of HIV/AIDS deaths can be expected to increase for many years. Clearly, AIDS represents an international health crisis that threatens to overwhelm even the best systems of health care delivery.

HIV is spread by sexual contact, by exposure to infected blood or blood products and perinatal transmission from mother to child. Heterosexual transmission remains by far the predominant mode of transmission worldwide (Quinn et al., 1996).

1.1.2.1 HIV-1 transmission by blood and blood products

HIV transmission by blood was first reported in 1983 (Jaffe et al., 1983). It is clear that both free infectious HIV and HIV-infected cells are present in blood, although HIV-
infected cells appear to be more infectious. Transfusion recipients and hemophiliacs have been infected by HIV present in blood and blood products such as factors VIII and IX, although heat treatment processes have virtually eradicated HIV transmission through this route (Levy et al., 1984b). Studies have demonstrated that the time period before development of AIDS in the donor can influence the risk of transmission (Perkins et al., 1987; Ward et al., 1989). Those who received blood transfusions from individuals who had already developed AIDS progressed to disease much more rapidly than those who received blood from an asymptomatic individual. In rare instances, transfusion recipients of contaminated blood do not become infected (Ward et al., 1989), possibly reflecting a very low virus load in the donors.

The use of illicit intravenous drugs is an efficient way of spreading HIV due to sharing of needles or syringes without sterilizing (Jarlais et al., 1992). Where drug-injecting equipment sharing is common, HIV infection may spread through drug-injecting populations.

Among health care workers, needle stick injury has not been considered a major risk factor, although it is still appreciable. About 1 in 800 puncture wounds have led to transmission (Gerberding, 1994).

1.1.2.2 HIV-1 transmission from mother to child

On average, vertical transmission leads to infection in about 25% of children born to HIV-positive mothers (Louis et al., 1993). The first children with HIV-1 infection were described in 1983. Reports suggest that if the mother is symptomatic with a characteristic high level of virus in the blood (virus load), transmission of HIV to their child can occur during pregnancy, at the time of delivery or even from breast feeding (Rossi et al., 1989; Scarlatti et al., 1991; Van de Perre et al., 1991; Weiser et al., 1994). Antiviral therapy taken by an HIV infected mother late in pregnancy significantly reduces transmission to her offspring (Louis et al., 1994). This latter finding has offered encouraging approaches to limiting HIV transmission to newborns.
1.1.2.3 HIV-1 in urine and other body fluids

It is unlikely that urine could serve as a vehicle for HIV-1 transmission. It has been shown urine contain none or only very few virus (Levy et al., 1985; Skolnick et al., 1989; Li et al., 1992). Skolnick et al. (1989) reported the absence of infectious HIV-1 in urine. However, a study reported HIV-1 DNA proviral sequences were detected in 53 of 80 (66.25%) fresh urine pellets from HIV-1-seropositive individuals (Li et al., 1992). Nevertheless, so far, there is no report showing transmission of HIV by urine (Flint et al., 2000).

HIV-1 was isolated from the saliva of HIV-1 infected patients. Early study showed the presence of low level of free HIV-1 particles or infected cells in saliva (Groopman et al., 1984; Ho et al., 1985; Levy et al., 1988). It was believed to be associated with the presence of large molecular weight glycoprotein in this body fluid, which could block HIV infection at the cell surface or lack of virus infection of salivary glands (Archibald and Cole, 1990; Amory et al., 1992). However, high prevalence of HIV-1 RNA in saliva has been demonstrated by using PCR method (Liuzzi et al., 1997; Pilcher et al., 2001, Shugars et al., 2000; 2001). A recent study showed HIV-1 RNA was detected in 44% of unfiltered saliva samples, 16% of filtered saliva samples, and 59% of saliva-derived cell pellets. Morphological characterization of cell pellets identified lymphocytes as a likely HIV-1 source. Thus substantial quantities of HIV-1 could be shed in the oral cavity, particularly when inflammatory conditions are present (Shugars et al., 2001). Therefore, the possibility that HIV can be transmitted by saliva should be considered. In addition, a small quantity of virus has been also found in tears but there has not been detection of virus in sweat and faeces (Levy, 1993c).

1.1.2.4 HIV-1 sexual transmission

There is no doubt that the most common way of spreading HIV is sexual transmission. Worldwide the vast majority of cases of HIV-1 infection have been acquired heterosexually (Overbaugh et al., 1999), despite the first case of AIDS being observed in the homosexual population. It has been demonstrated that HIV-1 virus can be cultured from the mononuclear cell fraction of semen from HIV-1-infected men (Zagury and Ho,
and subsequently HIV-1 cell free virus was detected in semen of HIV-1-infected patients (Borzy et al., 1988). Studies on artificial insemination demonstrated that semen could transmit HIV-1 (Stewart et al., 1985; Araneta et al., 1995). The first reports of the culture of HIV-1 from cervical and vaginal secretions of HIV-1 infected women demonstrated that virus was present in the female genital tract both as cell-associated and cell-free virus (Wofsy et al., 1986; Vogt et al., 1986). Generally, 10-57% of seminal and vaginal fluid samples were shown to have free infectious virus and/or virus-infected cells (Henin et al., 1993; Milman et al., 1994). A number of studies present evidence that both cell-associated and cell-free virus can be transmitted (Tan et al., 1996; Zhu et al., 1996; Sodora et al., 1998). However, whether HIV-1 is predominantly transmitted as a cell-free virus or in a cell associated form is not known.

It was reported that transmission rate is approximately two to three times higher from infected males to females than from infected females to males (Nicolosi et al., 1994). The reason for this phenomenon is not fully understood. It is possibly due to the fact that the transformation zone which is located between the squamous and glandular epithelium of penile urethra has a much smaller surface area compared to that in the female genital tract (Milman and Sharma, 1994). Sexual acts also play an important role in the efficiency of transmission of HIV-1. The risk of vaginal intercourse is considerably less than insertive anal intercourse (Lazzarin et al., 1991; Seidlin et al., 1993).

1.1.2.5 Mechanisms of HIV-1 mucosal transmission

1.1.2.5a Cellular target(s) for HIV-1 mucosal transmission

CD4+ lymphocytes, monocytes and macrophages are the principal cells for HIV-1 infection (Levy, 1993). In addition, HIV can also infect CD4+ cells, including epithelia (Zachar et al., 1991). Recently, it was reported that epithelial, stromal, and dendritic cells, as well as cells with CD14+CD4+, CD14+CD4+, and CD4+CD14+ phenotypes from the human female reproductive tract are infectable with HIV-1 (Yeaman et al., 1998), suggesting that multiple cell types may be targets for the initial infection by HIV-1.
However, little is known about potential target cells in the male genital tract (Pudney et al., 1993). In females, dendritic cells (DC) are localized in the skin and mucosal tissues such as uterus and cervix. There have been conflicting reports regarding the infection of DC with HIV-1. Langerhans cells (LCs), phagocytic dendritic cells found in the epidermis, have reported to be infected in vitro by HIV and can present HIV antigens to helper T cells (Tschachler et al., 1987; Ramazzotti et al., 1995; Spira et al., 1996). It has also been demonstrated that only early HIV genes are transcribed, indicating that incomplete replication of HIV-1 occurs in DC.

A model of virus dissemination during the sexual transmission of HIV is shown in Figure 1.1. For transmission of HIV-1 infection to occur, HIV-1 has to be disseminated from sites of infection on mucosal surfaces to secondary lymphoid tissues where viral replication can take place in T cells (Fauci et al., 1996a). A hypothetical model of the role of mucosal LCs in HIV infection has been proposed (Zambruno et al., 1995). This model suggested that for transmission to occur, HIV-1 penetrates through the mucosal epithelium (columnar or squamous) and infects LCs. These infected cells then migrate to the lamina propria and lymph nodes where they initiate a vigorous productive infection of T cells, allowing systemic dissemination of HIV. However, a recent study of mucosal infection with SIV showed that DCs were poorly infected (Granelli-Piperno et al., 1999). An in vitro study of HIV-1 infection also indicated that the virus poorly infects DC derived from skin and blood (Cameron et al., 1992; 1996). It has been proposed that DC have a unique ability to catalyze infection of T cells with HIV but do not become infected (Cameron et al., 1992). Recently, a DC-specific C-type lectin, DC-SIGN (DC-specific ICAM-3 grabbing nonintegrin) was found normally to be highly expressed on DC present in mucosal tissues but also binds to the HIV-1 gp120 (Geijtenbeek et al., 2000a; 2000b). However, DC-SIGN does not act as a receptor for viral entry into DC. During transmission of HIV, it is proposed that mucosal DC capture HIV-1 through DC-SIGN. These cells, carrying HIV, then migrate to secondary lymph nodes where they initiate highly efficient infection of T cells (Geijtenbeek et al., 2000a; 2000b).
Figure 1.1 Virus dissemination during the genital transmission of HIV. Adapted from Miller et al. (1992c).
1.1.2.5b Mechanism for cell-associated HIV-1 infection.

It was proposed that transmission of HIV-1 is mediated by HIV-1-infected T lymphocytes or macrophages in genital tract secretions (Phillips et al., 1994). In this model, the HIV infected T lymphocytes or macrophages in semen or vaginal secretions first adhere to the epithelium of the genital tract of the sexual partner. Adherence triggers rearrangement of cell cytoskeletal elements (microtubules and microfilaments) and results in directional secretion of HIV-1 onto the surface of the epithelium. HIV-1 particles are taken up by nonspecific phagocytosis into the epithelial cell, which then becomes infected. New virions released basally from the epithelial cell can infect CD4+ T cells or macrophages in the connective tissue below. It has been demonstrated that HIV-1 can infect vaginal epithelial cells by a CD4 independent mechanism (Furuta et al., 1994). This mechanism suggests that the HIV-1 envelope gp120 initially interacts with a cell-surface glycosphingolipid which could be blocked by antibodies raised against gp120.

1.1.2.5c Mechanism for cell-free HIV-1 sexual transmission

By comparing HIV-1 gp120 sequences in longitudinal samples from five acute seroconvertors with those from their corresponding sexual partners (transmitters) as well as compartmentalization of the virus in blood and genital secretions, Zhu et al. (1996) proposed a multiple-step process of HIV-1 sexual transmission: first, compartmentalization occurs between blood and semen within the donor, and then the selection for viral genotypes occurs between the donor semen and the recipient mucosa and blood. That is, those variants which have an advantage in infecting cells at entry can disseminate the virus in the new host and those strains which can replicate efficiently may become the dominant variants in blood as the virus spreads systematically from the mucosa. This hypothesis is also supported by the study in the SIV-Rhesus Macaque model (Miller, 1998). After intravenous inoculation of rhesus macaques, all the vaginally transmitting viruses produced plasma antigenemia and high levels of plasma viral RNA. In contrast, the infection that occurs after intravenous inoculation of nontransmitting viruses is characterized by a lack of viral antigen in plasma and low levels of plasma viral RNA. It is likely that the primary target cells for HIV heterosexual
infection in the recipient's genital tract and HIV-1 envelope sequences play an important role in this selection mechanism (Soto-Ramirez et al., 1996; Lu et al., 1996). Some authors found that the non-syncytia-inducing (NSI) isolates of HIV are preferentially transmitted (Roos et al., 1992) whereas others could not show a preferential transmission of HIV-1 NSI isolates (Albert et al., 1995).

1.1.3 Structure of HIV-1
HIV is a virus of the retroviral subfamily Lentivirinae, the members of which include the simian, bovine and feline immunodeficiency virus, caprine arthritis encephalitis virus (CAEV) and maedi-visna virus (MVV). HIV is an enveloped virus, with an RNA genome, and exhibits typical retrovirus morphology (Figure 1.2). The mature virion has a diameter of approximately 80-130 nm with a cone-shaped core composed of the viral gag protein p24. This nucleoid contains two identical RNA strands with which the viral RNA-dependent DNA polymerase (also called reverse transcriptase) (p66, p55), integrase (p32) and nucleocapsid protein (p7, p6) are closely associated. Gag protein p17 that provides the matrix (MA) for the viral structure lies just beneath the lipid membrane of the virus and is vital for the integrity of the virions. The viral surface is surrounded by 72 “knobs” consisting of the viral envelope glycoproteins, the external surface protein gp120 and the gp41 transmembrane protein (TM) (Ozel et al., 1988). Parts of the central and NH₂-terminal portions of gp41 are found on the outside of the virions and the central region of gp41 binds to the external viral gp120 in a noncovalent manner. The virion gp120 located on the viral surface contains the binding site for the cellular receptors and the major neutralising domains (Dalgleish et al., 1988).

1.1.4 The Structure of HIV-1 envelope glycoprotein
The HIV envelope glycoprotein plays a pivotal role in the early events of virus attachment and entry into the target cell. Neutralising antibodies found in the sera of infected individuals are primarily directed against this glycoprotein. The HIV env gene encodes a single-chain precursor gp160 that is cleaved by a cellular protease to yield the mature surface and transmembrane glycoproteins known as gp120 and gp41, respectively. At the virus surface, the gp120-gp41 complex is a noncovalently associated
Figure 1.2 Schematic diagram of the HIV-1 virion.

Each of the virion proteins making up the envelope (gp120 and gp41), capsid (p24), matrix (p17) and nucleocapsid (p9 and p7) is identified. In addition, the diploid RNA genome is shown associated with reverse transcriptase, an RNA-dependent DNA polymerase.
heterodimer, forming an oligomeric structure that probably comprises three gp120-gp41 heterodimers (Rao et al., 1995). However, there are multiple intra-gp120 disulfide bonds that keep the molecule in its proper conformation (Leonard et al., 1990). Even though the predicted molecular weight of the polyprotein is 88 kDa, the actual molecular weight of 160 kDa can be explained by its extensive glycosylation (Allan et al., 1985). There are 24 glycosylation sites in gp120 and four in gp41 so that the fully modified envelope is at least 45% carbohydrate in mass (Geyer et al., 1988).

gp120 consists of 551 amino acids and is delineated by eight disulfide bridges located within the primary amino acid sequence of the protein at highly conserved positions (Figure 1.3 and 1.4). Comparison of the gene sequence of divergent HIV-1 isolates showed significant diversity concentrated primarily in the gp120 coding region env, in five discrete regions named hypervariable regions (V1-V5). The regions between the variable regions are more conserved and were named constant (C1-C5) domains (Levy 1994). Immunisation with purified envelope proteins leads to neutralising antibodies capable of binding to the V3 hypervariable region, a loop of 33-35 amino acids confined by a conserved disulfide bond (V3 loop). The viral gp41 protein consists of a N-amino-terminal glycosylated extracellular region (Allan et al., 1985), a transmembrane region and an unusually long carboxy-terminal cytoplasmic region which anchors env in membranes.

The gp120 is involved in recognition and binding to the target cell receptors, and has therefore been identified as an antigenic target during immune responses to HIV infection. The gp120 molecule mediates the attachment of the virus to the cell-surface CD4 receptor, an interaction that is thought to trigger a series of conformational changes in the virus glycoproteins. This is hypothesized to result in exposure of the fusion peptide of gp41 and allows the fusion between the virion and the cell (Sattentau et al., 1993) (For HIV receptors, see section 1.1.6.2). Interactions between gp120 and CD4 have been extensively studied (Lasky et al., 1987; Leonard et al., 1990). Variable regions V3, V4 and V5 of gp120 interact with CD4 and antibodies against
Figure 1.3 Secondary structure of HIV-1 envelope protein gp120. Location of variable [V1-V5] and constants [C1-C5] domains are shown. N+ is the N-terminus and C+ is the C-terminus of the protein. Y indicates complex carbohydrates. (Taken from Levy, 1994).
Figure 1.4 Genome organization of human immunodeficiency virus-1. Location of regions encoding precursors for known virion proteins are shown.
V4 and V5 can block gp120-CD4 complex formation (Capon and Ward, 1991).

1.1.5 Molecular biology of HIV-1

HIV, like other lentiviruses, has a positive-stranded RNA genome that is approximately 9.7 kilobase (Kb) long and exists within the virion as a dimer, with two identical RNA strands (Muesing et al., 1985). An outline of HIV genome is shown in Figure 1.4. The genome of the virus contains the structural genes (gag, pol and env) common to all retroviruses. In addition to these genes, HIV genomes also encode regulatory genes tat, rev and the accessory genes nef, vif, vpu and vpr. SIV and HIV-2 genomes also encode a second, related gene, vpx, which appears to have arisen as a duplication of vpr (Tristem et al., 1990).

The genome of HIV-1 contains a repeated region (R) at the 5' and 3' ends, and untranslated 5' and 3' sequences (U5 and U3) that are important in the process of reverse transcription of the genomic RNA into DNA. These regions consist of the long terminal repeats (LTR) found in all other retroviruses (Figure 1.4). The viral RNA transcripts initiate transcription at the 5' LTR and terminate in the 3' LTR, at the polyadenylation signal.

1.1.5.1 Structural genes of HIV-1

The viral gag, pol and env, which are flanked by LTR, encode proteins required for production of infectious virus particles.

The gag gene encodes a 55 kD non-glycosylated precursor core polypeptide (p55) which is cleaved after translation from unspliced transcripts to form nucleocapsid: p17 (matrix, MA), p24 (capsid, CA), p7/9 and p6 (nucleocapsid, NC). The principal gag product p24 noncovalently associates with the viral genomes to form a nucleoid shell. The p55 Gag precursor alone is required for the assembly and release of viruslike particles (Gheysen et al., 1989).

The pol gene encodes the highly conserved enzymatic protein protease, reverse
transcriptase (RT), integrase (IN), and ribonuclease H. Because the \textit{gag} and \textit{pol} genes overlap and the \textit{pol} gene is encoded in a different reading frame from \textit{gag}, translational frame shifting must occur to produce the Gag-Pol polyprotein precursor p106 (Wilson \textit{et al.}, 1988). The p106 is cleaved by the viral protease to yield p10 (protease), p66/55 (RT), and p32 (integrase). Both the Gag and Gag-Pol precursors are cleaved by the viral protease during or after virus budding.

The \textit{env} gene of HIV encodes an 88-kDa precursor that is translated from a singly spliced mRNA. This precursor is glycosylated to give gp160 during its passage through the endoplasmic reticulum and the Golgi complex of the host cell. The gp160 is cleaved to produce an external glycoprotein (gp120) involved in recognition and binding to target cell receptors and a smaller, hydrophobic transmembrane protein (gp41) involved in membrane fusion (Barr \textit{et al.}, 1991). The gp120 is noncovalently bound to gp41 to form the knobs that coat the virion surface.

\subsection*{1.1.5.2 Regulatory and accessory genes of HIV-1}

In addition to three structural genes, HIV-1 has six regulatory and accessory genes (\textit{vif}, \textit{tat}, \textit{rev}, \textit{vpr}, \textit{vpu}, and \textit{nef}). Two regular genes (\textit{tat} and \textit{rev}) encode proteins Tat and Rev that are essential for viral replication. The remaining four accessory genes (\textit{vif}, \textit{vpr}, \textit{vpu} and \textit{nef}) encode the accessory proteins (Vif, Vpr, Vpu and Nef) which are essential for efficient virus production \textit{in vivo}. However, their precise roles remain ill defined.

The \textit{tat} (trans-activator of transcription) gene is believed to be necessary for efficient viral replication and enhanced viral transcription (Greene, 1991). It encodes a 14 kD protein which is synthesized early after infection (Arya \textit{et al.}, 1985). In HIV (as in SIV and EIVA), \textit{tat} protein enhances HIV genes expression by interacting with cellular factors and binds to a stem-loop structure known as \textit{trans}-activation-responsive element (TAR) RNA sequences located in the 5' end of all HIV transcripts (Barry \textit{et al.}, 1991). The recent identification of cyclin-T revealed a new mechanism by which Tat co-opts cyclin-T to increase processivity of LTR-driven transcription (Wei \textit{et al.}, 1998).
The *rev* (regulator of expression of virion proteins) gene encodes a protein (19 kD) translated from multiply-spliced RNAs (Sodroski *et al.*, 1986). The Rev protein functions to facilitate the export of unspliced and singly spliced viral mRNA from the nucleus. The *rev* protein binds to viral RNA in the nucleus through a highly structured RNA element known as the Rev-responsive element (RRE) located within the *env* gene (Greene, 1991, Levy, 1993). The Rev-responsive element is only present in unspliced and singly spliced viral mRNAs whose expression is regulated by *rev*. Consequently, Rev is required for the expression of structural proteins and indirectly downregulates its own expression, as well as that of *tat* and *nef*.

The *nef* (negative effector function) gene is present in all primate lentiviruses and is highly immunogenic. Nef was proposed as a specific repressor of HIV transcription (Ahmed *et al.*, 1988), although this result was not reproduced by others (Hammes *et al.*, 1989; Kim *et al.*, 1989). It has been shown that the Nef protein is not essential for viral replication in cell culture (Hammers *et al.*, 1989), although SIV Nef protein is required for efficient virus replication *in vivo* (Kestler *et al.*, 1991). It has been reported that the expression of HIV Nef protein results in reduction of CD4 expression on the cell surface (Aiken *et al.*, 1994). The Nef protein may, therefore, be associated with membrane structure and may function to downregulate surface expression of CD4 on infected cell by inducing endocytosis and degradation of CD4 in lysosomes (Garcia *et al.*, 1991; Aiken *et al.*, 1994). Therefore, Nef may be involved in both protein transport and transmembrane signaling. HIV-1 sequences containing deletions in *nef* were found in multiple blood samples over a period of 10 years in a patient with nonprogressive HIV-1 infection, suggesting that *nef* deficient strains are attenuated (Kirchhoff *et al.*, 1995). The mechanism for this function of *nef* has not been defined.

The *vif* (viral infectivity factor) gene encodes a late gene product found to be essential for the spread of HIV-1 in peripheral blood lymphocytes, primary macrophages (von Schwedler *et al.*, 1993) and some established cell lines (Sakai *et al.*, 1993). In addition to a role in viral particle formation, Vif appears to be important for both cell-to-cell and cell-free transmission (Fisher *et al.*, 1987). Three different functions of Vif have been...
described. First, Vif is required for the complete synthesis of viral DNA, which could be due to its effects on the internalization of the nucleocapsid, virion uncoating, activation of RT, or RNA processing (von Schwedler et al., 1993). The second proposes that Vif is a cysteinyl protease, which could play a role in the processing or conformation of Env (Guy et al., 1991). Finally, Vif might be required in the maturation process, since, in its absence, only low amounts of Env are expressed on the virion (Sakai et al., 1993).

The vpr (virion protein R) gene encodes a 15-kD protein associated with the nucleocapsid protein p6 (Paxton et al., 1993). Although dispensable for replication in CD4+ lymphocytes (Dedera et al., 1989; Balotta et al., 1993), Vpr appears to augment both HIV-1 and HIV-2 replication in macrophages (Westervelt et al., 1992) and has been implicated in nuclear localization of viral nucleic acids in nondividing cells (Heinzinger et al., 1994). Additionally, Vpr has weak transactivation properties for the HIV long terminal repeat (LTR) and other promoters (Cohen et al., 1990).

The vpu (virion protein U) gene is present in HIV-1 but not HIV-2 or SIV. Vpu is necessary for intracellular dissociation of gp160 and CD4 through degradation of CD4 in the endoplasmic reticulum (Willey et al., 1992a; 1992b). Vpu may play a role in proper and efficient virion assembly and release (Geraghty et al., 1993), and also facilitate the export of Env and the budding of mature virions (Yao et al., 1993).

1.1.6 Life cycle

1.1.6.1 HIV-1 life cycle

The genomes of HIV are single-stranded, positive-sense RNA. The virus replication is similar to that of other lentiviruses, which involves a requirement for RT enzymatic action and reverse transcription of the viral RNA, a multistep process involving "jump" or strand transfers (Goff, 1990; Kupiec and Sonigo, 1996), to produce double-stranded DNA provirus (Figure 1.5).

HIV infection is initiated by binding of the viral envelope to a cellular receptor, the CD4
molecule and coreceptor (see section 1.1.6.2). CD4 is expressed on the surface of the T-helper lymphocyte subset and, in lesser quantities, on mononuclear phagocytes. These are thus the principal target cells in vivo. There may be other cell surface molecules involved in virus binding and entry as HIV-1 infection has been detected in a variety of non-CD4-bearing cells in infected individuals (Nelson et al., 1988; Pomerantz et al., 1988). After the binding events, HIV enters cells through a pH-independent membrane fusion mechanism (Sinangil et al., 1988). During membrane fusion, the lipid molecules of the viral envelope and those of the cell plasma membrane can freely intermix and the two membranes eventually coalesce, allowing the viral nucleocapsid to enter the cell cytoplasm. Although HIV-cell binding and membrane fusion are prerequisite events in HIV infection, their occurrence does not guarantee infection (Potash et al., 1992; Stamatatos and Duzgunes, 1993).

Once inside the cell, virion-associated reverse transcriptase synthesizes a linear, double-stranded DNA copy of the viral genome. Then the viral core is actively transported to the cell nucleus through interactions with the gag matrix protein (p17), possibly involving Vpr (Bukrinsky et al., 1993; Heinzinger et al., 1994). Integration of full-length viral DNA occurs by colinear insertion into chromosomal DNA to form the provirus. Viral genes are replicated along with host chromosomal DNA and therefore persist for the life of the cell. Like other lentiviruses, HIV is able to replicate in non-dividing terminally differentiated cells as well as in dividing cells. It is still an open question whether this may require specific functions, supplied by the viral accessory genes, to permit translocation of the preintegration complex and integration into nonreplicating genomic DNA.

Expression of HIV genes involves both cellular and viral proteins. The retroviral DNA ("provirus") uses the cellular transcription machinery to express the viral RNA that has two essential roles. Firstly, it serves as the genomic RNA that is incorporated in the virion and secondly as the messenger RNA that produces all the viral proteins. Signals for initiation of transcription are found in the 5' LTR (long terminal repeats) region, which have important regulatory functions during reverse transcription. The viral
Figure 1.5 HIV-1 life cycle. After binding to the surface of the cell, the viral core enters by virus-cell membrane fusion. A double-stranded DNA copy is synthesized from genomic RNA by virion-associated reverse transcriptase. The viral core is transported to the nucleus, and the provirus is formed by integration into the host genome. Viral RNA can be expressed from the provirus, typically after cell activation, and viral proteins are synthesized on host ribosomes. Progeny virions are formed by budding through the membrane of infected cells (Adapted from O'Brien and Pomerantz, 1997).
precursor proteins are proteolytically cleaved and the virions, which are assembled in the cytoplasm are released from the cell by budding through the cell surface membrane. Cellular proteins have been found in association with virions and may be important for viral infectivity (Benkirane et al., 1994).

1.1.6.2 Viral co-receptors and cellular tropisms

The ability of HIV-1 to infect different types of cells varies from isolate to isolate and is referred to as cellular tropism (Figure 1.6 and 1.7). All HIV-1 strains infect primary CD4+ T lymphocytes. Many strains of HIV-1 replicate well in monocytes, but not in established T-cell lines (Cheng-Mayer et al., 1988), and are classified as monocyte-or macrophage-tropic (M-tropic). Other isolates, particularly those that have been passaged in lymphoid cells in vitro (T-cell-line-adapted strains), infect primary T lymphocytes but not monocytes or macrophages, and are referred to as T-cell-tropic (T-tropic) virus. M-tropic viruses are usually noncytopathic, that is, NSI, and T-tropic viruses are generally found to induce syncytia formation in vitro and are thus termed as syncytia-inducing (SI). Dual-tropic viruses that are M-tropic with SI characteristics may be produced during transition from M-tropic/NSI to T-tropic/SI (Doranz et al., 1996). Cellular tropism of HIV appears to be determined primarily by the gp120 subunit of the HIV-1 Env protein, particularly the third variable region (V3 loop) of gp120 (Cheng-Mayer, 1990).

Studies to investigate the basis of cellular tropism led to the identification of the co-receptor for HIV-1. In early 1996, Feng et al. reported that the receptor CXCR4 (also termed fusin) was an HIV-1 entry co-receptor (or cofactor) with CD4 for T-tropic strains. The sequence of CXCR4 indicated that it was a GTP-protein (G-protein)-coupled receptor with seven transmembrane helices. Shortly afterwards, several groups found that cysteine-cysteine (C-C)-linked chemokine receptor CCR5 (also called CC-CKR5, CKR5) functioned as a coreceptor for M-tropic HIV-1 but not for T-tropic HIV-1 strains (Alkhatib et al., 1996; Dragic et al., 1996). The C-C or β-chemokine receptors consist of seven transmembrane domains and typically contain no introns (Choe et al., 1996, Combadiere et al., 1996, Samson et al., 1996a). Primary CD4+ T lymphocytes
Figure 1.6 Coreceptors for M- and T-tropic strains of HIV-1. The T-tropic HIV-1 strain can infect host cells expressing CXCR4. The M-tropic HIV-1 strain can infect host cells expressing CCR5 (Adapted from Nagasawa et al., 1999).
Figure 1.7 The interaction of HIV-1 with CD4 receptor and coreceptor. The envelope glycoprotein of HIV exists as a heterodimer with the extra cellular component (gp120) interacting with the receptor and the transmembrane component (gp41) inducing fusion with the host membrane. Following HIV gp120 binding to CD4 a conformational change facilitates the interaction of gp120 with the chemokine receptor, exposes the fusogenic peptide on gp41 and allows it to insert into the cell membrane.
express both CXCR4 and CCR5, and transformed T-cell lines express CXCR4 but not CCR5 (Fig. 1.6 and 1.7). Other β-chemokine receptors (CCR2b and CCR3) can also act as co-receptors for M-tropic and dual-tropic HIV-1, but they are little used by M-tropic strains. Although HIV-1 isolates utilize both CXCR4 and CD4 as entry receptors, some HIV-2 isolates were found to use CXCR4 as an alternative receptor in the absence of CD4 (Endres et al., 1996).

Before the HIV-1 entry coreceptors were identified, the C-C chemokines RANTES, macrophage inflammatory protein (MIP)-1α, and MIP-1β, which are ligands for CCR5, were found to block infection with M-tropic HIV-1 (Cocchi et al., 1995). Then, the CXC chemokine stromal cell derived factor (SDF)-1 was shown to be the ligand for CXCR4 and to block infection with T-tropic but not M or dual-tropic HIV-1 strains (Bleul et al., 1996; Oberlin et al., 1996). Oravecz et al. (1996) showed that the β-chemokines seem to inhibit infection by blocking or down-regulating the receptor they share with virus. However, the role of intracellular signaling activated by co-receptors in HIV-1 entry is still poorly understood.

Although CD8+ cells are not considered to be prime targets of HIV-1 infection, several studies have demonstrated that HIV-1 is able to infect these cells. The presence of viral DNA in CD8+ cells of HIV-1-infected patients has been demonstrated (Livingstone et al., 1996). Flamand et al. (1998) showed that stimulation of CD8+ T cells with mitogens including plant phytohemagglutinin (PHA), bacterial staphylococcal enterotoxin B and anti-CD3 mAb, led to de novo expression of the CD4 antigen at the cell surface of CD8+ T cells, which results in susceptibility of CD8+ T cells to HIV-1 infection. Other study demonstrated that activated neonatal CD8+ T cell can be productively infected in vitro by M-tropic HIV-1 isolates, which are responsible for disease transmission (Yang et al., 1998). A recent study showed that mature CD8+ lymphocytes can be productively infected with HIV-1 in patients with AIDS (Saha et al., 2001). Infection of naïve CD8+ cells (CD3+CD8+CD45RA+) with HIV-1 in vivo has been also demonstrated (McBreen et al., 2001). These findings indicate that direct HIV-1 infection of CD8+ T lymphocytes may contribute to the decline in CD8+ T cells frequencies and functions on disease
progression in HIV-1 infected individuals.

1.2 The immune response to HIV-1

1.2.1 The humoral immune response to HIV-1
The humoral immune response, which is characterised by the production of large numbers of antibody molecules specific for antigenic determinants (epitopes) on a foreign pathogen, is uniquely adapted to the elimination of extracellular pathogens. This process requires the participation of macrophages, activated helper T cells and B cells.

Antibodies to HIV proteins can appear as early as 2 weeks and as late as 6 month after infection (Allain et al., 1986; Gaines et al., 1988), but generally within 1 to 3 months (Flint et al., 2000). Detection of serum antibodies to HIV is a common method of demonstrating HIV infection (Schochetman et al., 1989; Mylonakis et al., 2000; Flint et al., 2000). It was shown that the viral gp120 and gp160 proteins were the major targets for human antibody response (Allan et al., 1985; Barin et al., 1985, Moore et al., 1994; Binley et al., 1998; Parren et al., 1999). Antibodies specific for other HIV proteins, including p66, p55, gp41, p32, p24, and p17, have also been detected (Carlson 1988; Lewis et al., 1998).

1.2.1.1 Neutralising Antibodies to HIV-1
The most effective type of antiviral antibody is "neutralising" antibody, which usually binds to the viral envelope or capsid proteins and prevents the virus from binding and gaining entry to the host cell. HIV-1-infected people mount and sustain a vigorous antibody response to HIV-1. A portion of this response is neutralising. Neutralising antibody is first detectable in the serum within 1-2 months after infection. A variety of different observations show that early neutralising antibodies tend to be of low titer, are not present in all patients after primary infection, and are only able to neutralise a limited range of isolates (Moore and Ho 1995; Legrand et al., 1997; Moog et al., 1997; Pilgrim et al., 1997; Stamatos et al., 1998). Even following many years of HIV infection, most infected subjects mount only weak neutralising responses against
primary HIV-1 isolates (Sattentau et al., 1996; Burton et al., 1997). However, this does not mean that antibodies are powerless in controlling HIV infection. In the presence of natural killer (NK) effector cells HIV-1 specific antibody from patient with acute HIV-1 infection can inhibit HIV-1 replication (Forthal et al., 2001). Passive immunotherapy with IgG monoclonal antibodies against the HIV-1 gp120 CD4 binding region and the gp41 neutralising epitope protected intrarectally SHIV-infected macaques (Baba et al., 2000). Passive transfer of IgG from an SIV-infected long-term nonprogressor can delay disease progression when given to macaques at the time of SIV infection (Haigwood et al., 1996). Neutralising antibody against the HIV-1 Env glycoprotein is capable of protecting macaque monkeys from chimeric SIV/HIV-1 (SHIV-1) infection (Shibata et al., 1999). In vitro study showed that IgG₃ from plasma of HIV-1 infected donors has more potency in neutralising HIV-1 compared to that of IgG₁ and IgG₂. The result that IgG₃ F(ab')₂ was still more efficient in neutralisation than F(ab')₂ of IgG₁ indicated that the IgG₃ hinge region confers enhanced HIV-1 neutralising ability (Scharf et al., 2001). These results suggested that antibody specificity, such as subclass, may be crucial for neutralisation of HIV-1.

The vast majority of neutralising antibodies bind to gp120 and gp41 (Parren et al., 1999). Experimental stimulation of neutralising antibodies to HIV was first achieved by inoculation of animals with either genetically engineered or purified natural gp120 (Lasky et al., 1986; Robey et al., 1986). Matthews et al. (1986) found that HIV-specific neutralising antibodies react predominantly with the Env glycoproteins, similar to that observed in other retroviral systems. It has also been demonstrated that antibodies to p17 Gag protein and the immunodominant epitope of gp41 have neutralising activity (Sarin et al., 1986; Schrier et al., 1988; Parren et al., 1999).

The gp120-specific neutralising antibodies appear to be type specific when raised in goats immunised with either recombinant or purified viral gp120 (Matthews et al., 1986; Rusche et al., 1987). However, group-specific neutralising antibodies have been detected in human sera from HIV-infected individuals (Weiss et al., 1986) and chimpanzees (Goudsmit et al., 1988). Type specific antibody is only capable of
neutralising the viral strains used for immunisation, whereas the group-specific antibody can react with a range of viral strains.

The presence of neutralising antibodies has been shown to select for HIV variants in vivo (Robert-Guroff et al., 1986). Sera from an infected individual did not neutralise the recovered virus, however, they neutralised previous autologous virus or heterologous laboratory strains, reflecting the appearance of escape variants (McKeating et al., 1989). Emergence of these resistant strains may contribute to disease progression.

1.2.1.2 Neutralising epitopes of HIV-1

Epitopes or antigenic determinants are the immunologically active regions of an immunogen that bind to antigen-specific membrane receptors on lymphocytes or secreted antibodies. Epitopes are divided into two categories, sequential and conformational, depending on whether they are formed by the primary sequence or the three-dimensional conformation.

The elucidation of the X-ray crystal structure of HIV gp120 (Kwong et al., 1998), together with the mutagenic and antibody competition studies (Moore and Sodroski, 1996; Ditzel et al., 1997) make it possible to precisely characterise the neutralising epitopes on the gp120 molecule. Neutralising epitopes on gp120 map to the surface of the envelope glycoprotein and face the target cells. For T cell line adapted virus (TCLA) HIV isolates, the neutralising epitopes on the gp120 include the CD4 and co-receptor binding-sites, the V2 and V3 loops and the unique 2G12 epitope (Burton et al., 1994, Roben et al., 1994, Mo et al., 1997).

The V3 loop of gp120 was originally termed as the principal neutralising domain (PND) because of its role in the neutralisation of TCLA strains by sera from HIV-1 infected patients and gp120 vaccine recipients. PND contains a central amino acid sequence GPGRAF (amino acid 308-322) which is shared by many distinct HIV isolates (see Figure 1.8) (Broliden et al., 1991). Although monoclonal antibodies (mAbs) to V3 loop have a dominant role in neutralising TCLA strains, their role in neutralisation of primary
isolates is not significant (VanCott et al., 1995, Spenlehauer et al., 1998). Because of the hyper-variability of the V3 loop, neutralisation by mAb directed to this epitope is strain-specific. Some studies have shown that a broader neutralising response can be obtained when it is directed either to a relatively conserved motif at the crown of the loop (Conley et al., 1994), or to a discontinuous epitope spanning both sides of the loop (Moore et al., 1995). Anti-V3 loop antibodies neutralise TCLA stains by blocking the virus attachment to the target cells (Ugolini et al., 1997; Valenzuela et al., 1997). In addition, it was reported that mAb against the V3 loop inhibits the interaction of gp120-CD4 with co-receptor, suggesting neutalisation at a post-attachment stage (Wu et al., 1996; Trkola et al., 1996; Hill et al., 1997).

A second cluster of neutralising epitopes is located in the V1/V2 region, which constitutes a complex, disulfide-bonded arm of gp120. MAbs specific for this segment generally recognise conformational epitopes which are located in the centre region of the V2 loop (Gorny et al., 1994), and have been shown to neutralise TCLA viruses relatively well (Warrier et al., 1994). Two mAbs to the V2 loop have been reported to be able to neutralise primary isolates (Gorny et al., 1994; Vijh-Warrier et al., 1996) but the range of isolates that can be neutralised is limited (Pinter et al., 1998). So far mAbs directed against the V1 loop have not been identified.

A third epitope cluster that was first defined by Lasky et al. (1987) is a relatively well-conserved linear epitope within the C4 region, the gp120-CD4 binding domain. MAbs directed to this epitope neutralise a relatively broad panel of TCLA HIV-1 isolates (Sun et al., 1989), but neutralisation of primary isolates has not been observed. MAbs against this epitope was shown to block the interaction of the gp120-CD4 with CCR5 (Wu et al., 1996; Trkola et al., 1996), indicating that the epitope is located in or near the co-receptor binding sites.

A unique epitope defined by a single mAbs 2G12, termed 2G12-binding site, has been described (Trkola et al., 1996). To retain binding, this mAbs requires conservation of
Figure 1.8 Principal binding domain of neutralising antibodies on the V3 loop of the viral envelope gp120. Sequences between amino acids 296-331 are shown. The cysteines are joined by a disulfide cross-bridge (bar). The amino acid sequences in the box (crown) is neutralising region of V3. (Taken from Levy, 1994).
residues at the base of the V3 loop and the NH$_2$-terminal flank of the V4 loop; suggesting that the 2G12-binding site is adjacent to both the V3 loop and the C4 region. This mAbs potentially neutralises a percentage of primary isolates (Trkola et al., 1995; 1996).

An antigenic surface map has also been proposed for gp41 (Binley et al., 1996; Earl et al., 1997). Two neutralising epitopes have been described on gp41. One epitope is defined by the neutralising mAb 2F5 and is located in the membrane proximal part of the ectodomain of gp41, which is highly conserved in a broad spectrum of TCLA and primary isolates (Muster et al., 1993), and is generally accepted as having specific neutralising activity (D'Souza et al., 1995). The other epitope of gp41 is within the cytoplasmic domain of gp41 (Chanh et al., 1986), and mAbs to this domain of gp41 have been shown to bind equally to HIV-infected and uninfected cells, implying a non-HIV-related mechanism of neutralisation (Sattentau et al., 1995).

1.2.1.3 Mechanism of HIV-1 Neutralisation
The mechanism of antibody-mediated neutralisation for HIV-1 is to block the virus attachment to the target cells (Ugolini et al., 1997). For attachment of virus to the target cells to occur, multiple interactions in a localised area are required. Coating of virus surface with antibodies obstructs the virus approach to the target cells, thereby preventing attachment and initiation of a fusion event (Parren et al., 1998). It was found that the antibody-mediated neutralisation for HIV-1 is independent of the epitope cluster recognized by the neutralising mAb (Parrent et al., 1998).

For neutralisation of HIV-1, both blockage of gp120-CD4 binding and direct inactivation of HIV-1 infectivity after binding have been described (Layne et al., 1990; Moore et al., 1991; Orloff et al., 1993; McDoug et al., 1996) (Fig.1.9).

1.2.1.3a Inhibition of gp120-CD4 binding
Some reports have shown that antibodies neutralise HIV-1 by inhibiting gp-120-CD4 binding. These neutralising antibodies compete with monomeric gp120 binding to CD4
(Layne et al., 1990; Moore et al., 1991). It has been reported that most of the effective HIV-1 neutralising mAbs inhibit the attachment of the virus to the target cells by blocking the interaction with CD4 or co-receptor (Wu et al., 1996; Trkola et al., 1996; Ugolini et al., 1997). If the principal mechanism of neutralisation is not simply steric inhibition of virus binding to CD4+ cells, these mAbs may prevent recruitment of a sufficient number of CD4 molecules to form a fusion-competent complex, or may trigger a more complex pathway of antibody-induced conformational rearrangement within the envelope glycoproteins. Indeed it has been demonstrated that mAbs such as IgG1b12 which blocks the interaction of CD4 with monomeric gp120 can neutralise a TCLA HIV-1 clone without inducing any obvious conformational changes in the envelope glycoproteins (Poignard et al., 1996b).

1.2.1.3b Inhibition after gp120-CD4 binding
It is well-established that V3 mAbs inhibit TCLA HIV-induced fusion of CD4+ cells at a stage after virus binding (Nara et al., 1991). It has been shown that these neutralising antibodies increase gp41 exposure and induce dissociation of gp120 from gp41 in TCLA HIV-1 viruses (Poignard et al., 1996b) which, by analogy with sCD4-induced dissociation, will result in irreversible inactivation of the virus (Orloff et al., 1993).

1.2.1.4 Enhancing antibodies
Enhancing antibodies, i.e. those which enhance virus infectivity, have been found in HIV-infected individuals, particularly those at the late stage of disease (Homsy et al., 1988, 1990; Robinson et al., 1988). These antibodies were found to bind to gp120 and gp41 (Jiang et al., 1991). Mechanisms of enhancement for viral entry involve complement and Fc receptors (Homsy et al., 1989; Robinson et al., 1990; Takeda et al., 1990, 1992; Kliks et al., 1993). The neutralising and enhancing effects of anti-HIV antibody depend on the HIV strain involved and the concentration of the antibodies employed (Kliks et al., 1991). In some cases, a particular monoclonal antibody to gp120 will enhance virus infection, while another may neutralise it. It is also suggested that enhancing antibodies may induce gp120 conformational changes which can improve the efficiency of viral entry (Figure 1.9).
Figure 1.9 Scheme for potential mechanism of HIV neutralisation and enhancement.

(a) Conformational changes induced in the envelope glycoproteins mimic receptor binding and activate the fusion pathway. In the situation where this state is relatively stable, virus entry will be rendered more efficient, leading to enhancement of infectivity.

(b) Conformational changes induced in the envelope glycoproteins prevent virus binding or fusion, resulting in neutralisation.

(c) Antibody-induced dissociation of gp120 from gp41 leads to inappropriate exposure of the gp41 fusion domain and virus inactivation.

(Taken from Poignard et al., 1996a).
1.2.2 Cellular immune response to HIV-1
The cellular immune response is mediated by T lymphocytes that release cytokines and lyse target cells expressing foreign antigens. Cytotoxic T lymphocytes (CTLs) are major contributors to the antiviral T cell immune response. This T-cell population carries the CD8 cell surface glycoprotein and recognises peptide antigens which have been synthesised within cell’s nucleus or cytosol, and which have been degraded. They are presented at the cell’s surface as short peptides associated with self class I major histocompatibility (MHC) molecules of the immune system (Zinkernagel and Doherty, 1975). However, CD4+ CTL directed to HIV-1 specific antigens in a MHC-class II restricted fashion have also been described in the blood of infected patients (Liu et al., 1992), but their role in HIV infection is not clear.

1.2.2.1 T lymphocytes
T cells interact with invading antigens, in association with the MHC proteins, via their T cell receptor (TCR). Two classes of MHC exist, class I and class II. MHC class I is expressed on the majority of cells. In contrast, MHC class II is restricted to B cells, macrophages, monocytes, DC and activated T cells. CD4+ T cells respond to exogenous antigens presented in association with MHC class II in the surface of antigens presenting cells (APC). CD8+ T cells recognise endogenous antigens presented on cells in association with MHC class I.

1.2.2.2 HIV-1 specific cytotoxic CD8+ Cells
CTLs have two antiviral activities: First, they can kill virus-infected cells, and they can release cytokines and chemokines with antiviral activity (Morris et al., 1982; Zinkernagel et al., 1986; Cocchi et al., 1995; Guidotti et al., 1996; 1999; Appay et al., 2000).

HIV-specific CTLs were initially described in 1987. A remarkable feature was that human leukocyte (HLA)-restricted CTLs could be readily detected in freshly separated peripheral blood mononuclear cells or in alveolar lymphocytes from HIV-infected individuals, without prior in vitro stimulation with antigen (Plata et al., 1987; Walker et
al., 1987; Flamand et al., 1998; McBreen et al., 2001). Subsequent studies demonstrated that HIV-1 specific CTLs could be isolated from infected organs, such as spleen (Cheynier et al., 1994), lymph nodes (Hadida et al., 1995; Kuroda et al., 1999), and even from the vaginal mucosa of SIV-infected macaques (Lohman et al., 1995; Wilson et al., 2000). HIV-specific CTLs were also found in individuals exposed but not infected by HIV. The latter may reflect the ability to control virus early in infection (Langlade-Demoyen et al., 1994; Rowland-Jones et al., 1995; 1998; 2001; Kaul et al., 2001).

Studies of CD8+ CTL response at the different stages of disease provides evidence of the importance of HIV-1 specific CD8+ CTL in controlling HIV-1 replication and disease progression. Borrow and coworkers (1994) reported that the level of HIV-1-specific CTL activity in HIV-1 infected patients paralleled the efficiency of control of primary viremia. Patients with strong gp160-specific CTL responses showed rapid reduction of acute plasma viremia and antigenemia, while in contrast, primary viremia and antigenemia were poorly controlled in patients in whom virus-specific CTL activity was low or undetectable. This observation is consistent with other studies (Ariyoshi et al., 1992; Koup et al., 1994, Pantaleo et al., 1994), which demonstrated that the early decline in plasma viral levels shortly after HIV-1 infection coincides with the appearance of HIV-specific CD8+ CTLs. In addition, inverse correlations between frequency of peripheral blood HIV-1-specific CTL and plasma HIV load have been reported (Ogg et al., 1998). A recent study showed that during acute HIV-1 infection, broader HIV-1 specific CTL responses were correlated with lower viral load (Altfeld et al., 2001a). Similarly, in non-human primates models, the CD8+ CTL response is the best correlate of viremia control after SIV primary infection in macaques (Yasutomi et al., 1993; Reimann et al., 1994; Chen et al., 1995). Depletion of CD8+ cells results in SIV replication being uncontrolled during primary infection and high virus load in chronically infected rhesus monkeys (Schmitz et al., 1999; Jin et al., 1999). These data indicate the importance of CD8+ CTL responses that arise in response to HIV-1 infection in controlling HIV replication during primary infection and a role for cellular immunity in protection to HIV-1 in vivo. In addition to resolving the viremia of acute infection, early generation of HIV-specific CD8+ CTLs may influence subsequent disease course
It was reported that a more broadly directed CTL response correlates with slower disease progression (Pantaleo et al., 1997). There is an inverse correlation between disease progression and HIV-1-specific CTL activity in HIV-1 infected subjects (Musey et al., 1997). Moreover, HIV-1 infected long-term nonprogressors (LTNPs) maintained the established CTL precursors pool and the frequency of gag-specific CTL precursors were inversely correlated with virus load (Pontesilli et al., 1998). On the other hand, viral mutation in CTL epitopes can result in viral escape from CTL recognition, which contributes to disease progression (McMichael 1998; Goulder et al., 1997).

CD8+ class I-restricted CTLs have been demonstrated against a wide variety of the HIV-1 gene products, predominantly directed against Gag, Pol and Env but also targeting some regulatory proteins such as Nef, Tat, and Vif (McMichael and Walker, 1994; Autran et al., 1991, Novitsky et al., 2001). Within the envelope, both conserved and variable domains serve as CTL targets whereas those within Gag, Nef and Vif are relatively conserved. A striking feature of HIV-specific CTLs is that these effector cells from an infected person can be directed toward multiple epitopes in HIV viral proteins, but the range of HIV CTL epitopes recognised by infected individuals varies (Johnson et al., 1994). Phillips et al., (1991) reported that HIV-specific CTLs from an infected donor could be directed against three peptide epitopes in Gag. However, CTLs from HIV-1 infected hemophiliacs directed toward a single epitope in Gag were also reported (Goulder et al., 1997). It has been demonstrated that anti-HIV CTLs could switch from one epitope to the other in HIV infected individuals (Nowak et al., 1995) and that CTL activity may decline as disease progresses (McMichael and Walker, 1994; Wolinsky et al., 1996). Individuals who progress to AIDS may lose the CTL activity directed against the HIV Env, Gag, Pol, and Nef proteins despite maintaining a broad cytolytic function against other non-HIV antigens (Pantaleo et al., 1990). The loss of CTL activity is probably secondary to the loss of CD4+ T-cell population and impairment of their function (Fauci et al., 1996b). A recent study in the SIV macaques model of AIDS infection showed that initial immune pressure was directed mainly through an epitope in Tat, whereas on in Gag did not appear to be under strong immune selection pressure.
(Allen et al., 2000). During primary and late HIV infection in human, differences between the specificity of HIV-1 specific CD8⁺ responses has been noted (Altfeld et al., 2001a; Goulder et al., 2001). In subjects with early HIV infection, there were no detectable CTL responses towards the p17 Gag epitope which is recognised in majority of subjects with chronic infection (Goulder et al., 2001). HIV-1 infected long-term survivors presented a persistently predominant gag-specific CTL response (Pontesilli et al., 1998). In addition, recent date suggests that CTL responses directed against regulatory proteins Tat and Rev (Walker and Goulder, 2000; Allen et al., 2000; Addo et al., 2001) and accessory protein Vpr (Altfeld et al., 2001b) may also play a role in the HIV-1 specific immune responses. The identification of the regions of HIV-1 which induce HIV-1 specific CTL response may help better understand immune control of viremia and for the development of potential HIV vaccines.

1.2.2.3 CD8⁺ cell-mediated suppression of HIV-1 replication

In addition to the anti-HIV cytotoxic activity, CD8⁺ cells are capable of controlling HIV infection by a direct effect on viral replication. This was first demonstrated by Walker et al., (1986), who showed that HIV could be recovered from PBMCs of asymptomatic individuals only after the CD8⁺ cells were removed, and that adding back the CD8⁺ cells suppressed virus production in a dose-dependent manner. This anti-HIV activity is mediated by a nonlytic mechanism (Walker et al., 1991), is not MHC-restricted (Brinchmann et al., 1990; Levy et al., 1996), and can occur without contact between the target and effector cells (Walker and Levy 1989). However, the suppression of HIV replication is more efficient when the effector and target cells are in contact with one another (Levy et al., 1996; Brinchmann et al., 1990; Mackewicz et al., 1995). HIV-suppressing CD8⁺ cells have been shown to express certain cell surface markers: CD28⁺ (Landy et al., 1993), CD29⁺, CD45RA⁻, (Tsubota et al., 1989), suggesting that CD8⁺ clones with antiviral activity are phenotypically heterogeneous (Toso et al., 1995).

It was found that the suppression of HIV replication can occur across a semipermeable membrane that excludes cells but not fluids or by transfer of the supernatant from CD8⁺ cells, suggesting that it is mediated by release of soluble factor which is referred as
CD8\(^+\) antiviral factor (CAF) (Walker and Levy, 1989; Brinchmann \textit{et al.}, 1990; Mackewicz \textit{et al.}, 1994). CAF is active against a wide variety of HIV-1 isolates (both SI and NSI) and can suppress HIV-2 and SIV strains (Levy, 1993b). Furthermore, the suppression of HIV replication has been demonstrated to be at the level of viral transcription (Mackewicz \textit{et al.}, 1995).

Although cytokines, such as interferon-gamma (IFN-\(\gamma\)) and IL-16 produced by CD8\(^+\) cells, have viral-suppressive activity (Emilie \textit{et al.}, 1992; Baier \textit{et al.}, 1995), they do not have the properties attributed to CAF. Reports have identified the CC chemokines MIP-1\(\alpha\), MIP-1\(\beta\), and RANTES as potent suppressive factors produced by CD8\(^+\) T-cells (Cocchi \textit{et al.}, 1995). This anti-HIV suppressive activity can be explained by the blocking of HIV entry by competition for the viral coreceptor. HIV-1 T-tropic strains use the C-X-C chemokine receptor CXCR4 (Feng \textit{et al.}, 1996), the ligand for which SDF-1, blocks entry of T-tropic HIV isolates (Bleul \textit{et al.}, 1996; Oberlin \textit{et al.}, 1996). M-tropic isolates predominantly use CCR-5, a CC chemokine receptor that binds RANTES, MIP-1\(\alpha\), and MIP-1\(\beta\) which block entry of these isolates (Cocchi \textit{et al.}, 1995; Alkhatib \textit{et al.}, 1996). However, these chemokines cannot account for all the properties of CAF, such as the ability to suppress HIV replication by an effect on LTR-driven transcription (Mackewicz \textit{et al.}, 1995) and this process needs to be more fully explored.

1.2.2.4 CD4\(^+\) cell response

CD4\(^+\) T helper cells play a critical role in regulating production of antibodies, induction and maintenance of CTL responses, and activation of macrophages and natural killer cells (Abbas \textit{et al.}, 1996; Zajac \textit{et al.}, 1998; Walter \textit{et al.}, 1995; Ridge \textit{et al.}, 1998).

CD4\(^+\) cell response can be decreased early in HIV-1 infection (Levy, 1993; Rosenberg \textit{et al.}, 1997; Oxenius \textit{et al.}, 2000). The vast majority of HIV-1 infected individuals have weak or undetectable proliferative responses to HIV-1 antigens (Schrier \textit{et al.}, 1989; Clerici \textit{et al.}, 1989; Musey \textit{et al.}, 1999; Valentine \textit{et al.}, 1998; Rosenberg \textit{et al.}, 1998). But a minority of HIV-infected patients, who maintain high blood CD4\(^+\) T cell counts, have good proliferative responses to Gag p24 (Rosenberg \textit{et al.}, 1997; Pitcher \textit{et al.}, 1998).
1999). HIV-1-specific CD4⁺ T helper responses were observed in long-term nonprogressors and initiation of potent antiretroviral therapy early in the course of primary infection resulted in generation of strong HIV-1 specific CD4⁺ T helper responses to HIV-1 Gag 24 (Rosenberg et al., 1997; Musey et al., 1999; Altfeld et al., 2001a). This observation is consistent with the finding that HIV-1 specific CD4⁺ T cells were detected in early infection but disappeared in untreated patients (Oxenius et al., 2000).

Analysis of functional HIV-1-specific memory CTL precursor frequencies and p24-specific proliferative responses counts showed that levels of p24-specific proliferative responses positively correlated with levels of Gag-specific CTL precursors in a cohort of infected untreated persons with a wide range of viral loads and CD4⁺ cell, indicating that association between T helper cell and strong HIV-specific CTL responses (Kalams et al., 1999). An inverse relation between HIV-specific CD4⁺ T cell responses to HIV-1 p24 and viral load was also observed in persons with chronic infection (Rosenberg et al., 1997). HIV-1 long-term non-progressors have showed relatively strong CD4⁺ T cell antiviral activity compared with responses in disease progressors (Picker et al., 2000). These findings suggest that HIV-1 specific CD4⁺ cell response may contribute to the control of HIV-1 replication and be likely to be important in immunotherapeutic interventions and vaccine development. This concept is also supported by a study in non-human primates, which has shown that the level of SIV-specific CD4⁺ T cell responses induced during anti-viral treatment associated with the degree of control of viral replication (Lifson et al., 2000). A key feature of HIV-1 is its ability to infect and kill (either directly or indirectly) CD4⁺ T cells, particularly, activated CD4⁺ T cells (Veazey et al., 2000). There are several mechanisms proposed to explain HIV-mediated depletion of CD4⁺ cells including accelerated killing of mature T cells, altered movement leading to the appearance of circulating CD4⁺ T cell loss, impaired production of new T cells; HIV-1 associated apoptosis (reviewed in McCune 2001). Loss of these cell help may be responsible, in part, for the subsequent insufficient control of HIV infection by CTL (Picker et al., 2000).
The interaction of CD4+ T cells with MHC class II and peptide results in the activation of the cell, which in turn induces the secretion of cytokines. Cytokines represent major factors in development of immune responses including activation of B cells, CTLs and macrophages. Similar to the situation in mice, human CD4+ T cells can be separated into two distinct subsets of Th cell: Th1 and Th2. The different subsets differ in cytokine profiles and the subsequent immune response they aid. Th1 cells produce IFN-γ, IL-2, TNF-α/β, which promote cell-mediated immunity and delayed-type hypersensitivity (DTH) (Mosmann et al., 1986; Mosmann and Coffman, 1991; Romagnani, 1994). Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, which favour allergic reactions and provide the most efficient help for B cells (antibody production) (Mosmann et al., 1986; Romagnani, 1994). On the basis of the pattern of cytokines expressed, immune response can be divided into Th1 and Th2 (Clerici and Shearer, 1993). It has been reported that in certain pathologic conditions different patterns of cytokine expression may be related to different types of immune response (Mosmann et al., 1986; Romagnani, 1994).

With regard to HIV infection, a switch from Th1 to Th2 cytokine responses has been suggested to represent a critical step in the disease based on the pattern of cytokines production from PBMCs from HIV-infected individuals at different stages of disease progression (Clerici and Shearer, 1993). Patients with a Th1-type response, characterised by production of IL-2 and IFN-γ remain asymptomatic whereas those that switch from Th1 to Th2, as indicated by decreased secretion of IL-12 and increased production of IL-4, go on to be symptomatic (Clerici and Shearer 1993, Chehimi et al., 1994; Klein et al., 1997; Clerici et al., 2000). It is known that one cytokine which may contribute to sustaining and augmenting both HIV-specific CTL-activity and Th1 function is IL-12 (McFarland et al., 1998; Young et al., 2001). In vitro production of IL-12 in PBMC from HIV-1 infected patients has been shown to be decreased compared to that in HIV-1 seronegative individuals (Chehimi et al., 1994; Clerici et al., 2000; Ma et al., 2000). Dysregulation in IL-12 secretion by neutrophils from HIV-infected patients has been also observed (Vecchiarelli et al., 2000). Thus, it is possible that a primary IL-12 defect may contribute to the inability of HIV-infected individuals to sustain an effective antiviral immune response (Uherova et al., 1996; Nagy-Agren and Cooney 1999).
1.2.2.5 ADCC

Virus-specific antibodies may direct the effector cells which bear Fc receptors, such as natural killer (NK) cells and monocytes/macrophages, to lyse the infected cell through a process known as antibody-directed cellular cytotoxicity (ADCC). Infected cells expressing the HIV envelope or CD4\(^+\) cells pulsed with the gp120 molecule are effectively lysed by this process (Lyerly \textit{et al.}, 1987; Ahmad \textit{et al.}, 1994). Antibodies capable of triggering ADCC are predominantly of the IgG1 class and are directed against the envelope proteins (Ljunggren \textit{et al.}, 1988). Antibodies directed against Gag proteins are not as effective at inducing ADCC (Koup \textit{et al.}, 1989). HIV-1-specific antibodies capable of inducing ADCC decline as disease progresses. This may be related to the level of effector cell function (Tyler \textit{et al.}, 1990).

In addition, NK cells represent a discrete lymphocyte subset defined by CD16/56 expression. NK cells are spontaneously cytotoxic to tumor and virally infected targets. As such, they may play a role in natural resistance to HIV-1-associated disorders and other opportunistic infections. NK cells have been shown to be effective in killing HIV-infected cells (Brenner \textit{et al.}, 1993; Ullum \textit{et al.}, 1999; Hultstrom \textit{et al.}, 2000). However, peripheral blood NK activity is frequently reduced in HIV-1 infected-patients with HIV-1-induced disease (Brenner \textit{et al.}, 1993) and the impairment of NK cells function is observed throughout the course of HIV-1 infection (Cai \textit{et al.}, 1990; Hu \textit{et al.}, 1995). The causes of NK cell dysfunction in AIDS-related disorders remain unknown. An altered expression of important regulatory receptors have been observed on NK cells from HIV-infected patients. The inhibitory NK receptor CD94 was upregulated (Andre \textit{et al.}, 1999) and the NK activation molecule CD16 and CD56 downregulated (Hu \textit{et al.}, 1995). These phenotypical changes as well as the decreased number of NK cell in HIV infection may contribute to the decreased NK activity. This reduction in NK cells function may be one of reasons to cripple the immune response to infection. A recent study of HIV-1 exposed but uninfected individuals (HEPS) among Vietnamese showed the increased NK cell cytotoxicity present in these Vietnamese HEPS when compared to low-risk donors, indicating that NK cells may, in part, contribute to natural protection against HIV-1 (Scott-Algara \textit{et al.}, 2001).
1.3 Mucosal immunity to HIV-1

There is now evidence suggesting that the mucosal and systemic immune responses are elicited and regulated with a considerable degree of independence (Alley et al., 1988). Mucosal immune responses play an important role in protection against HIV infection. However, the immune response to this virus at the mucosal surface is poorly understood.

1.3.1 Overview of the mucosal immune system

The existence of a protective local immune system in humans was initially suggested by Davies (1922) based on his observation that bacterial agglutinins could appear in dysentery stools several days earlier than in the blood. The molecular basis for local immunity was established after the discovery of secretory IgA in external secretions (Tomasi et al., 1965).

The mucous membranes which in humans have a combined surface area of about 400m², are the major sites of entry for most pathogens (Miller et al., 1992c). The defence of these vulnerable membrane surfaces is provided by organised lymphoid tissues known as mucosal-associated lymphoid tissue (MALT). The mucosal immune system (termed a compartment) consists of the tissues, cells and effector molecules that protect mucous membranes (Figure 1.10). This immune system can be divided into two sites: inductive sites where antigen is encountered and initial responses are induced and effector sites where the production of secretory IgA (sIgA) results in local immune protection. The lamina propria of the gastrointestinal (GI) tract and upper respiratory tract are major mucosal effector sites. The mucosal inductive sites include gut-associated lymphoreticular tissues (GALT), brochus-associated lymphoreticular tissues (BALT), genital tract and secretory glands such as salivary gland.

The mucosal immune response is initiated by the uptake of antigen by microfold (M) cells in the columnar epithelium of the Peyer’s patches (PP) which consist of 30-40 lymphoid nodules on the outer wall of the intestines. M cells then pass the antigen to antigen presenting cells (APCs) in the lamina propria. The APCs process and present the antigen to antigen specific B and T cell precursors in PP and draining lymph nodes. The
Figure 1.10 The mucosal system and its cellular components. Adapted from Cruse and Lewis (2000).
activated B and T cells enter the efferent lymphatic, pass through the thoracic duct and reach the systemic circulating blood and distant mucosal sites. The activated B and T cells enter mucosal tissues by specific binding to adhesion molecules (addressins) on the cytoplasmic membranes of lymphocytes and the endothelium of postcapillary venules. This concept that immune responses may occur at mucosal surfaces which are distinct from the site of mucosal immunisation is known collectively as common mucosal immune system (Bienenstock, 1979; Mayer, 1997; Kelsall et al., 1998).

Since the lower female reproductive tract can initiate an immune response (Lehner et al., 1992; Parr et al., 1992; Hocini et al., 1995, Czerkinsky et al., 1999), the genital tract is part of a common mucosal immune system. The mechanism of immune response to antigen in the female reproductive tract has recently been elucidated. Langerhans cells (LCs), which are abundant in the vaginal and ectocervical mucosa have dendritic processes which extend to the lumen of the vagina, both LCs and macrophages act as APCs (Miller et al., 1992b; Lechner et al., 1999). To generate a primary immune response in the female genital tract, APCs take up the antigen, and migrate to the draining lymph nodes, where they activate B and T cells. These then enter the circulating blood stream via the efferent lymphatic and thoracic duct, and reach the genital tract. When re-exposed to the antigen, these cells induce a secondary immune response. The mucosal effector sites in the mucosal immune system of the female genital tract consist of Fallopian tubes, ectocervix, endocervix and perhaps the vagina (Mestecky et al., 1994; Yeaman et al., 1998; Czerkinsky et al., 1999).

In addition to antibody response, cell mediated immune responses, including CTLs, APCC and NK cells are also involved in protection of mucosal tissues. It has been demonstrated that functional CTL activity has been observed in subsets of intraepithelial lymphocytes (IELs) in lamina propria (Mayer, 1997; Yeaman et al., 1998). Induction of effective mucosal response to microbial attack is obviously a benefit to the host, since mucosal elimination of invading pathogens is the first line of immune defence to prevent subsequent systemic infection.
1.3.2 Secretory immunoglobulin A (sIgA)

1.3.2.1 Structure of sIgA

Although IgA constitutes only 10-15% of the total immunoglobulin (Ig) in serum, it is the predominant Ig class in external secretions including breast milk, saliva, tears, and secretions of the bronchial, genitourinary and digestive tracts. In contrast to IgG, human IgA displays a unique heterogeneity in its molecular forms. There are known to be two subclasses of IgA, IgA1 and IgA2. Serum IgA is predominantly monomeric IgA1. The IgA in external secretions, called secretory IgA (sIgA), consists of a dimer or tetramer with a relative increase in the proportion of IgA2. Monomeric IgA, like IgG, comprises two heavy and two light chains. The heavy or α chain is made up of four domains, a variable domain and three constant domains (CH1, CH2 and CH3). The heavy chains of human IgA1 and IgA2 differ in only 22 amino acids.

The molecules of sIgA contain a J-chain polypeptide and a polypeptide chain called secretory component (SC). SC is a 50 to 90 kDa polypeptide produced by the epithelial cells of the mucus membrane (Figure 1.11). It is found in secretions not only complexed to IgA and also as a free glycoprotein. SC consists of five immunoglobulin-like domains that covalently bind to the Fc regions of the IgA dimer. Immunochemical studies (Kerr, 1990) have suggested that SC interacts with CH2 and CH3 domains of both pairs of heavy chains. The fifth domain of the SC is disulphide-linked to the CH2 domain of one of the IgA monomers. This model indicates that much of the Fc region might be exposed.

1.3.2.2 Production of sIgA antibodies

Daily, humans produce 5-15g of IgA in mucous secretions, greater than that of any other Ig class. The production of sIgA involves the selective transport of sIgA to external secretions (Rudzik et al., 1975; Mestecky et al., 1987; Brandtzaeg, 1989; Mestecky et al., 1994; Cruse and Lewis, 2000) (Figure 1.12). Induction of the mucosal IgA response is known to be highly dependent on cognate help provided by T cells. In vitro studies
Figure 1.11 Theoretical model of secretory IgA. Secretory component (dark shading) interacting with $C_H 2$ and $C_H 3$ domains of both pairs of heavy chains. The fifth domain of the secretory component is disulfide-linked to the $C_H 2$ domain of one of the IgA monomers. (Adapted from Kerr, 1990).
Figure 1.12 Production of secretory IgA (adapted from Cruse and Lewis, 2000). The precursor of glycosylated transmembrane SC is produced on the rough endoplasmic reticulum, matured in the Golgi apparatus, and expressed on the basolateral plasma membrane of the mucosal epithelial cells as the poly-Ig receptor. J-chain containing polymeric IgA (pIgA) released by subepithelial plasma cells interacts with poly-Ig receptor on the surfaces of epithelial cells. This membrane complex is then endocytosed and transported in the form of a vesicle across the cells. These vesicles fuse with the plasma membrane at the luminal surface. Then the sIgA and free SC are released by cleavage.
indicated that cloned CD4+ T cells in MALT are able to induce sIgA+ B cells to become sIgA+ and enhance the production of IgA (Kawanishi et al., 1983; Mayer et al., 1985; Murray et al., 1987). These effects occur through either the direct contact between responding B cells and T cells or activated antigen-specific CD4+ cells (Hodgkin et al., 1991). The T cells can lead to secretion of appropriate cytokines for B cell responses and Ig synthesis. Th1-type cells selectively produce IFN-γ, IL-2, and TNF-β. Both IFN-γ and TNF-α can upregulate functional SC expression (Brandtzaey et al., 1988). Transforming growth factor β (TGF-β), mainly produced by macrophage and platelets, can induce sIgM+ B cells to switch to sIgA expression. Th2 cells produce IL-4, IL-5, IL-6 and IL-10, important lymphokines for IgA synthesis. IL-5 induces sIgA B cells to differentiate into cells secreting IgA. IL-6 induces increased numbers of IgA secreting B cells at higher levels of total IgA synthesis than IL-5, probably because of its known ability to stimulate the differentiation or proliferation of plasma cell precursors arriving at mucosal sites (Mulligan et al., 1993; Ramsay and Kohonen-Corish, 1993). In mice, both IL-4 and IL-6 are required for the development of a specific IgA response to T cell dependent antigens (Vajdy et al., 1995). The finding that the cytokines produced by cells isolated from mucosal lymphoid tissue are strongly geared towards a Th2 type response which promotes antibody responses may explain the predominance of the IgA in mucosal effector tissues (Else et al., 1994; Xu-Amano et al., 1994). Although it is clear that cytokines are essential for IgA expression and production, the role of cytokines in regulating mucosal immunity is not fully understood.

1.3.2.3 Function of sIgA antibodies
sIgA antibodies serve an important effector function in mucosal immunity, because they are present at mucous membrane surfaces, which represent the main entry sites for most pathogenic organisms. Since it is polymeric, sIgA can prevent virus attachment to epithelial cells by cross-linking antigens with multiple epitopes, then transporting these immune complexes from the lamina propria across the epithelium into the secretion, thus blocking their subsequent penetration (Mazanec et al., 1993). Another important function of sIgA is intracellular neutralisation of viruses in epithelial cells. The
mechanism of neutralisation is a transcriptional block of virus replication in the infected epithelial cells. For influenza virus the mechanism for this transcriptional block may involve a conformational change in hemagglutinin (HA) following sIgA antibody binding, which results in transmission of a signal to the transcriptase complex inside the virus (Dimmock, 1984). The epithelial cells infected by virus are involved with IgA transport, and are therefore the site of antigen-IgA neutralisation that results in blocked viral nucleotide transcription. The unique characteristics of sIgA, such as its polymeric nature, avidity for mucus and resistance to proteolytic enzymes which is reinforced by its association with SC, all contribute to the apparent efficiency of this antibody in virus neutralisation. In addition to virus, sIgA antibodies also neutralise other biologically active antigens such as bacterial toxins and enzymes. sIgA is also secreted in breast milk, and plays an important role in protecting the new-born during the first month of life (Miller et al., 1992c).

In summary, the mucosal immune response is a combination of humoral and cellular mechanisms. Exploitation of the origin of secretory antibodies and of specific antibody-containing cells in mucosal layers and of the development of a vaccine which is effective for the induction of mucosal immune responses may assist in reducing the incidence of many infectious disease, including AIDS.

1.3.3 HIV-1 mucosal immunity

1.3.3.1 Mucosal immune response to HIV-1 in the female genital tract
The investigation of mucosal immunity to HIV-1 has been mainly focused on the immune response to HIV-1 in genital tract of HIV-1 infected women. An early study (Archibald et al., 1987) has reported that antibodies to HIV-1 env proteins gp160, gp120 and pol proteins p55, p38 and gag protein p24 were detected by ELISA, Western blotting (WB) and radioimmunoprecipitation assay (RIPA) in cervical samples from HIV-1 seropositive women. But the presence of serum in the secretions did not have any apparent effect on the results of the RIPA. This finding suggest that these antibodies were of IgA class and produced locally. Later, IgA antibodies to HIV- glycoproteins
gp160, gp120 and core proteins p18, p24 were detected in female vaginal secretions from healthy HIV-1 seropositive women, HIV-1 sreonegative exposed women and AIDS patients. However IgA antibodies to HIV-1 glycoprotein gp120 in female vaginal secretions were rarely recovered (Bélec et al., 1989a, 1994a; Lu et al., 1991). Further investigations on the effects of HIV-1 infection on cervical/vaginal IgA synthesis were performed. Bélec et al., (1995a) have evaluated total IgA, IgA\textsubscript{1}, IgA\textsubscript{2} in paired sera and cervical/vaginal secretions from HIV-1 infected women. The results showed that total IgA, IgA\textsubscript{1}, IgA\textsubscript{2} increased proportionately in sera and cervical/vaginal secretions from early HIV-1 infection stages. However, the secretion rates of total IgA, IgA\textsubscript{1} and IgA\textsubscript{2} were markedly reduced in AIDS women. The data also demonstrated that healthy HIV-1 infected women contained higher IgA\textsubscript{2} subclass than IgA\textsubscript{1} in vaginal washings. The author suggested that the decreasing rate of secretion for IgA\textsubscript{2} subclass at early stage of HIV infection indicated impairment of IgA class-specific immunoglobulin production in the cervical/vaginal mucosa during HIV infection. Similar selective reductions in salivary IgA and in intestinal IgA-producing plasma cells have also reported (Kotler et al., 1987; Jackson et al. 1990; Sun et al., 1990; Müller et al. 1991; Janoff et al., 1994).

Since CD4\textsuperscript{+} T helper (T\textsubscript{H}) cells are essential to both induce and maintain appropriate IgA response in mucosa-associated tissue and since HIV-1 infection causes a loss of mucosal CD4\textsuperscript{+} lymphocytes (McGhee et al., 1989), it is conceivable that the number of CD4\textsuperscript{+} lymphocytes and subsequently IgA-bearing plasma cells within the cervical/ vaginal mucosa of HIV-1 infected women may be reduced. Overall, these findings are in keeping with the fact that female cervical/vaginal mucosa belongs to the mucosal immune system, with a predominance of IgA-producing cells and the IgA\textsubscript{2} plasma cells being more numerous than the IgA\textsubscript{1} plasma cells within vaginal mucosa (55% versus 45%) (Kutteh et al., 1994). Local antibodies in female genital tract could therefore play an efficient role in neutralising HIV-1 virus and limiting the virus infectivity on normal mucosa.

1.3.3.2 Mucosal immune response to HIV-1 in the male genital tract

Heterosexual intercourse is the major route of infection worldwide (Overbaugh et al., 1999). HIV-1 transmission from men to women has been well documented, whereas
studies for female-to-male transmission remain limited. Similar to the immune response pattern to HIV-1 in female genital tract secretions, both IgG and IgA antibodies to HIV-1 were detected in semen samples from healthy male HIV-1 seropositive individuals and male patients with AIDS-related complex (ARC) or with AIDS. 73% of these semen samples contained IgA antibodies to HIV-1 env glycoprotein gp160, although surprisingly, only 9% semen samples possessed IgA antibodies to gp120. Since HIV-1 env glycoproteins are the most external antigens, IgA antibodies to gp160 may significantly inhibit transmission of HIV-1 having been implicated in the neutralisation process (Bélec et al., 1989a; O'Shea et al., 1990; Wolff et al., 1992).

1.3.3.3 Mucosal immune response to HIV-1 in urine and other body fluids
Investigations into the mucosal immunity to HIV using urine samples from HIV-1 seropositive or exposed seronegative individuals are very limited. Most research on urine samples from HIV-1 infected subjects has been focused on the development of methodology for diagnosis of HIV-1 infection to replace the use of serum. The presence of antibodies to HIV-1 antigens p17, p24, p33, gp41, p51, p55, p61, gp120, and gp160 has been reported in unconcentrated and concentrated urine samples of HIV-1 seropositive individuals using ELISA kits and Western blot assay, the highest prevalence being in antibodies to HIV-1 gp120 and gp160 (Cao et al., 1989). The titre of the antibodies to HIV-1 in the urine specimens was parallel with the titre of antibodies to HIV-1 in the corresponding serum. IgA was also found in some urines of HIV-1 seropositive individuals by immunodiffusion analysis; IgM was not detected. IgG antibodies to HIV-1 (IIIB) gp160 were also found in the urine of children of HIV-1 infected women and it has been suggested that this could be an early indicator of vertically transmitted infection (Bauer et al., 1992). Constantine et al. (1994) reported that all tested urine samples in their study consistently exhibited reactivity to gp160. In common with semen (Wolff et al., 1992) and parotid saliva (Sun et al., 1990), many urine samples showed no, or very weak reactivity to p24, in contrast to the generally strong p24 reactivity found in serum samples. It is possible that antibodies to p24 may be either complexed with viral antigen and may not pass through the kidney glomerulus, or may be sequestered by reacting with cross-reactive antigens in the reproductive tract.
Testing antibodies to HIV in urine samples using enzyme immunoassay was more sensitive than conventional methods (Hashida et al., 1993, 1994a, 1994; Sterne 1993; Urnovitz et al., 1993; Conell et al., 1990; 1993). These results suggest that such assays may be used either as a primary screen in populations or as an alternative test for individuals unwilling to or with difficulty in providing a serum sample.

HIV-1 neutralising activity in serum and concentrated urine from HIV-1 seropositive individuals was also reported (Cao et al., 1990). HIV-1 neutralisation activity was detected in 23 of 56 of the serum samples and in 19 of 56 of the urine samples. The neutralising activity in the urine concentrates was generally associated with high titres of neutralising antibody in the corresponding serum samples. However 11 urine samples contained HIV-1 neutralising antibodies but the corresponding sera lacked neutralising activity. On the other hand, one urine concentrate lacked neutralising antibody, but the corresponding serum had high titres of neutralising antibody. The reason for the discrepancy between the frequency of neutralising antibodies in paired concentrated urine and serum is unclear.

Antibodies to HIV-1 in urine from seropositive or exposed seronegative subjects can reflect the mucosal immune response as well as systemic immune response to HIV-1. In spite of this, little work has been done in this area and further research is required. Mazzoli et al. (1997) reported that HIV-1 specific IgA but not IgG was present in urine and vaginal wash samples from 10 (5 males and 5 females) out of 16 (63%) HIV-exposed seronegative individuals, whereas both IgA and IgG were observed in their HIV infected partners; antibodies were not demonstrable in the urine of low-risk controls. These data indicating that the mucosal immune response is triggered when exposed to HIV-1, raise the possibility of a protective role for mucosal IgA in resisting HIV transmission and infection.

In addition to genital secretions and urine samples from HIV-1 infected subjects, specific antibodies to HIV-1, predominantly of the IgA isotype, have also been detected in other body fluids, such as tears, milk, nasal washing, intestinal fluid, cervical fluid.
and saliva (O’Shea et al., 1990; Sun 1990; Thongcharoen et al., 1992; Janoff et al., 1994; Mestecky et al., 1994). All these findings indicate that following HIV-1 infection, a mucosal immune response induced at one site, results in antibody secretion in a variety of external secretions.

1.3.3.4 Local IgG antibodies to HIV-1 within the genital tract
HIV-1-specific antibodies have been detected in the both male and female genital tract (Archibald et al., 1987; Bélec et al., 1989a; Wolff et al., 1992; Lu et al., 1993; Bélec et al., 1994b). Bélec et al. (1995b) have investigated local synthesis of IgG antibodies to HIV-1 within the genital tract of HIV-1 infected, clinically asymptomatic women and men. The results showed that the mean specific activities of IgG to gp41 and p24 in cervical/vaginal secretions and in seminal fluids were about 33-fold and 16-fold higher respectively than that of the corresponding sera, and the mean specific activities of IgG to gp160 in genital secretions were correspondingly about 17-fold and 10-fold higher than that of the sera. IgG antibodies to HIV-1 have been detected in genital secretions from HIV-1 infected individuals, and appear to be largely synthesised in situ within the genital tract of both genders. The authors therefore suggested that a strong, specific local production of IgG antibodies to HIV-1 occurs within the genital tract of both HIV-1 infected men and women. This hypothesis can fit with the fact that HIV specific IgGs are found in cervical/vaginal secretions of HIV infected women and seminal fluid of HIV infected men (Bélec et al., 1989b; O’Shea et al., 1990; Wolff et al., 1992; Lu et al., 1993). This concept was further supported by the another study by the same authors (Bélec et al., 1995c). Paired sera and cervicovaginal secretions (CVS) from 30 women infected with HIV-1 were analyzed for IgG and IgA antibodies to HIV and for IgG, IgA, and human serum albumin. The results showed that cervicovaginal immunoglobulins were markedly increased, and IgG predominated in HIV-infected women compared with that of the healthy controls. Furthermore, IgG excretion by reference to albumin was increased 1.9-fold, whereas the IgA secretion tended to decrease, suggesting a possible enhanced local IgG synthesis.

This is however in contrast to other opinions (Wolff et al., 1992) who claimed that HIV-1 IgG antibodies in semen of infected men were transudated from serum primarily via
the prostate. Thus, analysis of the different abilities of serum and local IgG antibodies to block transmission of HIV-1 is particularly important in the understanding both the basic mucosal immunology of HIV infection and the design of an HIV vaccine.

1.3.3.5 Cellular mucosal immune response to HIV-1

Little is known concerning the induction of an effective cell mediated immune response in the mucosal immune system. It has been demonstrated that HIV infection leads to a reduction in the number of CD4\(^+\) cells in mucosal sites. A lower ratio of CD4\(^+\):CD8\(^+\) cells also occurs in the intestinal lamina propria of subjects with either lymphadenopathy syndrome or AIDS (Rodgers et al., 1986). Data concerning the number of Langerhans cell (LC) in mucous membranes of HIV-1 infected patients are conflicting. One early study showed that the number of LC in oral mucus of HIV infected patients had not been reduced (Becker et al., 1988). However, a study reported a statistically significant reduction of LC (CD1a\(^+\)) found in oral mucosal washings of HIV-1 seropositive patients compared with healthy controls (Spörri et al., 1994). These abnormalities at mucosal surfaces may explain the pathogenesis of opportunistic infection in the gastrointestinal, respiratory and genitourinary tracts of infected patients. Miller et al. (1996) demonstrated that, in SIV-infected female rhesus monkeys, the vaginal CD8\(^+\) intraepithelial lymphocyte (IEL) population is moderately increased and more widely distributed compared with uninfected monkeys, and persistently infected monkeys had greater numbers of CTL precursors than the ones which were infected for only two weeks. SIV specific CTL have also been found in the vaginal mucosa of monkeys (Lohman, 1995). The above results may suggest that this immune effector function might provide a first-line defence against sexually transmitted intracellular pathogens.

In conclusion, although considerable progress has been made recently in understanding the mucosal immune response to HIV infection and in developing mucosal HIV vaccines, and it is generally accepted that mucosal immunity might be important in protection against HIV transmission, the types of anti-HIV mucosal immune responses required are still not fully understood.
1.4 HIV-1 HEPS individuals (EUs)

During the past ten years, AIDS researchers have recognised that some individuals are persistently HIV seronegative despite documented exposure to HIV, defined as highly exposed, persistently seronegative (HEPS) individuals or exposed uninfected individuals (EUs).

1.4.1 Host factors in HEPS individuals

Population analyses have shown that individuals homozygous for the 32-bp deletion in the gene encoding CCR5, which has been identified as a major coreceptor for M-tropic HIV-1 entry (see section 1.1.6.2) are rarely present in HIV infected populations, indicating that mutant CCR5 confers resistance to HIV-1 infection \textit{in vivo} as well as \textit{in vitro} (Dean et al., 1996; Samson et al., 1996b). It has been demonstrated that the mutant alleles harboured a 32-bp deletion ($\Delta 32$) in CCR5 gene which lead to a premature stop codon and encoded for a nonfunctional protein.

In 1996, Liu et al. reported that homozygous defect in CCR5 gene was found in two HEPS individuals. The encoded protein is severely truncated and cannot be detected at the cell surface. Furthermore, this defect had no obvious phenotype in the affected individuals. These data indicate that a CCR5$\Delta 32$ allele present in the human population appears to protect homozygous individuals from sexual transmission of HIV-1. Subsequent studies supported this conclusion by demonstrating that people who are homozygous for the deleted allele ($\Delta 32$) are highly resistant to HIV-1 infection \textit{in vivo} (Dean et al., 1996; Huang et al., 1996; Samson et al., 1996b; Michael et al., 1997), and cells from such people are resistant to the infection by HIV-1 M-tropic strains \textit{in vitro} (Connor et al., 1996; Liu et al., 1996; Rana et al., 1997). The hypothesis, that people who carry one deleted and one normal allele (heterozygous) $\Delta 32$ genotype can be partially protected against HIV-1 infection, is suggested and demonstrated by a couple of studies (Samson et al., 1996b; Hoffman et al., 1997). Recently, it has been confirmed that the presence of two CCR5$\text{wt}/\Delta 32$ heterozygous genotypes among 139 HEPS individuals, and PBMCs from these two heterozygous individuals were also found to be less susceptible to HIV-1 M-tropic isolates infection \textit{in vitro} (Kokkotou et al., 1998).
This result supports a role for 32-bp CCR5 deletion in HIV-1 resistance. However, despite the substantial protection it affords, the CCR5 Δ32 still fails to account for most HEPS cases (Dean et al., 1996; Huang et al., 1996; Smith et al., 1997; Zimmerman et al., 1997).

1.4.2 Systemic immune response in HEPS population

HIV-1-specific immune responses in HEPS population have been detected at both the cellular and humoral level. Several studies have demonstrated the presence of an HIV specific T cell response, in the form of T cell proliferation, interleukin 2 (IL-2) production induced by HIV peptide, detection in vitro of HIV specific cytotoxic T lymphocytes by HIV peptide and generation in vitro of HIV specific CTLs in cohorts of these individuals (Shearer et al., 1996). It was also reported that NK cells play a role in resistance to HIV infection (Krowka, 1996). In addition high levels of neutrophils and CD8+ cells were found in repeatedly exposed seronegative homosexual men (Detels et al., 1994), suggesting a hypothesis that CD8+ cells may modulate the outcome of HIV-1 exposure. It has been demonstrated that the anti-HIV-1 activity from CD8+ lymphocytes in multiple high-risk sexually exposed HIV-1 seronegative individuals was greater that that of CD8+ lymphocytes from nonexposed controls and their purified CD4+ lymphocytes were relatively resistant to HIV-1 infection in vitro (Paxton et al., 1996). This relative resistance did not extend to T cell line adapted strains, but was associated with the activity of the C-C chemokines RANTES, MIP-1α and MIP-1β. Therefore the authors suggested that this relative resistance of CD4+ lymphocytes may block HIV-1 transmission and contribute to protection from HIV-1 in multiply exposed HIV-1 seronegative persons. It also reported that anti-CD4 antibodies specific for defined epitopes of the CD4 molecule were detected in 6 of 18 individuals who were persistently seronegative, despite exposure to HIV-1. These antibodies could block HIV-1 driven syncytia formation, and inhibit the ability of CD4 binding to specific anti-CD4 monoclonal antibodies, but did not interfere with the CD4-gp120 interaction. The result indicated that these autoantibodies to CD4 may play a protective role in HIV-1 EUs. The possible mechanism for the resistance against HIV infection in multiply exposed individuals who exhibit strong HIV specific T cell immune activity, suggested by
Shearer and Clerici (1996), is acquired immunity through natural immunisation. Accidentally acquired immunity may therefore protect from HIV infection.

1.4.3 Mucosal immune response in HEPS population
Another clue to the explanation of natural resistance against HIV in HEPS individuals comes from studies in rhesus macaques and chimpanzees of resistance to low doses of HIV or SIV given intrarectally or intravaginally, indicated that there may be local cellular mucosal immune responses capable of protecting against low dose mucosal HIV or SIV challenges (Haynes et al., 1996). Recently, Mazzoli and his colleagues (1997) examined 16 Italian couples discordant for HIV infection, in which one member was HEPS. It was found that HIV-specific IgA but not IgG was present in urine and vaginal wash samples from HEPS individuals, whereas both IgA and IgG were observed in their HIV seropositive partners, Also, Env peptide-stimulated PBMCs from HEPS individuals produced more IL-2 and less IL-10 compared to those of HIV-infected individuals. These data demonstrated that a compartmentalised immune response to HIV is possible in humans, and mucosal IgA in the genital tract could offer a potent first-line defence against HIV sexual transmission. Recently, Kaul et al. (1998) reported that HIV-1 specific IgA was detected in the genital tract of 16 out of 21 (76%) HIV-1 EUs, 5 out of 19 (26%) infected women, and 3 out of 28 (11%) lower risk women (P<0.0001). T-helper lymphocyte response did not correlate with the presence or titre of HIV-1 specific mucosal IgA. These data suggest a role for mucosal HIV-1 specific response in HIV-1 resistance, independent of a systemic cellular response.

Since the development of vaccine which induces a specific mucosal immune response in genitourinary tract has far reaching implications for the prevention of HIV transmission, the need for determining the specificity of protective mucosal antibodies and understanding mucosal immunity status in multiply exposed HIV seronegative individuals is vital for developing HIV mucosal vaccines.

1.5 Vaccine development
The majority of HIV-seropositive individuals acquired their infection via mucosal
surfaces (Miller et al., 1992c; Phillips et al., 1994), which strongly implies that HIV-specific sIgA with neutralising activity in secretions on the mucosal surface is critical for control of infection in individuals exposed to HIV. Therefore, the ability of candidate HIV vaccines to induce mucosal responses is likely to be an essential feature of an effective HIV vaccine, not only because of the importance of mucosal transmission of HIV, but also because of the large pool of activated T cells in the gastrointestinal tract which are susceptible to HIV infection. Systemic immunisations are unable to efficiently induce immune responses in mucosal sites because of compartmentalisation of systemic and mucosal immune system, prompting an intensive effort to develop novel vaccine approaches able to induce a mucosal immune response. There is evidence showing that sIgA not only prevents viral adhesion to the mucosal receptors but also neutralises the virus (Mazanec et al., 1993).

1.5.1 Mucosal HIV-1 vaccines development

Mucosal HIV vaccines designed to be given by mucosal routes (oral, rectal, intranasal and intravaginal) to induce both mucosal and systemic immune response to HIV antigens are under development, although at present most are still restricted to animal models.

Oral vaccination is useful not only because of the generation of mucosal immunity but also because of the ease of administration and applicability for mass vaccination. Recent evidence demonstrates that oral immunisation can elicit sIgA. It has been reported that when mice and rabbits were orally immunised with HIV-immunsomes (gp160-coated liposomes) followed by parenteral immunisation with a low dose of the same antigens, high titres of serum IgA, IgM and IgG against HIV gp160, and sIgA in the saliva were detected (Thibodeau et al., 1992). These data suggest that oral immunisation can effectively induce a mucosal immune response upon re-exposure to antigen. The concept has been supported by other studies (Lehner et al., 1992; Mestecky et al., 1994; Morrow et al., 1994; Bond et al., 1995). There are recent studies showing that oral immunisation can induce systemic and mucosal immune responses to HIV (Bukawa et al., 1995; Wu et al., 1997). When mice were immunised orally with a multiple macromolecular peptide
vaccine candidate (VC1), composed of peptides from the V3 region of four subtypes of HIV-1, one CD4 binding site and one Gag region linked to cholera toxin, it stimulated a specific systemic and mucosal immune response to HIV. Strong and prolonged production of serum IgG as well as faecal and serum IgA antibodies were detected (Bukawa et al., 1995). The secretory component titre of HIV-1 specific faecal IgA antibody was $1:2^{11}$, which strongly implies that the HIV specific faecal IgA to VC1 vaccine was mainly in the secretory form. Neutralisation tests showed that this HIV specific faecal IgA was capable of neutralising HIV-1 IIIB, HIV-1SF2 and HIV-1MN. Moreover, the faecal IgA antibody response was greater that that of serum IgG. This result indicates that env-peptide delivered orally in combination with cholera toxin adjuvant can effectively induce HIV-specific immune response in mucosal tissues which may provide protection to mucosal surfaces against HIV-1 transmission.

The intranasal route of immunisation has been reported to induce mucosal immune responses (Muster et al., 1995; VanCott et al., 1998; Staats et al., 1996; 1997) as well as cellular immune responses (Morris et al., 2000) against the immunising antigen. Intranasal immunisations with oligomeric HIV-1 gp160 vaccine formulated with several adjuvants can elicit serum and vaginal IgG/IgA titres in mice (VanCott et al., 1998) and long-lasting sIgA was detected in the genital and intestinal tracts in mice after intranasal immunisation with chimeric virus that can express gp41 of HIV-1 (Muster et al., 1995). In 1996, Staats et al. reported that intranasal immunisation with hybrid HIV-1 peptide vaccine candidate T1SP10MN(A) and cholera toxin (CT) induced long-lived systemic and mucosal antibody responses as well as cell-mediated immunity to HIV. It has been further demonstrated that the intranasal route of immunisation is superior to gastric, vaginal and rectal routes for induction of specific anti-HIV immune responses by the same author (Staats et al., 1997). However, recent evidence demonstrates that rectal and vaginal immunisation with a multiple macromolecular peptide vaccine candidate VC1, mixed with cholera toxin strongly induces IgA antibody in faecal extract solution and vaginal washing (Kato et al., 2000).

A great deal of attention has focused on HIV DNA vaccines (Okuda et al., 1995; Askura
et al., 1997; Okada et al., 1997; Barouch et al., 1998, Kaneko et al., 2000). Intranasal immunisation with a DNA vaccine, which is constructed with the cytomegalovirus (CMV) promotor linked to env gp160 and rev genes, has been shown to induce the production of mucosal IgA in faeces and vaginal washings in mice. Similarly, oral immunisation with HIV env-encoded plasmid DNA encapsulated in microparticles induced HIV env-specific immune response in mice which elicited a significant level of protection against mucosal challenge with the vPE16 recombinant vaccinia virus (expressing the full-length HIV-1 IIIB gp160) (Kaneko et al., 2000). This result is consistent with the observation based on intrarectal immunisation with HIV env-specific peptide and cholera toxin (Belyakov et al., 1998a, 1998b). These data indicate that oral vaccination may represent a very effective approach in providing protection against HIV-1 infection acquired via mucosal surfaces.

Considerable progress has also been made recently in primate models for development of mucosal vaccines against HIV. It has been demonstrated that when rhesus macaques were sequentially vaginally, rectally and orally immunised with hybrid particles that carry the SIV gag protein p27 (SIVp27:Ty-VLP) prototype vaccine linked to the cholera toxin B (CTB) epitope followed by a oral boost, specific sIgA and IgG antibodies were detected in the urine, urethra washings, seminal fluid and vaginal secretions (Lehner et al., 1992). Use of recombinant adenovirus containing SIV envelope gene by oral and intranasal immunisation successfully elicited anti-SIV humoral, cellular and mucosal immune response in rhesus macaques (Buge et al., 1997). A study significantly extends these observations, by taking advantage of the lymphatic drainage from the rectum (Lehner et al., 1996). The iliac lymph nodes which drain the genitorectal mucosa were selected as the target of vaccine injection for the first time. It was found that cells secreting IgA antibody to p27, CD8-suppressor factor and the chemokines RANTES and MIP-1β significantly increased in the iliac lymph nodes and total protection was found in four out of seven macaques after rectal SIV challenge. These results have raised the possibility that application of HIV antigens to mucosal surfaces may be a fruitful approach of vaccination against AIDS.
Clinical trials of HIV-1 vaccines are being carried out in human volunteers (Kovacs *et al.*, 1993; Walker *et al.*, 1994; Kahn *et al.*, 1994; Mascola *et al.*, 1996; Keefer *et al.*, 1997; Nitayaphan *et al.*, 2000; Boyer *et al.*, 2000; Fazal *et al.*, 2000; Nilsson *et al.*, 2001) aimed at determining the humoral, cellular as well as mucosal immune responses to the vaccine product and the safety of the candidate HIV vaccines (Funkhouser *et al.*, 1993; Perry *et al.*, 1993; AIDS Vaccine Evaluation Group, 2001). Intramuscular immunisation with two recombinant gp160 candidate vaccines failed to induce detectable sIgA in the saliva and nasal washing samples although IgG was detected in nasal washing and sera. It has been suggested that vaccines inoculated via the parenteral route are unlikely to induce protective mucosal immunity. This is due to the different origin of precursor B and T cell which populate the systemic and mucosal immune compartment, a fact which should be considered when a mucosal vaccine is developed. It is generally accepted that formulation with novel adjuvants or antigen delivery systems could be the most likely to induce HIV-specific mucosal immunity (Mestecky *et al.*, 1994; McCormack *et al.*, 2000).

Recombinant envelope protein-based subunit vaccines have been extensively tested in phase I/II human clinical trials (Klein, 2001, Mascola and Nabel, 2001). Human trials of a recombinant subunit gp120 antigen (derived from HIV-1SF2 strain) have shown to be well tolerated, and antibodies with neutralising activity against HIV-1 can be generated and boosted, in immunised seronegative volunteers (Kahn *et al.*, 1994; Mascola *et al.*, 1996; Keefer *et al.*, 1997; Nitayaphan *et al.*, 2000). A bivalent subunit vaccines comprising recombinant gp120 proteins from HIV-1MN and a primary HIV-1 isolate are currently being tested in phase III trial since 1998 (in North America) and 1999 (in Thailand). Efficacy results of these phase III trials are expected to become available 2-3 years later (Migasena *et al.*, 2000; Mulligan *et al.*, 1999).

Formulation HIV-1 subunit vaccines with a combination of adjuvants to induce potent immune responses in immunised subjects was also investigated. HIV-1 seronegative volunteers received 200 µg recombinant monomeric HIV-1 rgp120W61D derived from a dual co-receptor tropism isolate HIV-1ACH320 in 50 µg 3D-MPL (3-deacylated
monophosphoryl lipid A) and 50 μg QS-21, a novel adjuvant QS-21 derived from the soapbark tree *Quillaja saponaria*, together with a novel oil and water emulsion (SB62) at 0, 4 and 28 weeks, induced antibody titres as high as those seen in HIV-1 infection, albeit the quality of the antibodies remained different in that there was no evidence of primary isolate neutralisation. Cellular immunity was also enhanced in immunised subjects in terms of lymphoproliferative responses, but HIV-1 specific CD8⁺ CTL was not demonstrated (McCormack *et al.*, 2000). Recently, a phase II study of two HIV-1 gp120 vaccines induced neutralising antibodies and envelope-specific lymphoproliferation in populations at risk for acquiring HIV-1 infection (McElrath *et al.*, 2000). Studies showed that envelope subunit vaccines induced neutralising antibodies that were generally type-specific and failed to neutralise primary HIV-1 isolates (Graham *et al.*, 1996, McCormack *et al.*, 2000; Nitayaphan *et al.*, 2000). Furthermore, phase I/II clinical trials have shown that envelope subunit vaccine rarely induce a CD8⁺ CTL response (Graham *et al.*, 1996; Locher *et al.*, 1999; McCormack *et al.*, 2000; Nitayaphan *et al.*, 2000), but lymphoproliferative responses to intact HIV-1 glycoproteins have been readily detected (McCormack *et al.*, 2000; Schooley *et al.*, 2000; McElrath *et al.*, 2000). Of note, some participants in Phase I/II trials who were immunised with HIV-1 gp120 vaccine had significant neutralising antibodies against vaccine isolates but still became infected during or following immunisation (Berman *et al.*, 1997; Connor *et al.*, 1998, Locher *et al.*, 1999). This feature remains a major obstacle of HIV vaccine development.

Live recombinant vaccinia and attenuated canarypox vectors (ALVAC) based HIV-1 vaccines evaluated in human clinical phase I/II trials have demonstrated safety profiles (Graham *et al.*, 1993; Pialoux *et al.*, 1995; Clements-Mann *et al.*, 1998; Belshe *et al.*, 2001). An ALVAC based HIV-1 vaccine encoding multiple HIV-1 gp120, p55 and protease (vCP205) has been initially assessed in a phase II trial. 435 volunteers including people at both higher and lower risk for HIV infection received vCP205 with/without gp120 boosting. More (94%) subjects given vCP205 plus gp120 than those (56%) who received vCP205 alone developed neutralising antibody to HIV-1MN. CD8⁺ CTL were detected in 33% of volunteers given vCP205, with or without gp120 (Belshe
et al., 2001). These data suggested that weak neutralising antibody responses induced by live recombinant viral vector, as has been demonstrated (Graham et al., 1992), can be heightened by boosting with recombinant envelope protein. In a recent study, a vaccination regimen consisting of a ALVAC based vector expressing HIV-1 Env, Gag, and Pol, in combination with a recombinant gp120 subunit protein resulted in neutralising antibodies in 91% of vaccinees and CD8+ CTL responses in 62% of subjects. Moreover, boosting with HIV-1 rgp120 did not increase the CTL responses to HIV-1 envelope proteins, but did enhance the magnitude and frequency of neutralising antibodies to HIV-1MN (AIDS Vaccine Evaluation Group 2001). Similar results were also demonstrated in other clinical trials including priming with HIV-1 gp160-vacinia and boosting with HIV-1 rgp120 (Corey et al., 1998); priming with ALVAC based vector expressing HIV-1 gp120, gp41, gag and protease followed by boosting with rgp120 in HIV-1 seronegative volunteers (Evans et al., 1999), and priming with ALVAC-HIV-1 gp160 and boosting with rgp120 in HIV-1-uninfected adults (Clements-Mann et al., 1998). More frequent and longer-lived (6 months after the last immunisation) HIV-1 CD8+ CTLs and/or neutralising antibodies were induced by priming with live recombinant viral vector, followed by boosting with recombinant HIV-1 envelope proteins than immunising with live recombinant vector or recombinant HIV-1 envelope protein alone (Ferrari et al., 1997; Verrier et al., 2000). Therefore, inducing protective humoral and cellular immune responses by HIV-1 vaccines might be achieved by formulating live recombinant viral vector based HIV-1 vaccines consisting of multiple HIV-1 genes with rgp120 booster regimen.

DNA vaccines encoding HIV-1 proteins have been tested in Phase I trials to evaluate the safety and immunogenicity as HIV-1 vaccine candidates in HIV-1 uninfected healthy volunteers. An HIV-1 env/rev DNA vaccine was administered intramuscularly to HIV-1 seronegative persons at a dose of 100 or 300 μg at 0, 4, 8, and 24 weeks. Subjects who received the 300 μg dose exhibited antigen-specific T cell proliferative responses and antigen-specific production of both INF-γ and β-chemokines. This study supports the idea that HIV-1 DNA vaccines can stimulate immune responses in naive volunteers (Boyer et al., 2000). Notably, a phase I trial of the first DNA vaccine specifically
designed to combat the clade A HIV-1 virus, which is the most prevalent strain in many parts of Africa, was officially launched in Oxford in 2000 and later in Kenya in 2001. The trial was conducted in low risk healthy volunteers and is to evaluate the safety and immunogenicity of the DNA vaccine. The results are expected to be ready in about two years (Fazal et al., 2000).

### 1.5.2 Edible vaccine development

By using recombinant DNA technology, a subunit vaccine is made by isolating genes from human pathogens (viruses and bacteria) and transferring the DNA into a transgenic (containing the transferred gene) host, which produces a protein (subunit of pathogen) that contains the antigenic fingerprint of the disease-causing agent but does not itself cause disease. These subunit vaccines are purified from the transgenic hosts and injected as vaccines to induce immunity against a specific disease.

Transgenic plants comprise a new production system that does not require vaccine purification or injection and provides the possibility that a fresh or pureed fruit such as banana or tomato could replace the needle as a means of vaccinating children and adults against deadly diseases. The vaccine-producing transgenic plant is created by placing the gene encoding the antigenic subunit under the control of plant-specific DNA regulatory sequences, which is then induced to integrate into the nuclear chromosome of the plant cell. Then, the antigen protein is produced and accumulated to a significant level in transgenic plants (Figure 1.13).

In 1992, Mason et al. first reported that the gene-encoding HBsAg of hepatitis virus B was successfully expressed in transgenic tobacco and that the HBsAg produced in transgenic plants is antigenically and physically similar to the HBsAg particles derived from human serum and recombinant yeast, which are currently used as vaccines. This findings led to the hypothesis that transgenic plants may offer a new way of generating vaccines, thereby allowing a very inexpensive means of oral immunisation simply through consumption of the edible vaccine. This concept is supported by the subsequent demonstration that the HBsAg expressed by transgenic tobacco was in a form that was
Figure 1.13 Schematic protocol for production of candidate vaccines in plants (Modified from Richter et al., 1996).
able to stimulate a B- and T-cell immune response in immunised mice (Mason et al., 1995). In 1996, the capsid protein of Norwalk virus, which causes epidemic acute gastroenteritis in humans, was expressed in genetically engineered tobacco and potato plants. When mice were either fed with the transgenic tubers which expressed recombinant Norwalk virus-like particles (rNV), or given extracts of tobacco leaf expressing rNV by gavage, serum IgG and secretory IgA specific for rNV were detected, indicating the potential usefulness of this subunit antigen as edible vaccine (Mason et al., 1996). In addition to virus, bacterial components have also been reported to have been successfully expressed in transgenic plants (Haq et al., 1995).

To investigate the possibility of transgenic plant derived oral vaccine which could be developed to combat enterotoxic *Echerichia coli* (*E. coli*) and cholera, which cause severe diarrhea and sometimes death, transgenic tobacco and potato plants expressing the binding subunit of *E. coli* heat-labile enterotoxin (LT-B), which is a highly active oral immunogen, were produced (Haq et al., 1995). Although the levels of expression in these plants were low, the proteins were immunogenic in mice fed with the raw potato tubers. Mice fed the plant derived antigen had higher levels of anti-LT-B serum IgG (humoral immune responses) and faecal IgA (mucosal immune responses) when compared with mice gavaged with authentic bacterial LT-B. Furthermore, the antibodies produced against the tobacco-derived LT-B were able to neutralise LT activity, indicating the potential protective value of the immune response. Recently, the LT-B produced in transgenic potatoes was found to be in an appropriate form for immunogenicity even after cooking (Arakawa et al., 1998). This advance may have the potential benefit for human beings as humans do not normally consume raw potatoes. These data support the concept that a food source containing a foreign antigen can result in oral immunisation (Haq et al., 1995; Mason et al., 1998). May et al. (1995) developed an *Agrobacterium*-mediated plant transformation system for the generation of transgenic banana system, which gives us encouragement that banana could be served as edible food vaccine to protect humans from infectious disease.

Based upon animal trials, the US food and drug administration approved human clinical
testing of raw potatoes containing LT-B in 1997. The results showed that edible vaccines are feasible for humans. Volunteers who consumed raw potatoes developed LT-B-specific IgA; the amplitude of the responses was comparable to a challenge with $10^6$ virulent enterotoxigenic \textit{E. coli} (an amount sufficient to induce severe diarrhea) (Tacket \textit{et al}., 1998). A companion paper reports that secretory antibodies produced in transgenic plants to passively immunise human volunteers prevent enterotoxigenic \textit{E. coli} infection (Ma \textit{et al}., 1998).

Transgenic plants as a vaccine production and delivery system to protect animals from infectious diseases has also been explored. A study showed that protection against a viral disease by immunisation with a transgenic plant expressed antigen was achieved (Carrillo \textit{et al}., 1998). The structural protein VP1 of foot-and-mouth disease virus (FMDV), which has frequently been shown to contain critical epitopes, has been expressed in different vectors and shown to induce virus-neutralising antibodies and protection in experimental and natural host. The transformed plants (\textit{Arabidopsis thaliana}) expressing VP1 were produced (Carrillo \textit{et al}., 1998). Mice immunised with leaf plant extracts elicited specific antibody response to synthetic peptides, FMDV VP1 and intact FMDV particles. Additionally, all of the immunised mice were protected against the challenge with virulent FMDV. Recently, the major structural protein VP60 of rabbit hemorrhagic disease virus (RHDV) has been successfully expressed in transgenic potato plants (Castanon \textit{et al}., 1999). The rabbits immunised with leaf extracts showed high level of anti-VP60 antibody titres and were fully protected against the hemorrhagic disease. Very recently the specific immune response to porcine transmissible gastroenteritis virus (TGEV) was achieved in pigs by immunisation with recombinant TGEV spike protein expressed in transgenic tobacco plants (Tuboly \textit{et al}., 2000).

There is likely to be immunological concerns about oral tolerance. In theory, too much orally administrated proteins delivered over an extended period might produce immune tolerance. But whether oral tolerance would develop if the amount of edible vaccine used was appropriate remains unclear. Because orally administrated proteins
theoretically need to overcome the oral unresponsiveness of the alimentary tract, which is the mucosal immune system's selective ability to not react immunologically against antigens of food and intestinal microorganisms even though it responds vigorously to pathogens (Cruse and Lewis, 2000). So far all the studies on edible vaccine have been reported to induce immune response against the immunising antigen in animals (Haq et al., 1995; Thanvala et al., 1995; Arakawa et al., 1998; Carrillo et al., 1998; Ma et al., 1998; Mason et al., 1995; 1998; Tracket et al., 1998; Castanon et al., 1999; Tuboly et al., 2000).

Taken together, the concept of edible vaccine is only a few years old but progress has been fast. Research on edible plant vaccines has moved from theory to proof-of-principle. Plant based oral vaccines may offer a new approach to vaccination strategies, in particular, in cases where a local immune response is crucial in the prevention of infections. The transgenic edible plant vaccines have shown to be a potential new generation of novel, encouraging and prospective vaccine production and delivery systems with its distinguishing characteristics including inexpensive and local production, unnecessary preparation or purification and easy transportation and storage.

1.6 Aims and work presented in this thesis
Since antibodies to HIV-1 in urine from HIV-1 seropositive or exposed seronegative subjects can reflect the mucosal immune response in the genital tract as well as the systematic immune response to HIV-1, my work in this thesis was aimed to firstly compare the specificity of anti-HIV antibodies of different isotypes present in paired urine and serum of HIV-1 seropositive and HEPS individuals, investigate the possibility of local antibody in urine samples from highly exposed, HIV-1 seronegative individuals and its role in mediating mucosal protection against HIV-1, and evaluate the theory that exposed seronegative individuals may develop a local anti-HIV antibody response, detectable in urine. Secondly it was to develop a novel method for the generation of Env protein of HIV-1 in transgenic plants to investigate the feasibility for creation of inexpensive and safe HIV "edible vaccine".
In order to investigate the local antibodies to HIV-1 in both HIV-1 seropositive and exposed HIV-1 seronegative individuals, the IgG, IgA and sIgA antibodies to different HIV-1 viral proteins in 3 paired urine and serum samples, and the corresponding 2 swab wash samples from HIV-1 seropositive individuals in Edinburgh and Uganda, and in 9 paired urine and serum samples from exposed HIV-1 seronegative individuals from Uganda was studied by WB. The antibody pattern in paired urine and serum or the corresponding swab wash samples from HIV-1 seropositive individuals, and in paired urine and serum samples from exposed HIV-1 seronegative individuals were, respectively, compared and the local immune response to HIV-1 in both HIV-1 seropositive and exposed HIV-1 seronegative individuals were analysed.

To investigate the feasibility of expressing HIV-1 gp160 protein in transgenic plants, both plant leaf and tuber expression vectors containing HIV-1 gp160 gene were constructed. To achieve this vector construction, the full-length of HIV-1 gp160 was amplified from DNA template by PCR and cloned into plant plasmids pIBT210.1 containing the plant leaf specific promoter 35S and pIBT240.1 containing the plant tuber specific promoter patatin, respectively. DNA fragments containing the required HIV-1 gp160 gene and the plant leaf specific promoter 35S or the plant tuber specific promoter patatin were then cut from the recombinant plasmids pIBT210.1 or pIBT240.1 with the appropriate enzymes and cloned into plant binary vector pGPTV-KAN. To obtain the transgenic plants, the desired recombinant plant expression vectors were transformed to Agrobacterium tumefaciens strain LBA4404 and the HIV-1 gp160 gene then transferred to potato and tobacco as well as to NT-1 plant cells by Agrobacterium-mediated plant transformation system. The transformants were cultured and selected on selection medium containing the appropriate amount of kanamycin. The transgenic tobacco plantlets, potato microtubers and NT-1 transformants were generated following the appropriate plant tissue culture and transformant selecting procedure. The integration of HIV-1 gp160 gene in the transgenic tobacco genome was tested by PCR. The transcription of HIV-1 gp160 gene in transgenic tobacco plantlets was analysed by RT-PCR. Expressions of HIV-1 gp160 protein in transgenic tabaco plantlet leaves, potato microtubers and NT-1 cell transformants were investigated by gp160/120 ELISA.
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2.1 MATERIALS

2.1.1 Chemicals
All chemicals were supplied by SIGMA Chemicals Co., England unless otherwise stated.

2.1.2 Specimens
Paired serum and urine samples from HIV-1 seropositive and seronegative individuals in Edinburgh and Uganda were collected and aliquoted. These samples containing 0.1% (w/v) sodium azide were then stored at —70°C until used. Prior to HIV-1 antibody analysis by Western blot, all samples used were inactivated at 56°C for 1 hour. The urine samples were then centrifuged at 500 x g for 15 minutes at room temperature. The urine supernatants were concentrated 100-fold using a Minicon B15 (Amicon) or later 200-fold with Miniplus™ concentrator (Amicon) for use in subsequent Western blots.

Vaginal swabs from HIV-1 seropositive women in Edinburgh were placed in 10 ml of PBS containing 0.1% sodium azide after swabbing the vaginas of the patients. The samples were aliquoted after collecting and stored at —70°C until used.

2.2 METHODS

2.2.1 Preparation of total HIV viral proteins

2.2.1.1 Cells for propagation of HIV
Both H9 and C8166 cells are non-adherent cells.

2.2.1.1a H9 cell line
H9 cells, which is human cutaneous T-cell lymphoma (Mann et al., 1989) clone derived from HUT78 cell line (human cutaneous T-cell lymphoma from peripheral blood of a patient with Sezary Syndrome) (Gazdar et al., 1980), are less susceptible to syncytium formation than C88166 cells (see 2.2.1.1b), but good for virus replication. This cell line was obtained from AIDS Reagent Project (kindly donated by Dr A Doyle), and cultures
were grown in RPMI (Hyclone) supplemented with 2 mM L-glutamine, 100 u/ml penicillin and 10 µg/ml streptomycin and 10 % fetal calf serum (FCS) (this medium will be referred to as RSA from now on) in 25 or 75 cm² sterile tissue culture flasks (Costar, High Wycombe, UK) at 37°C with 5 % CO₂ until approximately 5 x 10⁵ cells/ml, and then were split in 1: 10 for maintenance.

2.2.1.1b C8166 cell line
C8166 cells which is human T-lymphoblastoid cell line, carries a defective HTLV-1 genome. C8166 cells are very susceptible to syncytia formation and used as indicators of virus fusion and replication (Salahuddin et al., 1983; Lee et al., 1984). This cell line was obtained from AIDS Reagent Project (kindly donated by Dr P Clapham and Dr G Farrar). The C8166 cell line was cultured in RPMI (Hyclone) supplemented with 2 mM L-glutamine, 100 u/ml penicillin and 10 µg/ml streptomycin and 10 % fetal calf serum (RSA) in 25 or 75 cm² sterile tissue culture flasks (Costar, High Wycombe, UK) at 37°C with 5 % CO₂ and subcultured every 3-4 days for maintenance.

2.2.1.2 Production of virus stocks
HIV-1 IIIB is a syncytium-inducing (SI) strain and was obtained from the AIDS Reagent Project (kindly donated by Dr. R. Gallo and Dr. M. Popovic). The virus was passaged in the H9 culture maintained in RSA medium. 3 ml of H9 cells at a concentration of 7.5 x 10⁶ cells/ml were infected by adding 1ml of HIV supernatant (TCID₅₀ 4.25 x 10⁶) cultured in 6 ml of RSA medium and incubated at 37°C with 5 % CO₂ for 4-5 days. 1ml of infected cells were then cocultivated with C8166 cells at a concentration of 2.5 x 10⁶ cells/ml for overnight. The well-established infection was indicated by induction of numerous syncytia. Four volumes of uninfected H9 cells at a concentration of 5 x 10⁵ cells/ml were then added to one volume of the infected H9 cells, the mixture was cultured for 3-4 days. After titration, the virus containing supernatants were stored at – 70°C until used.

2.2.1.3 Titration of virus
C8166 cells were prepared at 4 x 10⁵ cells per ml in RSA medium. 133 µl of RSA
medium was added to the first six wells of a 96 well U-bottomed microplate (only central 60 wells of the plate used) and 150 μl to the rest, 67 μl of virus containing supernatants was added to the first six wells (1:3 dilution). 50 μl of this was transferred to then next round of wells and the processes repeated, until the final row where 50 μl was discarded. 50 μl of C8166 cell suspension was added (2 x 10^4 cells per well) making 1:4 dilution of the viral stock solution in row 1, 1:16 in row 2, etc. The plate was then incubated at 37°C, 5% CO₂ for 4 days. On day 4, 100 μl medium was removed and replaced with fresh RSA medium. On day 8, infected wells were assessed for the presence of syncytia. TCID₅₀/ml was calculated by Reed and Meunch's method (Dulbecco, 1988).

2.2.1.4 Preparation of viral lysates from culture supernatants
HIV-1IIIB infected H9 culture fluids as described above were centrifuged at 1500rpm for 10 minutes. The supernatants were filtered through a 0.45μm membrane microfilter (Sartorius) and inactivated at 56°C for 3 hours. They were then concentrated by passage through a 20% sucrose cushion in a SH-80 rotor (Dupont) at 20,000 rpm for 2.5 hours. The pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X 100, 1% Sodium deoxycholate, 0.1% sodium dodecyl sulphate) in a volume that was equal to 1 in 200 original volume, and then aliquoted and stored at -70°C until use. The antigen preparation was assessed by SDS-PAGE.

2.2.1.5 Preparation of HIV-1 IIIB lysates from infected cells
28 ml of the harvested HIV-1 IIIB infected H9 cell culture fluids were centrifuged at 1500rpm for 10 minutes. The pellet was washed twice with sterile PBS (Oxoid Limited, England) and then resuspended in lysing buffer (50 mM Tris; 5 mM EDTA; 150 mM NaCl and 0.5% (w/v) CHAPS which was added after the other reagents have dissolved) at a concentration of 1 x 10^8 cells/ml. To this was then added 1/10 volume of above lysing buffer and the mixture vortexed for 30 seconds. It was then placed on ice for 25 minutes prior to centrifugation at 1500rpm for 5 minutes at 4°C. The supernatant was collected, aliquoted and stored at -70°C for future use.
2.2.2 Immunoassays

2.2.2.1 Western blot

2.2.2.1a Sodium-dodecyl sulphate-polyacrylamide gel electrophoresis of proteins (SDS-PAGE)

The Hoefer Mighty Small SE 250/280 minigel apparatus (Hoefer Scientific Instruments, California, USA) and the Gradient Former model #GM-040 (C.B.S. Scientific Co., California, USA) were used. The HIV-1 virus preparations or recombinant proteins (AIDS Reagent Project, MRC) were separated by SDS polyacrylamide gel electrophoresis using a Tris-glycine buffer (50 mM Tris and 384 mM glycine) containing 0.1%SDS. The resolving gels consisted of 5%-20% linear gradient acrylamide gel made from stock solution (30% acrylamide/1.034% bis acrylamide, ScotLab) in 375 mM Tris-HCl buffer (pH 8.7) containing 0.1% SDS. 25 µl of 10% ammonium persulfate and 2.6 µl of TEMED (N,N,N',N'-tetramethylethylenediamine) were added to each of 8 ml of 5% light gradient gel solution and 20% heavy gradient gel solution. The filling flow from the gradient maker to gel sandwich was adjusted to 2 ml per minute. 1 ml of Methanol was applied to the top of the gel gently, and the gel allowed to polymerise at room temperature for 1 hour. Then the resolving gel was overlaid with 3% acrylamide (stacking gel) in 125 mM Tris-HCl buffer (pH 6.8) containing 0.1% SDS. Prior to loading, samples were mixed with an equal volume of 2 X sample buffer (125 mM Tris-HCl; 20% (v/v) glycerol; 4% SDS; 2% (v/v) 2-mercaptoethanol and 0.001% bromophenol blue) and boiled for 3 min. 20 µl of the mixture was loaded onto a well of an analytical gel. Gels were run at 20 mA of constant current until the bromophenol blue tracking dye entered the resolving gel and then increased the current to 30 mA until the bromophenol blue tracking dye reached the bottom of the resolving gel (1 hour). The gel was used for the subsequent immunoblotting or staining with Coomassie Blue.

2.2.2.1b Western blot

Separated proteins were transferred from the gel to pre-wetted nitrocellulose membranes.
(Sartorius) at 0.8mA/cm² for 1 hr using a semi-dry electroblotter (Ancos, Denmark) in transfer buffer (48 mM Tris, 39 mM glycine and 20% methanol). After transfer, the nitrocellulose membranes were blocked using 10% dried skimmed milk (J Sainsbury plc, Stanford, UK) in PBS with constant agitation at room temperature for 2 hours before incubation with either primary antibodies or the samples (see section 2.1.2 for details), diluted in antibody dilution buffer (PBS with 0.5% Tween 20 and 1% dried milk), for 3 hours at room temperature or overnight at 4°C with constant agitation, were diluted with PBS/T. Unbound primary antibody was removed by washing in PBST (PBS containing 0.05% Tween 20) three times. Blots were then incubated for 2 hrs with sheep anti-human IgG (SAPU) or sheep anti-human IgA (SAPU), which was prediluted 1 in 300 with antibody dilution buffer, at room temperature with agitation. The blots were again washed three times with PBST prior to incubation with horseradish peroxidase (HRP) conjugated donkey anti-sheep IgG, diluted 1:300 with antibody dilution buffer, at room temperature for 2 hours under agitation. After washing, the peroxidase activity was detected colorimetrically by the addition of a fresh solution of 3,3-diamonobenzidine (DAB) tetrahydrochloride (10 mg (1 tablet) was dissolved in 15 ml of PBS, pH7.6; 12 µl of 30% hydrogen peroxide was added to the DAB solution prior to use). After incubation at room temperature for 15 to 30 minutes, the reaction was stopped by washing the strips 2 times with distilled water.

2.2.2.1c HIV-1 western blot kit
The samples of serum, urine and vaginal swab (see section 2.1.2 for details) were diluted 1:50 by adding 40 µl samples or control to the 2.0 ml sample buffer (PBS containing 0.03% Tween-20) containing 3% powered milk supplied. The presence of antibodies to HIV-1 proteins was then detected using HIV-1 Western Blot Kit (Epitope, Inc., Beaverton, Oregon). The assay was performed by following the manufacturer’s instructions.

2.2.2.2 HIV-1 gp160/120 ELISA (Enzyme linked immunosorbant assay)
This assay is modified from the method basically developed by Moore and Jarrett
(1988).

2.2.2.2a Coating of plates

Immunosorbant removablewell strips (Dynatech laboratories, Inc., Chantilly, VA, USA) were fitted into the corresponding MicroELISA strip holder (Dynatech laboratories, Inc., Chantilly, VA, USA). These strips were coated with sheep polyclonal antibody (Ab) to HIV-1 gp120 (D7324, AAalto BioReagents Ltd, Dublin, Ireland) at a concentration of 4 μg/ml in coating buffer (100 mM NaHCO₃, pH 9.6) by incubation overnight at room temperature. The wells were washed 4 x 200 μl/well with wash buffer (0.05% Tween20 in 1x TBS (50 mM Tris-HCl, pH7.6; 145 mM NaCl)), blocked for 1 hour at 37°C with 100 μl 2% BSA (Bovine serum albumin) in 1 X TBS, and then washed again with wash buffer. The plates were then sealed into plastic bags with silica gel sachets (BDH laboratory supplies, Poole, England, UK) as desiccant and kept at -20°C until use.

2.2.2.2b ELISA

The coated strips were removed from the -20°C freezer and equilibrated to room temperature. Samples were diluted two fold with 0.1% Empigen (Calbiochem) in 1xTBS. To obtain a standard curve, recombinant gp160 (AIDS Reagent Project, MRC, UK) was diluted to 25, 12.5, 6.25, 3.125 and 0 ng/ml with 0.1% Empigen (Calbiochem) in 1 X TBS. 100μl of the samples and different dilutions of recombinant gp160 were added to the duplicate wells and the plates were incubated for 3 hours at 37°C. Unbound proteins were removed by washing 4 times with 200 μl wash buffer per well.

100μl of anti gp120 monoclonal antibody (AIDS Reagent Project, MRC, UK) which had been diluted to a concentration of 1 μg/ml with wash buffer containing 1% BSA, was added to the wells (except blank wells) and incubated for 1 hour at 37°C. The wells were washed 4 x 200 μl/well as above.

100μl of biotinylated F (ab') 2 Goat anti-human IgG (Zymed, San Francisco) diluted 1:1000 with wash buffer containing 1% BSA was added and incubated for 1 hour at
37°C. The plates were washed as before.

100 µl/well of extravidin alkaline phosphatase (EA-AP, 1:1000 in wash buffer containing 1% BSA) was added to the wells (except blank wells) and incubated for 1 hour at 37°C. Unbounded EA-AP was removed by washing 4 x 200 µl/well with the wash buffer. The bound EA-AP was detected with SIGMA FAST™ p-Nitrophenyl phosphate (pNPP) substrate system (one pNPP tablet and one Tris Buffer tablet were dissolved in 5 ml distilled water) using 100 µl/well of substrate solution for all wells including the blank. The plates were kept in the dark for 15-30 minutes. The reactions were stopped with 50 µl of 0.4 M NaOH and the optical density (OD) determined at 405nm using a Labsystems Multiskan Bichromatic Reader. The machine was zeroed with blank wells (two empty wells per plate). Concentration of samples was estimated by reference to a standard curve (see section 5.3.4.1).

2.2.3 General molecular biology methodology

2.2.3.1 Polymerase chain reaction

Plasmids (see Chapter 4) containing the whole env160 sequence or DNA extracts from transgenic plants leaves were used as template for amplification of HIV-1 specific env DNA fragments. The PCR reaction conditions were as follows: 4 ng of plasmids or 5µl of DNA extracts and 0.25 µM of each primer were used per reaction. PCR mixture consisted of 50 mM Tris HCl (pH 9.1), 16 mM ammonium sulphate, 3.5 mM MgCl2, and 150 µg/ml nuclease-free BSA, 3.3 µM dNTPs and 2 units (for extending DNA strands less than 1Kb (kilobase pairs)) or 5 units (for extending a strand by 2.6Kb) of Taq DNA polymerase (purchased from either Sigma Chemicals Co., England or Helena BioSciences, Sunderland, UK). All the reaction mixtures were made up to 20 µl with DEPC (Diethyl procarbamine) treated distilled water (dH2O) and overlaid with paraffin oil to prevent evaporation. They were then subjected to thirty cycles consisting of 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 3 minutes, followed by a single cycle of 72°C for 10 minutes, on either a GENEE Thermal Cycler (Techne) or PCR Express (Hybaid). 10 µl samples of each PCR reaction were analysed on a 1% TAE agarose gel
electrophoresis (see section 2.2.3.2).

2.2.3.2 Agarose gel electrophoresis of DNA

1% agarose (Flowgen, Staffordshire, UK) in 1 X TAE (40mM Tris-acetate, 1mM EDTA (Ethylenediamine tetraacetic acid), pH8.5) was melted by microwave for 4 minutes. After cooling to 60°C, ethidium bromide (EB) was added at a final concentration of 0.3 μg/ml. The gel then was poured into gel cast and allowed to set. Electrophoresis running buffer (1 X TAE) was added to the tank to just cover the gel. Then DNA samples (PCR product and plasmid DNA) were loaded, and the gel was run at 100 volts for about 1 hour until DNA fragments were well resolved. 1 Kb DNA ladder (GIBCOBRL) or Lambda/HindIII DNA marker (Promega) 1Kb DNA ladder (Gibco) was run alongside the samples as DNA molecular weight marker. DNA bands in the gels were visualized on a ultra-violet (UV) transilluminator, and photographed using a Polaroid CU-5 land camera and black and white Polaroid 667 film or Eagle Eye™ system (Stratagene).

2.2.3.3 Digestion of DNA with restriction endonucleases

Restriction endonuclease cleavage was accomplished simply by incubating the enzyme(s) (including EcoRI, HindIII, NcoI, SacI, SalI, SmaI and XbaI) with the DNA in appropriate reaction conditions supplied by the manufacture (see Chapter 4 for details). Generally, 20 μl digestion mixture consisted of 2 μl of 10 X digestion buffer supplied with each enzyme (Promega or NewEngland Biolabs), 0.2 μl of 100 X BSA, 1 μg DNA samples, 10 units of each enzymes (0.5-1 μl), and dH₂O was added to make up to 20 μl. The reaction mixture was incubated at 37°C (for EcoRI, HindIII, NcoI, SacI, SalI and XbaI) or 25°C (for SmaI) in a water bath for 2 hours. When the double digestion (cleaving a DNA substrate with two restriction endonucleases simultaneously) was performed, the digestion buffer that resulted in at least 75% activity for both enzymes was used. When the two combination enzymes required different optimal digestion temperature, such as SmaI and SacI, the double digestion was performed sequentially. Restriction enzymes were inactivated by heating at 65°C for 15 minutes and the digestion reaction was either used for subsequent agarose gel electrophoresis, phenol extraction or kept at -20°C until use.
2.2.3.4 Purification of PCR products

PCR products were purified using QIAquick PCR purification kit (QIAGene, Germany), according the manufacturer's manual. In brief, 5 volumes of buffer PB (supplied by manufacturer) was added to the PCR reaction and mixed. Then mixture was applied to the QIAquick column and centrifuged for 1 minute at 13,000rpm. After washing with 0.75ml washing buffer PE (supplied by manufacturer), the DNA was eluted with 30 μl DEPC treated dH2O and kept at -20°C until use.

2.2.3.5 Phenol extraction and ethanol precipitation of DNA

An equal volume of 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol was added to the DNA solution and mixed by vortexing vigorously for 30 sec. After microcentrifuging for 5 minutes at room temperature, the upper (aqueous) layer was transferred to a new tube taking care to avoid any material at the interface. If a white precipitate was present at the interface, the DNA solution was repeatedly extracted with phenol/chloroform/isoamyl alcohol a second time. 0.1 volume of 3M sodium acetate (pH5.2) and then 2-2.5 volume of ice-cold 100% ethanol were added and mixed by vortexing. To precipitate the DNA, the tube was placed at -20°C overnight or at -70°C for 30 minutes or on dry ice for 15-20 minutes and pelleted by microcentrifuging for 30 minutes at 4°C. The pellet was washed twice with 1ml 70% ethanol and dried in a Speedvac evaporator or on a 65°C heat block for 5-15 minutes depending the size of the pellet. The DNA pellet was dissolved in 10-20 μl of DEPC treated dH2O.

2.2.3.6 Recovery of DNA from agarose

DNA was separated on a 1 % (w/v) low melting point agarose (Flowgen) or routine 1% agarose gel made up in 1 X TAE buffer. DNA in the gel was visualized by UV illumination and the DNA fragment of the predicted size was cut out of the gel. DNA was then extracted and purified by using the Geneclean II kit (BIO 101 Inc., Stratech Sci., England) following the manufacturer's instruction (see section 2.2.3.6a). Later samples were extracted and purified by using Prep-A-Gene DNA purification Systems (BIO-RAD) (see section 2.2.3.6b).
2.2.3.6a Geneclean

3 volumes of 6M sodium iodide (NaI) were added to the gel slice containing DNA and the gel was melted in a 55°C water bath. 5 μg of Glassmilk suspension (silica) was added and mixed by inverting the tube several times. The mixture was then placed on ice for 15 minutes, mixing every 2 minutes. The Glassmilk/DNA complex was pelleted by microcentrifuging for 5 seconds at 13000 rpm. The pellet was washed three times with 500μl NEW WASH (NaCl, Tris, EDTA and ethanol). The DNA was then eluted from the Glassmilk by incubating the suspension at 55°C for 5 minutes in 8 μl of DEPC treated dH2O and microcentrifuging for 30 seconds. The elution was repeated once more and the two supernatants were pooled and centrifuged for 5 minutes at 13000rpm to remove any traces of matrix. The DNA concentration in the supernatant was estimated by running 1.0 μl of sample on 1% agarose gel alongside Lambda/HindIII DNA marker (see section 2.2.3.7).

2.2.3.6b Prep-A Gene DNA purification

The desired DNA band containing 1 μg of DNA required was excised from an agarose gel. The volume of the gel slice was estimated by weighing and converting to milliliters (ml). Conversion of grams (g) to ml was accomplished by assuming 1 g of a gel slice is equal to 1 ml. 3 volumes of binding buffer (6 M sodium perchlorate; 50 mM Tris (pH 8.0); 10 mM EDTA (pH 8.0)) was added to the gel slice and heated at 50°C on a water bath for 5 minutes to dissolve the agarose. To this was added 5 μl of Prep-A-Gene matrix and mixed by inverting the tube several times. The mixture was then incubated for 15 minutes at room temperature, mixing every 5 minutes. The DNA/matrix complex was pelleted by centrifuging at 13,000rpm for 30 seconds. The pellet was rinsed with 200 μl of binding buffer (6 M sodium perchlorate; 50 mM Tris (pH 8.0); 10 mM EDTA (pH 8.0)) and centrifuged for 30 seconds at 13,000 rpm. The pellet was then washed twice with 200 μl of wash buffer (400 mM NaCl; 20 mM Tris (pH 7.5); 2 mM EDTA (pH 7.5) and 50% ethanol (v/v)). After the second wash, all traces of liquid were carefully removed by using an automatic with a fine pipette tip. The bound DNA was eluted by incubating the DNA/matrix complex at 50°C for 5 minutes in 10 μl of dH2O.
Following centrifugation at 13,000rpm for 2 minutes, the supernatant containing DNA was collected and re-centrifuged for 5 minutes at 13000 rpm to remove any traces of matrix. 1 μl of the supernatant was used for checking DNA concentration by running on 1% agarose gel alongside Lambda/HindIII DNA marker (see section 2.2.3.7).

2.2.3.7 Measurement of DNA and RNA concentration

Concentration and purity of DNA and RNA was determined by measuring the absorbance at 260nm and 280nm in a spectrophotometer (CE 594, Jencons (Scientific) Ltd, UK). An absorbance of 1 unit at 260nm is equivalent to 50 μg of double stranded DNA per ml or 40 μg of single stranded DNA or RNA per ml. Purity of the DNA or RNA was determined by calculating the ratio between the absorbance values at 260nm and 280nm, where pure DNA or RNA has a ratio of 1.5-2.0. Thus the concentration of DNA or RNA was obtained by using the following formula.

\[
\text{Concentration of DNA (μg/ml) = Absorbance at 260nm x dilution factor x 50}
\]

\[
\text{Concentration of RNA (μg/ml) = Absorbance at 260nm x dilution factor x 40}
\]

For small amount of DNA, estimates were taken by visualizing the intensity of the fluorescence under UV illumination compared to Lambda/HindIII DNA marker of the known concentration.

2.2.3.8 Cloning PCR products into pCR-Script\textsuperscript{TM} Amp SK(+) cloning vector

To insert the PCR products (the whole HIV-1 env gene) into a vehicle for subsequent cloning into plant plasmids pIBT210.1 and pIBT240.1 (see Chapter 4 for details), PCR-Script\textsuperscript{TM}SK(+) cloning vector (see Figure 4.1) (Stratagene, Cambridge, UK) was employed to clone the PCR product containing HIV-1 env gene and was used according to manufacturer's instruction. In brief, ligation reactions were set up at molar ratios of approximately 40-100:1 (PCR product: cloning vector) and the reaction mixture consisted of 10 ng of pCR-Script\textsuperscript{TM} SK(+) cloning vector, 0.5 μl of 10 mM ATP, 5 units of Srf I restriction enzyme, 4 μl of PCR product and 4 units of T4 DNA ligase in 1 X pCR-Script reaction buffer supplied. Ligation reactions were incubated for 2 hours at
room temperature and heated to 65°C for 2 minutes. After cooling on ice for 5 minutes, 2 μl of ligation mixture was used to transform 40 μl Epicurian Coli XL 1-Blue MRF’ KAN supercompetent cells (Stratagene) in a ice-chilled 5 ml Falcon 2054 polypropylene tube (Falcon) containing 25 mM β-mercaptoethanol. The transformation reaction was incubated on ice for 45 minutes and heat pulsed on a 42°C heat block for 45 seconds. After cooling on ice for 2 minutes, 0.3 ml prewarmed SOC medium (see Appendix I) was added to the transformation reaction. The tube was then incubated at 37°C for 1 hour with shaking at 225-250 rpm. 50 μl and 100 μl of the transformation reaction was spread onto separate LB agar plate containing ampicillin (20 μg/ml), 20 μl of 0.2 M IPTG (isopropyl-β-D-thio-galactopyranoside) and 20 μl of 10% (w/v) X-gal (5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside). The plates were incubated at 37°C for 14-16 hours. Next day, the white colonies which indicated possible successful the insertion (see section 4.3.2) were chosen for examination.

2.2.3.9 Ligation of DNA fragments into plasmids
The ligation of restriction endonucleases digested DNA/PCR fragments into the specific plasmid required were performed following the manufacturer’s instructions by using either rapid DNA ligation kit (Boehringer Mannheim) or T4 DNA ligase and buffer purchased from New England Biolab (UK). The ligation molar ratio (ratio) of insert DNA to cloning vector DNA (vector) was generally 3:1. 100 ng purified digested vector was used for each ligation reaction and the amount of purified digested insert DNA in nanograms (ng) was optimized by using the following equation:

\[
\frac{\text{no. of bps of insert DNA} \times \text{ng of vector}}{\text{no. of bps of vector}} \times \text{ratio}
\]

Therefore, for ligation of HIV-1 env gene consisting of 2596 base pairs (bps) into 100ng cloning vector pIBT240.1 consisting of 5554 bps, and the molar ratio of insert DNA to cloning vector was 3:1, the amount of purified HIV-1 env gene fragment should be
calculated as following

\[
\frac{2596 \times 100}{5554} \times 3 = 140.2 \text{ ng}
\]

2.2.3.9a Rapid DNA ligation

The vector DNA and insert DNA were combined in one tube containing 2 μl of 5 x DNA dilution buffer and sufficient DEPC treated dH2O to make the final volume of reaction mixture to 10 μl reaction mixture. 10 μl of 2 X T4 DNA ligation buffer (supplied with the kit) and 5 units T4 DNA ligase were added to the tube and mixed thoroughly by flicking the tube with fingers for several times. Then the reaction mixture was incubated for 15-30 minutes at room temperature. 2 μl of each ligation reaction was used directly for the transformation of DH5α competent cells (see section 2.2.3.10).

2.2.3.9b Overnight ligation

In this method, insert DNA and cloning vector were combined in one tube. To 1 μl of 10 X ligase buffer (500 mM Tris-HCl, pH7.5; 100 mM MgCl₂; 100 mM dithiothreitol (DTT), 10 mM rATP, 250 μg/ml bovine serum album (BSA)), 0.5 μl of T4 DNA ligase (400 u/μl) (New England Biolabs) and 8.5 μl of DEPC treated dH2O or dH2O were added and mixed well by gently flicking the tube with fingers several times. The reaction mixture was then incubated at 16°C overnight. 2 μl of each ligation reaction was used directly for the transformation of DH5α competent cells (see section 2.2.3.10).

2.2.3.10 Transformation of competent cells

Transformation of MAX efficiency DH5α™ competent cells (GIBCO BRL) was performed by adding the 1-2 ng plasmid or 2-5 μl ligation mixture to the 40 μl cells in a ice-chilled Falcon 2054 polypropylene tube (Falcon) and incubated on ice for 45 minutes followed by heat shock on 42°C heat block for 45 seconds. The cells/DNA was placed on ice for 2 minutes, and 300μl 42°C prewarmed SOC medium (see Appendix I) was added. Then cells were incubated at 37°C for 1 hour under shaking at 225 rpm. 50-100 μl cells were spread on each LB/agar plates (see Appendix I) containing either 100
μg/ml ampicillin or 50 μg/ml kanamycin depending on the specific selection marker of the plasmid. The plates were incubated for 16 hours at 37°C, after which those containing colonies were removed and stored at 4°C for up to one month.

### 2.2.3.11 PCR screening of recombinant plasmids

This methodology was kindly supplied by Dr. Hugh Mason (Boyce Thompson Institute for plant research, Cornell University, USA).

Single colonies of presumed transformants were picked up with a sterile plastic pipette tip and dispersed in 50 μl sterile dH₂O. 18-20 μl of this bacterial suspension was then used in a 25 μl PCR reaction for 25 cycles using the PCR program and conditions described in 2.2.3.1. 15 μl of each reaction was run on 1% agarose gel to examine products. The remainder of the recombinants bacterial suspensions identified as recombinants was added to 3 ml of LB containing 100 μg/ml ampicillin or 50 μg/ml kanamycin and cultured overnight.

### 2.2.3.12 Minipreparation of plasmid DNA

#### 2.2.3.12a Alkaline lysis miniprep.

Transformants grown on selective agarose plates were inoculated into 3 ml LB/amp and grown up overnight at 37°C with shaking. 2.5 ml of overnight culture was pelleted by centrifuging at 6000 rpm for 2 min and re-suspended in 100 μl of Soln I (50 mM Tris-HCl pH7.5; 10 mM EDTA; 10% Glucose; 1 μg of RNase per sample) and placed at room temperature for 5 min. 200 μl of Soln II (0.2 M NaOH; 1% SDS) was added, mixed gently, and placed on ice 5 min (inverted every 2 min). To this 150 μl Soln III (3M KAOc, pH 4.9) was added, mixed gently by inverting tube several times and placed on ice for 5 min prior to the mixture being centrifuged at room temperature for 5 min at 13000 rpm. The supernatant was recovered into a fresh eppendorf tube taking care to avoid any floating or pelleted material. 1ml of cold 100% ethanol was added and mixed by inverting the tube for 5 times. After incubation at room temperature for 2 minutes, plasmid DNA was pelleted by centrifuging at 12000rpm for 10 min at room temperature.
The pellet was washed once with 70% cold ethanol, centrifuged for 5 min at 13000 rpm, dried at 65°C and then resuspended in 30-40 μl dH₂O according to the pellet size. The plasmid DNA was stored at -20°C.

2.2.3.12b Isopropanol miniprep
This methodology was kindly supplied by Dr. Hugh Mason (Boyce Thompson Institute for Plant Research, Cornell University, USA).

Transformants grown on selective agarose plates were inoculated into 3 ml LB/amp and grown up overnight at 37°C with shaking. Next day, 2.5ml of overnight culture was centrifuged at 6000 rpm for 30sec and the pellets was re-suspended in 50 μl water. 300 μl TENS (0.1N NaOH, 0.5% SDS in TE), mixed gently several times, and placed at room temperature for 5 min. 200 μl of ammonium acetate was added, mixed gently and placed on ice for 5 minutes, prior to the mixture being centrifuged at 4°C for 3-5 min at 13000 rpm. The supernatant was recovered into a fresh eppendorf tube taking care to avoid any floating or pelleted material. 0.6 volume of isopranpanol was added and mixed. After incubation at room temperature for 5-10minutes, plasmid DNA was pelletted by centrifuging at 13000rpm for 10 min at room temperature. The pellet was washed once with 70% cold ethanol, air-dried and then resuspended in 30-40 μl dH₂O. The plasmid DNA was stored at -20°C.

2.2.3.13 Midipreparation of plasmid DNA
3 ml of overnight culture prepared as previously described as 2.2.3.12a was inoculated into 100 ml of LB/amp or LB/kan (see Appendix I) and incubated at 37°C for 16 hours with shaking. Next day, This culture was centrifuged at 4000 rpm for 20 minutes and the resulting cell pellet was completely re-suspended in 3 ml of Cell Resuspension Solution (50 mM Tris-HCL, pH7.5; 10 mM EDTA; 100 μg/ml RNase A). 3 ml of Cell Lysis Solution (0.2 M NaOH; 1% SDS) was added and mixed by inverting the tube several times until the cell suspension became clear. 3 ml of Neutralisation Solution (1.32 M potassium acetate, pH4.8) was then added and mixed by inverting the tube several times. The lysate was centrifuged at 4000 rpm for 30 minutes at 4°C. The supernatant was
carefully collected to a new centrifuge tube, avoiding the white precipitate. 10 ml of the resuspended resin (Promega) was added and the resin/DNA mix was then transferred to a Wizard Midicolumn per preparation. A vacuum was applied to pull the resin/DNA mix into the Midicolumn. The column was washed twice with 15 ml of Wash Solution (200 mM NaCl; 20 mM Tris-HCL, pH 7.5; 5 mM EDTA (concentration prior to ethanol addition) 55% final concentration of ethanol). After the second wash, the resin was dried by continuing to draw a vacuum for 30 seconds after the solution had been pulled through the column. The Midicolumn was separated from the reservoir and transferred to a 1.5 ml microcentrifuge tube, and then centrifuged at 13000 rpm for 2 minutes to remove any residual Column Wash Solution. After the Midicolumn had been transferred to a new microcentrifuge tube, 300 µl of preheated (65-70°C) DEPC treated dH2O was added to the Midicolumn and incubated for 1 minute. The Midicolumn was spinned at 13000 rpm for 2 minutes. The eluted DNA was then centrifuged at 13000 rpm for 5 minutes to remove resin fines which could be observed as small translucent particles in the DNA solution. The supernatant containing plasmid DNA was carefully transferred to a new tube. After measuring the DNA concentration on spectrophotometer (see section 2.2.3.7), the DNA solution was aliquoted and stored at -20°C until use.

2.2.3.14 Storage of bacterial stocks
2 ml overnight culture was grown up in LB with 100 µg/ml ampicillin or 50 µg/ml kanamycin. The bacteria were spun down for 2 minutes at 500-1000 rpm. The supernatant was removed and the pellets were resuspended in 1 ml LB containing 20% glycerol and transferred to Eppendorf tube. Then the tube was snap frozen on dry ice and kept at -70°C.

2.2.3.15 Nested reverse transcription (RT)-PCR
Nested PCR is a more sensitive form of PCR involving two rounds of amplifications with two sets of primers: an outer pair for the first round, with a second pair nested within the out pair, for the second round. This double action increases the sensitivity and specificity and allows the amplification from low level of target DNA.
2.2.3.15a cDNA synthesis

6.5 µl RNA extracts (see section 2.2.4.5) from transgenic tobacco leaves was added to the tube containing 2µl 5 X RT buffer (250 mM Tris-HCl; 200 mM KCl; 25 mM MgCl2; (Promega) and 1 µl (1 unit) of RNase-free DNase (RQ1; Promega). The mixture was incubated for 30 minutes at 37°C. Then, the RQ1 was inactivated in the mixture by incubating at 65°C for 15 minutes. 5 µl of RQ1 treated RNA solution was added to a tube containing 1.5µl (0.75µg) of oligo (dT)15 primer (Promega) and the tube was incubated at 65°C for 10 minutes to denature secondary structure of mRNA. The tube was then immediately cooled on ice. 14.5 µl premix consisting of 3 µl of 5 X RT buffer; 4 x 2 µl of each of 10 mM dATP, 10 mM dGTP, 10 mM dCTP, and 10 mM dTTP; 2 µl of 100 mM DTT and 1.5 µl (75 units,) of Expand™ reverse transcriptase (Boehringer Mannheim) was added to the tube containing RNA and oligo (dT)15 primer (Promega). This reaction was incubated for 1 hr at 42°C. Subsequently, the reaction mixture was either used directly for PCR or stored at ~20°C until use.

2.2.3.15b Primary PCR

4 µl of cDNA reaction as template; 0.2 µl of HIV-1 gp4l primer 254 and 0.2 µl (0.2 µg) of oligo (dT)15 as outer pair of primers and 30 cycles of PCR program was performed (see section 2.2.3.1).

2.2.3.15c Secondary PCR

3 µl of primary PCR product was used as PCR template and HIV gp4l primer 518 and 256 (see section 2.2.3.18) which nested within the outer pair primers used in the primary PCR was used as inner pair of primers, and 30 cycles of PCR program was performed (see section 2.2.3.1).
2.2.3.16 Protein assay

The total protein concentration in the extracts of transgenic plant leaf, microtuber and plant NT-1 cells was assayed by using protein assay kit II (Bio-Rad) following the manufacturer's microassay instruction with some modifications. Briefly, 20 ml of dH2O was added to the lyophilised BSA (supplied) to yield a concentration of approximately 1.4 mg/ml. BSA solution was diluted with 1 X TBS to produce concentrations of 0.5 mg/ml; 0.25 mg/ml; 0.125 mg/ml; 0.0625 mg/ml; 0.03125 mg/ml; 0.015625 mg/ml; 0.0078125 mg/ml and 0 mg/ml (blank) as protein standards. Protein samples were diluted 1:40 with 1 X TBS. 40 µl of each of protein samples and standard dilutions were added into duplicate wells of a 96 microtiter plate. 160 µl of a 1:4 dilution of dye reagent concentrate was added to each well of the plate and mixed well using a multi-channel pipette. The plate was incubated at room temperature for 15 minutes to 30 minutes and the optical density (OD) determined at 595nm using a Labsystems Multiskan Bichromatic Reader. OD595 value was plotted versus concentration of standards and the unknowns were read from the standard curve.

2.2.3.17 Sequencing of HIV-1 env gene

Sequencing of the HIV-1 env gene insert was performed by using ABI PRISM™ dye terminator cycle sequencing ready reaction kit (for Applied Biosystems 373 DNA sequencer) (Perkin Elmer, UK) or ABI PRISM™ BigDye™ terminator cycle sequencing ready reaction kit (for ABI PRISM™ 310 genetic analyser) (PE Applied Biosystem, USA) with ampliTaq® DNA polymerase.

2.2.3.17a Sequencing PCR

For each reaction, the following reagents were added: 6.0 µl of terminator ready reaction mix; 1 µg plasmid DNA and 0.2 µl primer (5 pmol-10 pmol). Appropriate amount of DEPC treated dH2O or dH2O was added to make the volume up to 20 µl and mixed reactions overlayed with 20 µl of mineral oil. The tubes were then placed on the thermal cycler. The following program performed for 25 cycles: 94°C for 30 sec; 55°C for 30 sec; 60°C for 3 minutes. PCR products were then used for ethanol/NaAc precipitation
prior to sequencing.

2.2.3.17b Ethanol/NaAc precipitation of sequencing PCR products
For each sequencing reaction, 2.0 µl of 3M sodium acetate (NaAc), pH4.6 or 3.0 µl of 2M NaAc, pH4.6 and 50 µl of chilled 95% ethanol (EtOH) was added to 1.5-ml microcentrifuge tube. 20 µl contents of the sequencing PCR reaction was transferred to the tube of NaAc/EtOH mixture and mixed thoroughly. The tube was incubated at room temperature for 15 minutes and covered with aluminium foil to avoid the light. After spinning for 30 minutes at 13,000 rpm, the pellet was rinsed with 250 µl of chilled 70% EtOH and dried at 90°C for 2 minutes. Then, the tubes were placed on ice until loading.

2.2.3.17c Electrophoresis on the ABI PRISM 310 Genetic Analyser
For analysing the sequence on the ABI PRISM 310 Genetic Analyser (Perkin-Elmer), POP-6 polymer was set as run module; BD Set-any primer was set as dye set/primer file and Taq terminator matrix was set as matrix file.

Before loading, each sample pellet was re-suspended in 20 µl of template suppression reagent (TSR, supplied by the manufacturer). After heating at 95°C for 2 minutes to denature, the tube was briefly spun and placed on ice for a while. The tubes were placed on the sample tray in the order the same as sample sheet. The sample tray was put back into the analyser and the sequencing was initiated simply by click “run” icon on the menu. As the run completed (one hour per sample), the data was collected and printed out.

2.2.3.17d Electrophoresis on the Applied Biosystems 373A DNA sequencer

Polyacrylamide sequencing gel
The gel plates were cleaned thoroughly with Alconox (Alconox, USA), rinsed with hot water and dH2O, and then air dried before assembling. 6% sequencing gel (240mm in length and 0.4mm in thickness) was made by dissolving 30g of Urea in 9 ml of 40% (w/v) acrylamide/bis (19:1) solution (Bio-Rad), 0.5 g of Amberlite™ MB-150 resin (Supelco, Bellefonte, USA) and 20 ml of dH2O for 1 hour. The gel solution was then
filtered and degassed through a 0.2 μm cellulose acetate filter which was prewetted with 6ml of 10 X TBE (890 mM Tris-borate; 20 mM EDTA, pH 8.0). 300 μl of freshly prepared 10% (w/v) ammonium persulphate and 33 μl of TEMED (N,N,N',N'-Tetramethylethylenediamine, IBI, Eastman Kodak Company, UK) were added to the 6% acrylamide/urea/TBE gel solution. The gel was poured and allowed to set for 2 hours.

**Electrophoresis on ABI 373 DNA sequencer**

The sample sheet was set up on the Macintosh (MAC), and on the ABI machine, filter set A was chosen for Taq sequencing. Then the gel plate was scanned before the 24 sharks-tooth comb was inserted and the gel equipment was assembled. 1.5 litre of 10 X TBE was made with dH₂O to fill up the upper and lower buffer tanks. The wells were rinsed with 1x TBE buffer to remove excess urea.

The purified and dried sequencing PCR products were resuspended in 4 μl of FE (5 volume of deionized formamide to 1 volume of 25 mM EDTA (pH8.0) containing 50 mg/ml blue dextran) by pipetting up and down for 5sec. The tubes were heated at 90°C for 2 minutes exactly and put them on ice immediately. The wells of sequencing gel were rinsed with 1x TBE buffer again to remove excess Urea, and then the odd wells were loaded with samples. The gel was run for 5 minutes and the even samples were loaded into the wells. The machine was left to run at 2500volts, 40mA at 40°C for 12 hours.

**Data analysis**

The sequencing data was collected from the MAC and preliminarily edited within TED (part of the Staden computer package) using Seqprocess (written by Dr. C. Wade, Centre for HIV Research, University of Edinburgh). The sequences were then assembled using Xbap software (Staden computer package) and Seqedit script (written by Dr. C. Wade, Centre for HIV Research, University of Edinburgh). Subsequently, sequences were aligned and translated within version 2.0 of the Genetic Data Environment (GDE) package.
2.2.3.18 Primers

2.2.3.18a HIV-1 gp 120 V1/V2 primers

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2.2.3.18b HIV-1 gp120 V3/V4 primers

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2.2.3.18c HIV-1 gp41 primers

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<td>(-8079*)</td>
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2.2.3.18d Plant TEV/vsp primers

TEV/vsp primers were kindly supplied by Dr Hugh Mason (Boyce Tompson institute for plant research, Cornell University, USA).

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<tr>
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<tr>
<td>Vsp</td>
<td>5'-GTGCATATCAGCATAACC-3'</td>
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*position of primer on HIV-HXB2 sequence.

**position of primer on plant plasmid pIBT240.1 sequence.

2.2.3.18e N160F/S160R primers

This pair of primers was designed by Y Li to introduce NcoI site and SacI site at the 5' and 3' of HIV-1 env gene respectively.
N160F sense : 5'-ATACTCCATGGAATGAGAGTGAAGGAG-3'
S160R antisense : 5'-ATCTAGAGCTCTCTTATAGCAAAATC-3'

2.2.4 Plant molecular biology methodology
All the plant molecular biology and plant tissue culture methodology, plant explants and NT-1 cell line used in this thesis were kindly supplied by Dr. Hugh Mason (Boyce Tompson Institute for Plant Research, Cornell University, USA) and his co-workers.

2.2.4.1 Electroporation transformation of Agrobacterium tumefaciens
The Gene Pulser™ (Bio-Rad) was set to 2.5KV. 1 µl of plasmid DNA was added to 50 µl of Agrobacterium tumefaciens strain LBA4404 and mixed gently in an ice-cold electroporation cuvette. The outside of the cuvette was cleaned by wiping with Kimwipes and placed into the sample chamber of the machine. Electroporation was performed by applying the pulse for 5 milliseconds (msc). The cuvette was removed and 1ml of ice-cold YENB medium (see Appendix I) was added to the cuvette immediately. The mixture was transferred to a sterile culture tube and incubated at 30°C for 1 hour with shaking at 250 rpm. The transformation culture was plated on LB/kan agar plates (see Appendix I) and incubated at 30°C for 48-72 hours.

2.2.4.2 Plant transformation and regeneration
To produce transgenic plants, the target gene was introduced into plants by Agrobacterium tumefaciens mediated plant transformation. The plant leaves from plantlets were cut with surgical blade into small strips (approximate 5 x 8 mm). The cut leaf strips were inoculated with Agrobacterium tumefaciens strain LBA4404 transformed with the recombinant pGPTV-KAN vector containing HIV-1 env 160 gene, and during the subsequent coculture on regeneration medium for 2-3 days, the bacteria bind to the plant cells around the wounded edge of the explant and the gene transfer process occurs.

After the transformation had occurred, the explants were transferred to regeneration/selection medium containing 500 µg/ml carbenicillin to kill the bacteria and 50 µg/ml or 300 µg/ml kanamycin to inhibit untransformed plant cells. During the next 3 weeks, the
transformed cells grew into calli and differentiated into shoots via organogenesis. After 3-6 weeks, the shoots were excised from the explants and induced root on rooting medium containing 500 µg/ml carbenicillin and 50 µg/ml or 100 µg/ml (see the following sections and Appendix I for details).

Due to the low efficiency of plant transformation and foreign protein expression, transformation of plant tissue was performed 2-4 times per *Agrobacterium tumefaciens* culture at 1-2 week intervals. All the plant cultures were grown in medium plates or Magenta boxes sealed with Micropore tape (General Medical Corp.) under the mixture of cool and warm white fluorescent lights (GE 40 Watt bulbs) on racks at 26°C for 16 hour day length, and subsequently at 22°C for 8 hour night time conditions.

2.2.4.2a Tobacco ("sumsun") transformation and regeneration

*Inoculation*

*A. tumefaciens* strain LBA4404 transformed with the recombinant plant expression vector pGPTV-KAN-210.1S-envl60 containing HIV-1 *envl60* gene (see section 4.3.7.1 for details) was used for tobacco transformation. 3ml of *A. tumefaciens* culture was grown in YENB with 50µg/ml kanamycin at 30°C with shaking at 250 rpm for 48 hours. 0.5 ml of the culture was taken to measure OD at 600 nm using spectrophotometer (DU-64, Beckman). The optimum OD<sub>600</sub> reading should be 0.6-0.7 (approximately 6 x 10<sup>8</sup> cells/ml). If OD<sub>600</sub> was over 1.0, the culture was diluted until the OD<sub>600</sub> reading was below 0.5 and cultured for another hour. The OD<sub>600</sub> reading was checked periodically. The overnight culture was centrifuged at 3,500 rpm for 30 minutes. The pellet was resuspended in 20ml MSO medium (see Appendix I).

Healthy, unblemished leaves were harvested from young tobacco (*Nicotiana tobaccum* strain "sumsun") plants and cut into 3-5 millimeter strips on the sterile paper towel under sterile conditions in a laminar flow cabinet to produce a wounded edge. The cut leaf strips were then inoculated by immersion in 50 ml of the *A. tumefaciens*/MSO solution for less than 2 minutes with gently shaking. Each leaf strip was gently blotted dry on
sterile paper towels and placed upside down on MS104 medium plate (see Appendix I). Approximately 30-40 leaf strips were placed on each petri dish and 3-4 petri dishes used for each transformation.

**Shooting**

After incubation for 2 days under the above conditions, the inoculated explants were transferred to MS selection media (see Appendix I) and incubated for 2 weeks under the above conditions. The explants were then removed and placed upside down on fresh MS selection medium plates (see Appendix I). This process was repeated every 2 weeks until shoots appeared, generally, this would take 4-6 weeks.

**Rooting**

From the callus of an explant, when shoots from a callus were approximately 1-2 cm tall and had at least 1 node they were cut cleanly and placed upright in MS rooting medium (see Appendix I) in a Magenta box (5 plants per box) to root. Care was taken to take only one shoot from each explant to ensure no sibs were propagated. When more than 3 leaflets were observed on the plantlet (generally this would take 3-6 weeks), the leaflets were collected for analysis of DNA, RNA and protein expression (see Chapter 5).

2.2.4.2b Potato "FL1607" transformation and regeneration

**Preculture explants**

Potato cultivar, *Solanum tuberosum* L. cv. "FL1607" plantlets were maintained by serial propagation on CM media in Magenta boxes. Leaves from young plants were cut into 3-5 millimeter strips on the sterile paper to produce a wounded edge and precultured upside down in petri plates containing solid LC1 media (see Appendix I) for 3-4 days until callus formation was observed around the wounded edges. Approximately 30-40 leaf strips were cultured on each petri dish and 6-7 plates set up per transformation.

**Inoculation**

2 x 3ml of *A. tumefaciens* strain LBA4404 culture was grown for 48 hours in YM
medium (GIBCO/BLR) with 50 \( \mu \text{g/ml} \) kanamycin at 30\(^\circ\)C with shaking at 250 rpm. 0.5 ml of the culture was taken to measure OD at 600 nm using a spectrophotometer (DU-64, Beckman). The optimum OD\(_{600}\) reading should be 0.6-0.7 (approximately \( 6 \times 10^8 \) cells/ml). If the OD\(_{600}\) reading was below 0.5, the culture was grown for another hour. The OD\(_{600}\) readings were checked periodically. The overnight culture was centrifuged at 3,500 rpm for 30 minutes and the pellet was resuspended in 100 ml MSO medium (see Appendix I).

Co-cultivation of leaf strips was performed by adding approximately 150 precultured leaf strips (described as above) to 50 ml of above suspension of \( A. \) \textit{tumefaciens}. This mixture was incubated at room temperature for ten minutes with occasional inversion. After blotting on sterile paper towels to remove excess bacteria, the leaf strips were placed upside down on fresh LC1 plates and incubated under the above conditions until a ring of bacteria could be observed around the wounded edges. This usually took 3-4 days. The strips were transferred to fresh plates containing LC1 media supplemented with 500 \( \mu \text{g/ml} \) carbenicillin and 50 \( \mu \text{g/ml} \) kanamycin (LC1 c/k) every week until green callus formed on the cut edges of the leaf strips indicating that they were ready for regeneration. This would take approximately 2-3 weeks.

**Shooting**

Shoots were regenerated by transferring callus formed from leaf strips to fresh petri plates containing solid LC2 c/k (see Appendix I) media every week until shoots were at least 2 cm and included at least one node. This would generally take 3-6 weeks.

**Rooting**

Shoots were removed from the leaf strips after the second axillary bud had formed and were placed in Magenta boxes containing CM media with 50 \( \mu \text{g/ml} \) kanamycin to induce roots. The leaf strips were then transferred to a new LC2 c/k plates.

**Microtuber induction**

When the rooted plantlets were about 5-6 cm high, a process which would take 3-6
weeks, the stem which included one node, was cut from the plantlet and placed upright on a petri dish containing Tuberization medium (see Appendix I) and incubated at 18°C in the dark until the microtuber was formed and grown up to 3-6 mm in diameter, a process which generally took 4-6 months. The microtubers were then harvested for analysis (see Chapter 6)

2.2.4.3 NT-1 cell Transformation

2.2.4.3a NT-1 cell
NT (Nicotiana tabacum)-i cells were cultured in NT-1 media (see Appendix I) and incubated at 26°C in the dark with shaking. The cell cultures were then subcultured (1:40) and fed with fresh medium every week. Before transformation with Agrobacterium tumefaciens strain LBA4404, the NT-1 cell suspension was split 1:1 with NT-1 medium and cultured for 3 days under the above condition. 1 μl of 20 mM Acetosyringone (3',5'-Dimethoxy-4'-hydroxy-acetophenone, Aldrich Chem. Co.) freshly made in 100% ethanol was added to per ml of cells before inoculation.

2.2.4.3b Inoculation
3 ml of the Agrobacterium tumefaciens strain LBA4404 culture was grown up in YENB/kan at 30°C with shaking at 250 rpm for 48 hours.

Acetosyringone treated NT-1 cells were pipetted up and down about 20 times to induce small lesions in the cells and increase the efficiency of the transformation. 0 μl, 50 μl, 75 μl, 100 μl and 200 μl of bacteria culture were added to separate petri dishes containing 4 ml of NT-1 cells, and mixed thoroughly. The plates were wrapped with parafilm and incubated for 3 days at 26°C in the dark.

2.2.4.3c Generation of NT-1 transformants
10 ml of NT-1 medium containing 500 μg/ml carbenicillin (NTC) was added to each of above inoculated NT-1 cell plates. The cells were then pipetted into 50 ml conical tubes containing 35 ml of NTC medium (see Appendix I). The tubes were centrifuged at
1,000rpm for 5 minutes at room temperature. The pellets were resuspended in 50 ml NTC medium and spun a second time. The cells were then resuspended in approximately 4 ml of NTC medium and plated out on NTKC (see Appendix I) at 2.0 ml per plate. The plates were left open in the hood for 10-15 minutes until there was no longer excessive surface fluid on the plates prior to being sealed with parafilm. The plates were then incubated at 26°C in dark for 3-4 weeks to allow the transformants to appear. The transformants were then transferred to fresh NTKC plates. After incubation for 4-5 weeks under the above condition, colonies were collected for protein expression analysis (see Chapter 6).

2.2.4.4 Leaf DNA preparation
Two leaflets of transgenic plants were collected in a 1.5 ml tube and immediately placed in liquid nitrogen for 5 minutes, the tube was then removed and 500 μl extraction buffer (200 mM Tris-HCl pH7.5; 250 mM NaCl; 25 mM EDTA; and 0.5% SDS) was added. The sample was ground with pellet pestle (VWR Scientific Products, PA, USA) and vortexed briefly before incubation at room temperature for at least one hour. The ground sample was centrifuged at 13,000 rpm for 5 min, and the supernatant was transferred to a new tube containing an equal volume of isopropanol. After mixing, the tube was incubated at room temperature for 10 minutes. The DNA/isopropanol mixture was then centrifuged at 13,000 rpm for 5 minutes, and the pellet washed once with 1 ml of 75% ethanol. After air drying for 5-10 minutes, the pellet was dissolved in 40 μl of dH₂O and stored at –20°C until use.

2.2.4.5 Leaf RNA preparation
Two leaflets of transgenic tobacco were placed in a 1.5 ml microcentrifuge tube and frozen immediately in liquid nitrogen. The frozen leaflets were then placed on ice and ground to a fine powder using a precooled pestle (VWR Scientific Products, PA, USA). 700 μl (2 ml/g) of RNA extraction buffer (0.2 M sodium acetate, pH5.0; 0.1% SDS; and 0.01 M EDTA) was then added and mixed while the samples were kept on the ice. An equal volume of TE (Tris/EDTA) equilibrated phenol (pH8.0) (Sigma) was added and mixed vigorously for 3-5 minutes. The sample was centrifuged at 3,000 rpm for 10
minutes at 4°C. The aqueous phase was extracted with an equal volume of phenol:chloroform (1:1), and mixed and centrifuged as above. The aqueous phase was then extracted with an equal volume of chloroform and centrifuged as before. 1/3 volume of 10 M LiCl was added to a final concentration of 2.5 M and the RNA was precipitated at 4°C overnight. The precipitate was collected by centrifuging at 13,000 rpm for 30 minutes at 4°C, and washed once with 1 ml of 2.5 M LiCl and twice with 1 ml of 70% ethanol. Subsequently, the pellet was dried under vacuum and kept at −70°C. The RNA pellet was resuspended in 50 μl of DEPC treated dH2O on ice before use.

2.2.4.6 Leaf protein extraction
Two leaflets of transgenic tobacco plantlets were placed in 1.5 ml microfuge tubes and immediately frozen on dry ice. 200-400 μl extraction buffer (25 mM NaPi, pH6.6; 100 mM NaCl; 1 mM EDTA; 0.1% Triton X-100; 10 μg/ml leupeptin; 50 mM Sodium ascorbate (this was always added freshly, as it is unstable and will cause a dark orange color with time)) was added and the leaf sample was homogenised with a pestle (VWR Scientific Products, PA, USA) until the leaf material was finely divided. The extracts were then microcentrifuged at 13,000rpm for 5 minutes at 4°C. The supernatant was removed and aliquoted for ELISA or total protein assay, or stored at −70°C.

2.2.4.7 Potato microtuber protein extraction
The transgenic potato microtubers were homogenised with 400 μl of iced cold extraction buffer (25 mM NaPi, pH6.6; 100 mM NaCl; 1 mM EDTA; 1% Triton X-100; 10 μg/ml leupeptin; 50 mM Sodium ascorbate (added freshly as previous section) using pestle (VWR Scientific Products, PA, USA) or glass homogeniser (JENCONS, England, UK). The homogenate was then centrifuged at 13,000 rpm for 5 minutes at 4°C. The supernatant was removed and aliquoted for ELISA or total protein assay, or stored at −70°C.

2.2.4.8 NT-1 cells protein extraction
NT (Nicotiana tabaccum)-1 cell transformants were collected from NTKC plates and weighed. NT-1 cell transformants were homogenised in 2 ml/g of iced cold extraction
buffer (25 mM NaPi, pH6.6; 100 mM NaCl; 1 mM EDTA; 1% Triton X-100; 10 μg/ml leupeptin; 50 mM Sodium ascorbate (added freshly as previous section) using a glass homogeniser (JENCONS, England, UK). The homogenate was centrifuged at 13,000 rpm at 4°C for 5 minutes. The supernatant was aliquoted for ELISA, or total protein assays or stored at −70°C.
Chapter 3
HIV-1-Specific Mucosal Immune Responses In HIV-1 Infected And HEPS Individuals
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3.1 INTRODUCTION

Susceptibility to HIV-1 varies between individuals and some individuals are able to resist to infection despite multiple and repeated exposure to HIV (Rowland-Jones & McMichael 1995a; Shearer et al., 1996). This condition is likely to depend on the features of both the host and the virus (Shearer et al., 1996).

There is increasing evidence that an unconventional immune response, which is HIV-1 specific but detected in HIV-1 uninfected individuals, at both the cellular and humoral levels might be associated with HEPS status (Shearer et al., 1996; Burastero et al., 1996; Mazzoli et al., 1997). HIV-1 specific cell-mediated immunity has been detected in a high proportion of HEPS individuals (Rowland-Jones et al., 1995b, 1998, 2001). Paxton et al. (1996) found that the anti-HIV-1 activity of CD8⁺ lymphocytes in HEPS is greater than that of CD8⁺ lymphocytes from nonexposed controls and that their purified CD4⁺ lymphocytes were relatively resistant to HIV-1 infection in vitro. Studies based on rhesus macaques and chimpanzees and their resistance to low doses of HIV or SIV given intrarectally or intravaginally indicated that there may be local cellular mucosal immune response capable of protecting against challenges (Haynes et al., 1996). This leads to the hypothesis that a compartmentalised immune response to HIV is possible in humans. HIV-1 specific IgA was detected in the genital tract of 16 out of 21 (76%) HIV-1 EUs, 5 out of 19 (26%) infected women, and 3 out of 28 (11%) lower risk women (P<0.0001) (Kaul et al., 1999), indicating a role for mucosal HIV-1 specific response in HIV-1 resistance. Mucosal IgA, especially sIgA which is produced locally on mucosal surface (see section 1.3 for details), is present at mucous membrane surfaces, which are the main entry sites for most pathogenic organisms. Therefore the presence of these antibodies in the genital tract could offer a potent first-line defence against heterosexual transmission of HIV, and may contribute to HIV resistance in HEPS population.

In addition, in a proportion of HEPS, the resistance to infection has a genetic basis. This is suggested by the observation that a mutant form of the HIV coreceptor, CCR5, on the surface of monocyte/macrophages is present in a subset of such individuals (Liu et al., 1996). CCR5 has been identified as a major coreceptor for M-tropic HIV-1 entry (see
section 1.1.6.2) Population analyses have shown that individuals homozygous for the 32-bp deletion in the gene encoding CCR5 are rarely present in HIV infected population, indicating that mutant CCR5 confers resistance to HIV-1 infection in vivo as well as in vitro (Samson et al., 1996; Dean et al., 1996).

The majority of worldwide HIV-1 infections are the result of unprotected heterosexual contact (d'Cruz-Grote, 1996). The initial host-virus interaction in these populations will therefore occur at the level of the genital mucosa where the first defensive barrier consists of mucosal immunity, particularly IgA. It has been demonstrated that alteration of the normal genital mucosal barrier alters susceptibility to HIV-1 infection (Simonsen et al., 1988). HIV infects susceptible cells such as Langerhans cells, macrophages and T cells in the genito-urinary tract (Miller et al., 1996), stimulating a local mucosal immune response (Bélec et al., 1989a; Cao et al., 1990). In the most infected individuals, HIV-1 specific cytotoxic T cells (Musey et al., 1997) as well as IgG and IgA (Artenstein et al., 1997) are present in genital tract. Data concerning the contribution of mucosal immunity to protection against HIV-1 infection in HEPS population are limited. Therefore, an investigation of subtypes of antibodies, especially sIgA, to HIV-1 in genital secretions from seropositive and exposed seronegatives would be significant in understanding of mucosal immunity to HIV-1 and the mechanism of limiting HIV-1 transmission. A recent study has shown that HIV-1 specific IgA was detected in the genital secretions of HIV-1 resistant Kenyan sex workers. This strongly suggests that local mucosal immunity may have an important role in HIV-1 resistance (Kaul et al., 1999). The aim of this study was to evaluate the theory that HEPS individuals may develop a local anti-HIV antibody response.

3.2 MATERIALS AND METHODS

3.2.1 Materials

3.2.1.1 HIV-1 seropositive samples
All paired urine and serum and/or vaginal swab samples from HIV-1 seropositive individuals were collected by Dr David Yirrell (Centre for HIV Research, Edinburgh
All 3 HIV-1 seropositive serum samples (S1590, V3255 and D5206) for optimising Western blot experiments were kindly given by Dr David Yirrell (Centre for HIV Research, Edinburgh University, Edinburgh, UK) and are all from Scotland.

3.2.1.2 HEPS subjects and samples

One of the projects undertaken by the Medical Research Council Programme on AIDS in Uganda has been to study sexual mixing patterns between high HIV-1 prevalence (40% in general adult population (Nunn et al., 1996)) trading town, surrounding rural areas and fishing villages on Lake Victoria (Pickering et al., 1997a). HIV-1 high risk 143 men and 81 women were involved in a longitudinal study lasted for more than 18 months. All female individuals were known for selling sex and all men for high rates of partner change (Pickering et al., 1996; 1997b; 1997c). The men recorded an average of 3.1 sexual contacts a week, and the woman 5 sexual contacts a week (Pickering et al., 1997a).

9 of the above HIV-1 high risk individuals studied, including 2 females and 7 males who had reported HIV-1 seropositive regular partners, were selected for this study. An reported average unprotected sexual contacts among these subjects was 29 times over a period of 6 months (Pickering, H., personal communication). HIV-1 status was determined serologically using 2 EIA systems, Recombigen HIV-1 EIA (Cambridge Biotech Corporation, MA, USA) and Wellcozyme HIV-1 Recombinant (Wellcome Diagnostics, Dartford, England). All these 9 subjects remained HTV-1 seronegative, despite high exposure to HIV-1 as described above, and were thus defined as HEPS individuals.

All paired urine and serum samples from 9 HIV-1 HEPS individuals were collected by Dr David Yirrell (Centre for HIV Research, Edinburgh University, Edinburgh, UK). The details of subjects and samples are summarised in Table 3.1A. Control serum and urine samples were kindly donated by uninfected low risk individuals.
It has been reported that cell-debris in urine might cause false-positive HIV-1 antibodies in immuno assay (Kostolansky F and Friedman-Kien A.E, 1993). To avoid this false-positive reaction, urine samples were centrifuged and the concentrated supernatants (see section 2.1.2) were applied to Western blotting assay in this study.

3.2.2 Methods

3.2.2.1 Virus culture
HIV-1 IIIB was passaged in the H9 cell culture as described in section 2.2.1.2. After titration as described in 2.2.1.3, the virus stock, with TCID₅₀ from 10⁵ to 10⁶, was kept at -70°C until used.

3.2.2.2 EIA test
All serum samples used were tested with Wellcozyme HIV 1+2 EIA Kit (Murex Diagnostics Limited, Dartford, England) immediately after collection and were later re-tested by the same method at the Hepatitis and HIV Reference Lab of Medical School, Edinburgh University following the manufacturer’s instructions.

3.2.2.3 Western Blot
Both commercial and “in house” Western blotting strips were used to detect antibodies to HIV as described in 2.2.2.1.

For detection of sIgA, after incubation with serum, urine or swab samples, the nitrocellulose strips were incubated with the secondary antibody (mouse monoclonal anti-human secretory component (SC), pre-diluted 1:500 with antibody dilution buffer or sample buffer (supplied with the HIV-1 Western blot kit)) for 2 hours at room temperature under agitation. The strips were then washed and incubated with sheep anti mouse IgG (SAPU) (diluted 1:200 with antibody dilution buffer or sample buffer (supplied with the HIV-1 Western blot kit)) for 1.5 hours at room temperature with constant agitation. Then the HRP conjugated donkey anti sheep IgG was added and the peroxidase activity was detected colorimetrically by the addition of a fresh solution of
Table 3.1 Details of subjects and samples studied

A: HEPS subjects

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<td>41095</td>
<td>ND</td>
</tr>
<tr>
<td>134M</td>
<td>M</td>
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</tr>
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<td>111095</td>
<td>ND</td>
</tr>
<tr>
<td>C3221</td>
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<td>Edinburgh</td>
<td>B</td>
<td>90694</td>
<td>+</td>
</tr>
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<td></td>
<td></td>
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<td>90694</td>
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<td></td>
<td></td>
<td>S</td>
<td>90694</td>
<td>ND</td>
</tr>
<tr>
<td>14581</td>
<td>F</td>
<td>not known</td>
<td>Edinburgh</td>
<td>B</td>
<td>90295</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>U</td>
<td>90295</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S</td>
<td>90295</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Identification number of subjects studied.
*B=Serum; U=Urine; S=Swab.
*Date of sampling.
*Age at sampling
*Result of serum test by HIV 1+2 EIA Kit.
ND=not done
diamonoenzidine.

3.2.2.4 Dot immunobinding assay (DIA)
5 µl of different dilutions of recombinant HIV-1 gp120 (AIDS Reagent Project, MRC, UK) were dotted onto a strip of nitrocellulose (Sartorius, UK) and air dried at room temperature for 10 minutes. After incubation with mouse mAb to HIV-1 gp120 (AIDS Reagent Project, MRC, UK). The strips were washed as described in 2.2.3.1 and incubated with either HRP-conjugated sheep anti-mouse IgG (SAPU, Scotland) or sheep anti-mouse IgG (SAPU, Scotland) followed by HRP-conjugated donkey anti-sheep IgG (SAPU, Scotland). After washing, the peroxidase activity was detected colorimetrically by the addition of a fresh solution of 3,3-diamonobenzidine (DAB) tetrahydrochloride (10 mg (1 tablet) was dissolved in 15 ml of PBS, pH7.6; 12 µl of 30% hydrogen peroxide was added to the DAB solution prior to use). After incubation at room temperature for 15 to 30 minutes, the reaction was stopped by washing the strips 2 times with distilled water.

3.3 RESULTS

3.3.1 Optimisation of western blot
Western blot allows the detection of antibody directed to individual structural proteins or recombinant polypeptides of HIV and it therefore is widely used to analyse the antibody response to viral infection. In order to analyse the antibody recognition pattern in urine, serum and swab samples collected from MV-1 seropositive and HEPS individuals (see section 3.2.1.), Western blot was initially optimised as follows.

3.3.1.1 Polyacrylamide gel electrophoresis
Viral proteins were separated by SDS polyacrylamide gel electrophoresis with a Tris glycine buffer (50 mM Tris, 384 mM glycine) containing 0.1% SDS (Laemmli 1970). In order to separate wider size range of viral proteins, a 5-20% linear gradient polyacrylamide gel (30% acrylamide +1.08% bis acrylamide) in 375mM Tris, pH 8.7 containing 0.1% SDS was used. Samples, mixed with an equal volume of sample buffer, were boiled for 3min. 20µl of the mixture was loaded onto a well. Gels were run at 180v
for 1 hour and then stained by Coomassie Blue (in 20% methanol, 5% acetic acid) staining for 15 min at room temperature. Gels were destained in several changes of 20% methanol and 5% acetic acid. The result presented in Table 3.2 showed that gradient gel appeared to be effective in separating HIV-1 proteins.

3.3.1.2 Amplification system
In a further experiment, two amplification systems were compared using DIA to optimise the sensitivity of Western blot for detection of antibodies to individual HIV-1 viral proteins. After the nitrocellulose strip bearing viral recombinant HIV-1 gp120 was incubated with mouse mAb to HIV-1 gp120, the specific antibody was detected by incubation with either HRP-conjugated sheep anti-mouse IgG (SAPU, Scotland) (primary amplification) or being incubated with sheep anti-mouse IgG (SAPU, Scotland) followed by HRP-conjugated donkey anti-sheep IgG (SAPU, Scotland) (secondary amplification). The results presented in Table 3.3 demonstrated that the secondary amplification proved to be more sensitive than the primary amplification system. Therefore, the secondary amplification system was chosen for use throughout the project.

3.3.1.3 Antigens preparation
To obtain the whole range of individual viral proteins, HIV-1 IIIB was passaged in the H9 culture and then the viral antigens were purified from HIV-1 culture supernatant and infected cells (H9) as described in 2.2.2. The reactivity of the antigens obtained with HIV-1 seropositive serum (see section 3.2.1.1) was evaluated by Western blot. The results are summarised in Table 3.4. Both antigens made from culture supernatant and from infected cells were reacted with HIV-1 positive serum (see section 3.2.1.1). Antigens made from culture supernatants, were filtered through a 0.45 μm membrane microfilter (Sartorius) and then concentrated by passage through a 20% sucrose cushion (see section 2.2.1.4). Only two reaction bands corresponding to p65 and p55 appeared when the antigens were 2.5-fold concentrated. Reaction bands corresponding to p65, p55, p51, gp41, p31, and p24 appeared when the antigens were 10- and 80-fold concentrated, respectively, in the absence of bands corresponds to gp160, gp120 and
p17. However when antigens were 200-fold concentrated after purification by passaging through a 20 % sucrose cushion (see section 2.2.2.3) the specific bands associated with gp160, gp120, p65, p55, gp41, p24 and p17 were clearly observed. The antigens made from HIV-1 IIIB infected cells yielded a full range of viral polypeptides of HIV-1 including gp160, gp120, gp41, p65, p55, p24 and p17 but showed a strong unacceptably high background (data not shown). Therefore, the viral lysate from an HIV-1 IIIB culture supernatant was chosen to use as viral antigens for Western blotting assay.

3.3.2 HEPS state confirmation

Serum samples from HEPS population and serum controls were retested by HIV EIA to confirm HEPS state before performing Western blotting analysis. No HIV-1 specific antibodies were detected in the serum from either HEPS individuals or controls sera (Table 3.1).

3.3.3 Analysis of antibody recognition patterns in HIV-1 seropositive and HEPS individuals

3.3.3.1 Antibody response to HIV-1 antigens in HIV-1 seropositive individuals

To analyse the pattern of immune response to HIV, three paired urine and serum, and two corresponding vaginal swab samples were collected from HIV-1 seropositive individuals. The antibodies against individual viral polypeptides of HIV proteins were determined using Western blot assay.

The specific IgG and IgA to HIV-1 were analysed in paired sera, and urine (n=3) from HIV-1 positive individuals (Table 3.1). The results showed that multiple bands were detected in all the samples. Of three serum samples (n=3), specific IgG reactive bands to HIV-1 viral proteins gp160, gp120, p55, p51, gp41, p24 and p17 were detected in all samples with specific IgG reactivity bands to gp65 and p31 in two out of three tested samples. For IgA reaction, all serum samples (n=3) showed specific bands corresponding to viral protein p51 in the absence of reactivity to gp160; two of three serum samples showed IgA reactivity to gp120, p55, p31, p24 and p17, and IgA reactivity to either p65 or gp41 was detected in only one of three serum samples (Figure 3.1, Table 3.5 and 3.6). No bands were detected in the serum control samples.
**Table 3.2** Detection of HIV-1 proteins following SDS-PAGE (5-20%)

<table>
<thead>
<tr>
<th>HIV-1 proteins</th>
<th>5-20% gradient SDS-PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp 160</td>
<td>+*</td>
</tr>
<tr>
<td>gp120</td>
<td>+</td>
</tr>
<tr>
<td>p65</td>
<td>+</td>
</tr>
<tr>
<td>gp41</td>
<td>+</td>
</tr>
<tr>
<td>p31</td>
<td>+</td>
</tr>
<tr>
<td>p24</td>
<td>+</td>
</tr>
<tr>
<td>p17</td>
<td>+</td>
</tr>
</tbody>
</table>

*+ = positive

**Table 3.3.** Comparison of amplification system by DIA

<table>
<thead>
<tr>
<th>Amplification system</th>
<th>Dilution of recombinant HIV gp120</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/5</td>
</tr>
<tr>
<td>Primary amplification*</td>
<td>++++</td>
</tr>
<tr>
<td>Secondary amplification**</td>
<td>++++</td>
</tr>
</tbody>
</table>

* HRP conjugated sheep anti-mouse IgG as secondary antibody.

** Sheep anti-mouse IgG as secondary antibody.

+ = positive; the numbers of plus signs indicates strength of reaction.

- = negative

**Table 3.4** Qualitative comparison of viral proteins present in culture supernatants (spts) and cell lysates of HIV-1 IIIB-infected H9 cells

<table>
<thead>
<tr>
<th>Concentration of HIV-1 IIIB culture spts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral proteins</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>160</td>
</tr>
<tr>
<td>120</td>
</tr>
<tr>
<td>65</td>
</tr>
<tr>
<td>55</td>
</tr>
<tr>
<td>51</td>
</tr>
<tr>
<td>41</td>
</tr>
<tr>
<td>31</td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>17</td>
</tr>
</tbody>
</table>
In corresponding urine samples (n=3) multiple HIV specific bands were also observed. All three urine samples had the specific IgG reactive bands corresponding to HIV-1 viral protein gp41, p31 and p24. Of three urine samples, two had IgG reactive bands to gp160, gp120, p55, p51 and p17, and one had IgG reactive bands to p65 (Table 3.5). For IgA reaction, specific IgA bands corresponding to viral proteins gp41 and p24 were detected in all of 3 urine samples but no reactive bands to gp160, p65 and p17 were observed. Both urine samples from patients C3321 and I4581 had IgA reactive bands to p55 and p51 (Table 3.6), and IgA reactive bands to gp120 and p31 were only detected in urine samples from patient I4581 (Figure 3.1, Table 3.5 and 3.6).

In addition to the result in serum and urine samples, specific IgG and IgA to HIV were also analysed in two corresponding vaginal swab samples from HIV-1 seropositive individuals C3321 and I4581 (Table 3.1). As shown in Figure 3.1, Table 3.5 and 3.6, both vaginal swab samples had the specific IgG bands corresponding to HIV-1 viral proteins gp120, p55, p51, gp41, p31 and p24 in the absence of bands corresponding to gp160 and p65. Specific IgG band corresponding to p17 was detected in only one of two samples tested. For IgA reaction, two bands corresponding to viral proteins gp41 and p24 were detected in both vaginal swab samples, and bands corresponding to viral gp120, p51 and p31 was detected in one of two samples, respectively.

The mucosal immune response to HIV in these samples was investigated by measuring sIgA, because sIgA serves an important effector function in mucosal immunity (see section 1.3.2). The results showed that specific sIgA to HIV-1 p24 were detected in all of three urine samples, two out of three serum samples and one out of two vaginal swab samples (Figure 3.1, Table 3.5 and 3.6). Specific sIgA to HIV-1 gp160, p65 and gp41 was only detected in urine sample from patient 134M but not C3321 and I4581, and sIgA to p31 was only detected in urine sample from patient I4581 but not the others.
For patient C3321, specific sIgA to HIV-1 p24 were found in all of urine, serum and vaginal swab samples collected. In contrast, specific sIgA reactive bands to HIV-1 p31 and p24 were detected only in urine sample but not in the corresponding serum and vaginal swab samples from patient 14581. For patient 134M, specific sIgA antibody to HIV-1 viral gp 160, p65, p55, p51, gp41, p31, p24, and p17 were detected in the serum but only specific antibodies to HIV-1 gp160, p65, gp41 and p24 were detected in the corresponding urine samples.

3.3.3.2 Antibodies to HIV-1 antigens in paired serum and urine samples from HIV-1 HEPS individuals

To investigate the systemic and mucosal humoral immune responses in HEPS individuals, anti-HIV-1 IgG, IgA and sIgA antibodies were analysed in paired serum and urine samples collected from 9 of HIV-1 HEPS individuals (Table 3.1) using Western blot assay. Healthy control (low risk) samples were used as negative control.

The multiple HIV specific bands were detected, as shown in Figure 3.2, 3.3, Table 3.7 and 3.8. Of 9 serum samples from HEPS individuals, 4, 4 and 5 showed IgG reactive bands to HIV-1 gp160, gp120 and p51, respectively. One and one showed IgA reactive bands to HIV-1 gp120 and p51, respectively. A similar pattern of IgG reactivity in urine samples from HEPS was found. Of 9 concentrated urine samples (see section 2.1.2), IgG reactive bands corresponding to HIV-1 gp160, gp120 and p51 were detected in two samples from individuals 100M and 008C; and IgA reactive bands corresponding to HIV-1 p51 viral protein was detected in two samples from 085W and 130M.

In exploring the mucosal immunity, only one single sIgA band corresponding to HIV-1 p51 was detected in one serum sample but not in the corresponding urine sample. However, specific IgG antibodies to HIV-1 gp160, gp120 and p51 were only detected in urine but not serum sample from individual 008C. Moreover, specific IgA antibody to HIV-1 p51 was detected only in urine not in the
corresponding serum sample from 130M. Surprisingly, of 4 control urine samples, one showed IgA reactive band corresponding to HIV-1 p51. No bands were present in the control serum samples (Figure 3.2, 3.3; Table 3.8).

3.4 DISCUSSION

3.4.1 Optimisation of western blot

There are a variety of methods for HIV antibody detection, currently including EIA and Western blot. In this project, Western blot was used to analyse the antibody pattern to individual antigens of HIV-1 in paired urine and serum or corresponding vaginal swab samples from both HIV-1 infected and HEPS individuals.

The quantity of antigens is critical to produce western blotting of great specificity. To obtain good discrimination between viral proteins, viral antigens were made from the virus culture supernatant and infected H9 cells. The result showed that HIV gp160, gp120, p65, p55, p51, gp41, p31, p24 and p17 were released from both supernatant lysate and infected cell lysate. However, it is clear that supernatant lysate was superior to cell lysate because the cell lysates produced more non-specific bands and dark background caused by nonviral proteins derived from the host cells (data not shown). These nonviral protein bands made interpretation of results difficult by producing nonspecific reactions. The supernatant lysate was thus chosen as a source of viral antigens and thereafter applied to the SDS-PAGE and blotting in this study.

To obtain the greater sensitivity of Western blot, two amplification systems were investigated using DIA. HRP-conjugated secondary antibody and HRP-conjugated third antibody were used as detection systems in primary amplification system and secondary amplification system, respectively. The results showed that the sensitivity of DIA employing the secondary amplification system could be increased more than 5 fold over that of DIA employing the primary amplification system. This is due to one more antibody involvement
Figure 3.1 Analysis of different classes of anti-HIV antibodies in serum, urine and vaginal swab from HIV-seropositive individuals by Western blot

Western blot was performed as described in Chapter 2.

A: Serum, urine and vaginal swab samples from two HIV-1seropositive individuals
B: Serum and urine samples from one HIV-1seropositive individual
A

<table>
<thead>
<tr>
<th></th>
<th>I4581</th>
<th>C3321</th>
</tr>
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<tbody>
<tr>
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<td></td>
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<tr>
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B

<table>
<thead>
<tr>
<th></th>
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<th>134M</th>
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</thead>
<tbody>
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</tr>
<tr>
<td>p17</td>
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</tr>
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</table>
Figure 3.2 Analysis of different classes of anti-HIV antibodies in the paired serum and urine from five HEPS individuals by Western blot.

Western blot was performed as described in Chapter 2.

M: Blotting broad range protein markers.
Table 3.5 Presence of IgG, IgA and sIgA antibodies to HIV-1 antigens in 3 paired serum and urine, 2 corresponding vaginal swab samples from HIV-1 seropositive individuals

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<tr>
<th>HIV-1 Antigens</th>
<th>Number of samples</th>
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<tr>
<td></td>
<td>IgG</td>
</tr>
<tr>
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<td>p55</td>
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<tr>
<td>gp41</td>
<td>3</td>
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<td>p24</td>
<td>3</td>
</tr>
<tr>
<td>p17</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 3.3 Analysis of the paired serum and urine from four HEPS individuals by Western blot.

Western blot was performed as described in Chapter 2.

N: Healthy (low risk) control.
P1: Low positive control.
P2: High positive control.
Urine Samples

Serum Samples
<table>
<thead>
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<th>Viral antigen</th>
<th>gp160</th>
<th>gp120</th>
<th>p65</th>
<th>p55</th>
<th>p51</th>
<th>gp41</th>
<th>p31</th>
<th>p24</th>
<th>p17</th>
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<td>+</td>
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<td>Serum IgA</td>
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<td>Serum sIgA</td>
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<tr>
<td>Serum sIgA</td>
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<tr>
<td>Swab* IgG</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>Swab* IgA</td>
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<tr>
<td>Swab* sIgA</td>
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</tr>
</tbody>
</table>

*Vaginal swab samples

Table 3.6 Antibody patterns in HIV-1 seropositive individuals
Table 3.7 The presence of IgG, IgA and sIgA antibodies to HIV-1 in paired serum and urine samples from 9 HEPS individuals and 2 serum, 4 urine controls

<table>
<thead>
<tr>
<th>HIV-1 Antigens</th>
<th>IgG</th>
<th>IgA</th>
<th>sIgA</th>
<th>IgG</th>
<th>IgA</th>
<th>sIgA</th>
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Table 3.8 Antibody patterns in HEPS individuals

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amplifying a weak reactive signal which could not be detected by primary amplification system.

3.4.2 Antibody response to HIV-1 in seropositive individuals

As initial exposure to HIV occurs most frequently at the genital mucosa, it emphasises the need to understand the nature of HIV-specific mucosal immune response, especially those that could lead to protection against HIV infection. To address this, the mucosal antibody responses to HIV in both HIV-1 seropositive and HEPS individuals were investigated. The results showed that HIV-specific IgG, IgA and sIgA antibodies can be present in urine and vaginal swab samples of HIV-1 infected individuals, although the antibody pattern to individual viral polypeptides of HIV-1 varied with sample tested by Western blot assay.

SIgA is locally produced in mucosal epithelial cells and then transported into external secretions selectively (Rudzik et al., 1975; Mestecky et al., 1987; Brandtzaeg 1989) (see section 1.3.2.2). In this study, sIgA bands corresponding to viral gp160, p65, gp41, p31 and p24 were shown in urine samples and sIgA to p24 in vaginal swab sample from HIV-1 seropositive individuals (Table 3.5 and 3.6). These findings demonstrated that a mucosal immune response was induced by HIV infection. These results also supported previous reports that local antibody was produced in male and female genital tracts as a result of HIV infection and could therefore play an efficient role in neutralising HIV virus and limiting the virus transmission on normal mucosa (Bélec et al., 1989a; 1994a; 1995a; Kutteh et al., 1994; Archibald et al., 1987; O'shea et al., 1990; Wolff et al., 1992; Hocini et al., 1997).

Interestingly, specific sIgA antibodies to HIV-1 viral gp 160, p65, p55, p51, gp41, p31, p24, and p17 were also detected in the serum sample from patient 134M and sIgA against p24 in C3321 (Table 3.6). In contrast to IgG, human IgA displays a unique heterogeneity in its molecular forms. There are known to be two subclasses of IgA, IgA1 and IgA2. Serum IgA is predominantly monomeric IgA1. The IgA in external
secretions, called sIgA consists of a dimer or tetramer with a relative increase in the proportion of IgA2 which are produced locally on mucosal surface (See section 1.3 for details). The reason for the presence of sIgA in serum in the present study is unknown. It is possible that the presence of specific sIgA antibodies in serum in this study could be the result of a backflow of sIgA locally synthesised in genital mucosa by an unknown pathway. It has been reported that the increased level of polymeric IgA in serum during lactation can be the result of a backflow of IgA produced in the mammary glands (Halsey et al., 1982).

IgA antibody is the predominant immunoglobulin on the mucosal surface (Mestecky et al., 1987). However, it may not be true for HIV infection. The results of this study showed that IgG is the predominant isotype in urine and vaginal swab samples where IgG antibodies against gp160/120, p55, p65/51, p41, p31, p24 and p17 were present. As the HIV specific IgG antibody pattern in paired urine and vaginal swabs samples were not similar to that in the corresponding serum samples, it is unlikely that the source of these HIV-1-specific IgG antibodies are through serum transudation as suggested by some reports (Wolf et al., 1992; Black et al., 1997). It has been demonstrated that IgG could be produced locally (Morgan et al., 1980; Heinen et al., 2000). Therefore it is assumed that the predominant IgG antibody to HIV-1 present in urine and vaginal swab samples in the present study was locally produced. This finding is similar to the previous reports that IgG antibodies to HIV-1 appeared to be largely synthesised in situ within the genital tract of both genders (Bélec et al., 1995b; Hocini et al., 1997) and IgG excretion was increased in HIV-1 infected women, whereas the IgA secretion tended to decrease, suggesting a possible enhanced local IgG synthesis (Bélec et al., 1995c; Lu et al., 1993). This hypothesis was also supported by the observations in animal models that nonclassical mucosal antibodies of the IgG isotype were found to be the predominant antibody in the saliva, rectal swabs, vaginal washes, semen and urethral washes in HIV-1 infected chimpanzees (Israel and Marx, 1995), and specific SIV IgG antibodies were found to be predominant immunoglobulin in vaginal swab samples of chronically infected rhesus macaques (Miller et al., 1992a). It has also been
reported that the genital secretory immune response of rhesus macaques following intravaginal exposure to antigens consists primarily of IgG (Yang and Schumucher, 1979). Thus it is possible that IgG is the predominant antibody response on the mucosal surface in human subjects in response to HIV infection.

3.4.3 Antibody response to HIV-1 antigens in HIV-1 HEPS individuals

The natural immune protection mechanisms preventing HEPS population becoming infected are still unclear. But it has been suggested that mucosal immunity may play an important role in protecting from HIV infection (see section 1.1.2.4). To address this, the humoral immune responses in paired serum and urine samples of 9 HEPS individuals were investigated. Their seronegative status was demonstrated by immunoassay (EIA) after collecting the paired serum and urine samples, and furthermore confirmed before performing Western blot in this study. Systemic and mucosal immunity in these HEPS individuals were investigated by profiling the specific IgG, IgA and sIgA antibodies to viral proteins of HIV-1 in paired serum and urine samples.

In exploring HIV-1 specific sIgA in HEPS individuals, it was observed that no HIV specific sIgA bands were common to all 9 urine samples. However, specific IgG antibodies to HIV-1 gp160, gp120 and p51 were detected in urine but not the corresponding serum sample from individual 008C. Similarly, specific IgA antibody to HIV-1 p51 was detected only in urine not in the corresponding serum sample from individual 130M (Table 3.8). These results suggest that mucosal immunity was present in some HEPS individuals. The role of local anti-virus antibodies is unknown. It is generally believed that Langerhans cells located in the mucosal epithelium are the mucosal target cells for HIV-1 and thus attract HIV-1 infection following sexual exposure (Pope, 1999; Chou et al., 2000). The presence of anti-HIV-1 local antibodies in the genital tract may act as an effector mechanism to prevent HIV-1 infection, and therefore, play an important role in protecting HEPS individuals against sexual transmission of HIV-1.
There were more bands recognised by IgG antibody than IgA, suggested that IgG is the predominant isotype in genitourinary tracts in these subjects. These findings are similar to the antibody production pattern in immunised animal model that a higher level of vaginal IgG than that of vaginal IgA was induced in mice when inoculated vaginally with a DNA vaccine, suggesting that IgG producing B cells within the vaginal mucosa may be preferentially stimulated by HIV-1 envelope antigen (Wang et al., 1997). A similar result was also observed in a macaque model in which the iliac lymph node was immunised with a subunit SIV envelope and core vaccine (Lehner et al., 1996). Together with increasing IgA antibody-secreting cells to p27, IgG antibody-secreting cells to p27 and gp129 were also increased in the iliac lymph nodes of the immunised and protected macaques. However, this observation is not the same as previous studies on HEPS population where HIV specific IgA but not IgG was present in urine and vaginal wash samples from HIV-exposed seronegative individuals and HIV-1 resistant sex workers (Mazzoli et al., 1997; Kaul et al., 1999). The reason for this discrepancy remains unclear but may be related to the sample used and the background of individual studied. It should be considered that both HIV-1 specific IgG and IgA may be produced locally and associated with protection from HIV infection in HEPS population.

Interestingly, a weak sIgA band to HIV-1 viral p51 was detected in one of 9 serum samples (Table 3.7). It is clear that sIgA is secreted at mucosal sites (Rudzik et al., 1975; Brandtzaeg et al., 1989). Therefore, this HIV-1 specific sIgA antibody found in serum sample can be considered to be synthesised locally in genital mucosa and transported back to serum from the mucosal production site by an unknown mechanism. Furthermore, this finding also demonstrated that mucosal immunity was induced in this HEPS individual. The reason for absence of sIgA in the urine sample is unclear but might related to the low concentration of secretory antibody present in urine samples.

Surprisingly, HIV-1 specific IgA band to viral p51 was found in one urine control sample from a low risk uninfected individual. This might be due to non-specific cross reactive antibodies. The reason for producing this non-specific band is unclear. It was
reported that the presence of non-specific cross-reactive antibodies in HIV-1 WB was possibly due to recent immunisation; autoantibodies (i.e. antinuclear antibodies and rheumatoid factor); lymphoma or liver disease (Ranki et al., 1988; Kleinman et al., 1988; Dock et al., 1988; 1991; Diagnostic test for HIV, 1997). There also appear to be healthy persons with antibodies that cross-react with specific HIV-1 peptides or recombinant antigens (Busch et al., 1991; Blomberg et al., 1990). These antibodies may have arisen because of molecular mimicry by chance or by functional selection or immunisation following activation and noninfectious exposure (Blomberg et al., 1990).

Specific IgG antibodies to HIV-1 viral antigens gp160, gp120 and p51 were found in four serum samples from HEPS subjects, unexpectedly (Figure 3.2 and Table 3.8). These results may be interpreted as indeterminate according to CDC (US Centre for Disease Control, Atlanta, GA) Western blot criteria which require reactivity to at least two of the following antigens: gp160/120, gp41 and p24 for a positive classification. This positive criterion is currently followed by most institutions. However, it is dissimilar to most indeterminate Western blot results which show only weak reaction to the gag proteins (mostly p17, p24 and/or p55) (Constantine, 1998). There is evidence that an indeterminate Western blot result is not predictive of early infection. Some individuals may have the identical profile for long periods of time (years) but never seroconvert (Constantine, 1998). Jamjoom et al. (1997) reported that two hundred and fourteen Western blot-indeterminate samples were followed up by retesting the subsequent samples from the same patients with Western blot over a 3-12 month period. The results showed that 66.4% samples gave negative results and 30% samples remained indeterminate. Thus the gp160, gp120 and p51 bands, even when they appear as indeterminate results, may indicate a humoral immune response induced by exposure to HIV and might play a role in protecting HEPS individuals from infection with HIV. However, these indeterminate results need to be repeated over years and other tests should be involved, so that the above suggestion could be further investigated.
3.4.4 Conclusion

The systemic and mucosal humoral immunity in HIV-1 infected and HEPS individuals was profiled using Western blot in this study. The results demonstrated that a mucosal immune response was induced by HIV-1 infection and HIV-1 specific IgG is the predominant isotype present in genitourinary tract of HIV-1 infected individuals, which may play an efficient role in neutralising HIV virus and limiting the virus transmission on normal mucosa.

Mucosal humoral immunity was demonstrated in some HEPS individuals. Also, HIV-1 specific IgG antibodies were the predominant isotype present in genitourinary tract of HEPS individuals. These results suggested that anti-HIV-1 specific mucosal immune response may be associated with a natural protection mechanism in HEPS populations and provide insight into development of a HIV-1 mucosal vaccine.
Chapter 4

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4.1 INTRODUCTION

The HIV env gene encodes a single-chain precursor gp160 which is subsequently cleaved to external surface envelope glycoprotein (gp120) and transmembrane glycoproteins (gp41). As described in Chapter 1, the HIV gp160 plays a pivotal role in the early events of virus attachment and entry into the target cell. The gp120 molecule mediates the attachment of the virus to the cell-surface CD4 receptor and is involved in recognition and binding to the target cell receptors (Sattentau et al., 1993). Therefore, HIV-specific neutralising antibodies found in the sera of infected individuals are predominantly directed against this glycoprotein (Matthews et al., 1986), and antibodies to HIV gp160 were detected in genital secretions, urine samples, tears, milk, nasal washing, intestinal fluid, cervical fluid and saliva (Bélec et al., 1989a; O'shea et al., 1990; Sun 1990; Thongcharoen et al., 1992; Wolff et al., 1992; Constantine et al., 1994; Janoff et al., 1994; Mestecky et al., 1994), suggesting that gp160 is an antigenic target during immune responses to HIV infection. The presence of neutralising antibodies might inhibit the transmission of HIV infection in vivo. Based on these studies, HIV gp 160 was selected as an immunogenic antigen for a HIV vaccine.

Considerable progress has been made in the development of vaccines against HIV. In animal models, it has been shown that oral and intranasal immunisation with HIV gp160 induced high levels of IgA, IgM and IgG in serum, and secretory IgA at the mucosal surfaces (Thibodeau et al., 1992; Muster et al., 1995; VanCott et al., 1998; Staats et al., 1996), indicating that mucosal immunisation could induce a mucosal immune response (Lehner et al., 1992; Mestecky et al., 1994; Morrow et al., 1994; Bond et al., 1995; Bukawa et al., 1995; Wu et al., 1997; Kaneko et al., 2000). Neutralisation tests showed that HIV specific IgA was capable of neutralising HIV-1 IIIB, HIV-1 SF2 and HIV-1 MN (Bukawa et al., 1995). This result suggests that Env protein delivered orally in combination with cholera toxin adjuvant can effectively induce HIV-specific immune response in mucosal tissues and may thus provide protection against HIV-1 transmission to mucosal surfaces.
Transgenic plants comprise a new vaccine production system that does not require purification or injection and provide the possibility that a fresh or pureed fruit such as banana and tomato could replace the needle as a means of vaccinating children and adults against deadly diseases. Progress in the development of transgenic plants can be attributed to the exploitation of the Agrobacterium transformation mechanism and has resulted in the design of modified, non-tumourigenic Agrobacterium Ti plasmids for the engineering of transgenic plants. Agrobacterium tumefaciens is the causal agent of plant crown gall disease and can genetically transform wounded plant cells, and thus provides an ideal vector systems for the generation of transgenic plants. The abnormality of the plant growth is due to a relatively small discrete portion of large tumour-inducing (Ti) plasmids which are transferred to plant cells. Integration to the plant genome and expression of this transferred DNA (T-DNA) results in the crown gall phenotype (Binns and Thomashow, 1988; Klee et al., 1989; Depicker et al., 1991). Two regions were found critical for gene transfer in Ti plasmids. The first region is known as the virulence (vir) region containing genes which are essential for gene transfer, but are not themselves generally transferred to the plant genome. The second important region is the T-DNA. The Agrobacterium/plant interaction is coordinated by a biochemical communication process between susceptible plant cells and the infecting bacteria that is mediated by the gene products from the vir region on the Ti plasmid. Sensing of the plant cell-derived signal molecules by the bacteria leads to the excision of the T-DNA from the Ti plasmid, followed by subsequent transfer and integration in the infected plant cell genome (Zambryski, 1992). Exploitation of Agrobacterium transformation mechanism resulted in the design of modified, non-tumourigenic Agrobacterium Ti plasmids for the engineering of transgenic plants. Basically, two types of non-oncogenic Agrobacterium vectors are presently in use. Vectors which recombine via DNA homology into a resident Ti plasmid are often referred to as integrative or intermediate vectors (Fraley et al., 1983). The finding that the T-DNA could be separated from the vir genes onto two separate replicons in Agrobacterium led to the development of the second type of vector which do not need to recombine into the resident Ti plasmid and
are referred to as binary or autonomous vectors (An et al., 1985; Klee et al., 1985). Binary vectors are now the most commonly used vector system for creating and regenerating transgenic plants. These vectors contain a number of unique restriction endonuclease sites which can be used for inserting the foreign genes of interest and plant selectable marker(s) between the T-DNA borders and a cloning vehicle capable of replicating in *E. coli* and *Agrobacterium*. The recombinant plasmids containing the foreign gene of interest are transformed into *E. coli*, and subsequently mobilised into an *Agrobacterium* strain harbouring a Ti plasmid (either wild-type, or a "disarmed" Ti plasmid in which the T-DNA was deleted). They are replicated in *Agrobacterium* usually with a copy number of 5-10 relative to the Ti plasmid and bacterial chromosome. The vir genes resident within *Agrobacterium* can process and mobilise the T-DNA from the bacterium to a plant cell (Ooms et al., 1982; Hood et al., 1986).

As discussed in Chapter 1, the transgenic plant edible vaccines have shown to be a potential new generation of novel, encouraging and prospective vaccine production and delivery systems with its distinguishing characteristics including inexpensive production, unnecessary preparation or purification and easy transportation and storage. Plant based oral vaccines may offer a new approach to vaccination strategies, in particular, in cases where a local immune response is crucial in the prevention of infections. So far viral antigens from hepatitis virus B (HbsAg) (Mason et al., 1992; 1995), norwalk virus (Mason et al., 1996), foot-and-mouth disease virus (Carrillo et al., 1998), hemorrhagic disease virus (RHDV) (Castanon et al., 1999), and porcine transmissible gastroenteritis virus (TGEV) (Tuboly et al., 2000) have been successfully expressed in transgenic plants. Immunisation with recombinant viral proteins expressed in transgenic plants induces a protective immune response in animals. The aim of this work was to clone the whole of the region encoding HIV-1 gp 160 and construct a recombinant transgenic plant expression vector containing this gene. Plants expressing these recombinant proteins would than be used for studies on the immunoreactivity.
4.2 MATERIALS AND METHODS

4.2.1 Materials

4.2.1.1 pCR-Script™ Amp SK(+) cloning vector
The pCR-Script™ Amp SK(+) cloning vector (Stratagene Ltd., Cambridge) is a 2961 base pair (bp) plasmid, derived from the pBluescript® II SK(+) phagmid. The pCR-Script™ Amp SK(+) cloning vector contains an ampicillin-resistance gene, a lac promoter for gene expression and the SK multiple cloning site (MCS), which is modified to include the novel rare-cleavage restriction-enzyme Srf I target sequence 5'-GCCC|GGGC-3' (Figure 4.1).

4.2.1.2 HIV-1 plasmid pRNBXX
The HIV-1 plasmid pRNBXX was made by Dr Sarah Ashelford (Centre for HIV research, Edinburgh University). This 6073 bp plasmid contains the HIV-1 env 160 sequence, an ampicillin-resistance gene and the target sequences of restriction-endonucleases Not I, BstE I, XhoI and XbaI (Figure 4.2).

4.2.1.3 Plant plasmids
Plants plasmids pIBT210.1, pIBT240.1, pIBT110 and pIBT140 were a kind gift from Dr Hugh Mason (Boyce Thompson Institute for Plant Research, USA).

pIBT210.1 (4051 bp) contains the cauliflower mosaic virus 35S promoter (5' CaMV 35S), which is plant leaf specific. pIBT240.1 (5554 bp) uses a potato tuber-specific expression promoter, patatin. Both pIBT210.1 and pIBT240.1 have a tobacco etch virus 5' untranslated region (TEV 5' UTR) which mediates enhancement of translation initiation, a polyadenylation signal of a soybean vegetative storage protein gene (3'vspB) which is needed to mediate mRNA 3' end processing, an ampicillin-resistance gene and multiple cloning sites which include NcoI, SmaI, BamHI, KpnI and SacI (Figure 4.3 and Figure 4.4).
Both pIBT110 and pIBT140 are T-DNA binary vectors and contain the left and right T-DNA borders (LB, RB) which denote the limits of the DNA that is integrated into the plant genomic DNA via Agrobacterium tumefaciens-mediated transformation. They also contain the neomycin phosphotransferase \((nptII)\) for selection of plant transformants using kanamycin, a viral (TEV) 5'-UTR for translation enhancement, the 3' end from soybean \((3'vspB)\) and multiple cloning sites which include SmaI, BamHI, KpnI and SacI. pIBT110 (13150 bp) contains a CaMV 35S promoter that is plant leaf specific (Figure 4.5), while plasmid pIBT140 (14652 bp) uses a potato tuber-specific expression promoter \(pata\)tin (Figure 4.6).

4.2.1.4 Plant expression vector pGPTV-KAN

Plant plasmid pGPTV-KAN (glucuronidase plant transformation vector) was kindly given by Dr Elke Kemper (Max-Planck-Institut für Züchtungsforschung, Carl von Linné Weg 10, D-5000 Köln 30, Germany). This plasmid is a 13387 bp plant binary T-DNA vector derived from the plant binary vector pBIN19 which is the most widely used binary vector for Agrobacterium-mediated plant transformation (Bevan 1984). The β-glucuronidase \((uidA)\) reporter gene it contains can be used for promoter studies, or may be exchanged for any other gene of interest as desired. The plant selectable marker gene \(nptII\), which confers kanamycin resistance to plants, is driven by the nopaline synthase (nos) promoter and located near the T-DNA left border so that the selectable marker and \(uidA\) reporter genes are divergently organized for efficient expression (Becker et al., 1992). The plasmid contains unique cloning sites EcoRI, SmaI, XbaI, SalI and HindIII (Figure 4.7).

4.2.2 Methods

The general molecular biology methods were performed as described in 2.2.3.

4.2.2.1 \(Pfu\) PCR

Plasmid pRNBXX containing the whole \(env160\) sequence was used as template for
Figure 4.1 Circular map of the pCR-Script Amp SK(+) cloning vector

The pCR-Script Amp SK(+) cloning vector is a 2961 bp plasmid designed to insert blunt-ended PCR products at SrfI site. CoIE1 origin: Plasmid origin of replication. LacZ gene: allowing blue-white screening of recombinants. MCS: Multiple cloning site. Ampicillin: ampicillin-resistance gene (Amp') for antibiotic selection of the cloning vector (adapted from instruction manual of pCR-Script™ Amp SK(+) cloning kit, Stratagene Ltd., UK)

Figure 4.2 Diagrammatic representation of plasmid pRNBXX

Plasmid pRNBXX is a 6073 bp plasmid containing the whole env160 sequence (481-3054) of HXB2.

Figure 4.3 Diagrammatic representation of plant plasmid pIBT210.1

pIBT210.1 (4051 bp) contains the cauliflower mosaic virus 35S promoter (35S 5') which is plant leaf specific, a tobacco etch virus 5' untranslated region (TEV), a polyadenylation signal of a soybean vegetative storage protein gene (3'vspB) and the multiple cloning sites including NcoI, BamHI, SmaI, KpnI and SacI.

Figure 4.4 Diagrammatic representation of plant plasmid pIBT240.1

pIBT240.1 (5554 bp) contains a potato tuber-specific expression promoter patatin, a tobacco etch virus 5' untranslated region (TEV), a polyadenylation signal of a soybean vegetative storage protein gene (3'vspB) and multiple cloning sites including NcoI, BamHI, SmaI, KpnI and SacI.
Plasmid pIBT110 is a 3150 bp plant binary vector. This plasmid contains an expression cassette for neomycin phosphotransferase \( (nptII) \) located near the right border of T-DNA for selection of plant transformants using kanamycin, cauliflower mosaic virus \( 35S \) promoter \( (35S\ 5') \), a tobacco etch virus \( 5' \) untranslated region (TEV), a polyadenylation signal of a soybean vegetative storage protein gene \( (3'vspB) \) and multiple cloning sites including Smal, KpnI and SacI. RB and LB indicate the right and left transferred DNA (T-DNA) borders.

Plasmid pIBT140 is a 14652 bp plant binary vector. This plasmid contains a expression cassette for neomycin phosphotransferase \( (nptII) \), located near the right border of T-DNA for selection of plant transformants using kanamycin, the plant tuber-specific promoter \( patatin \), a tobacco etch virus \( 5' \) untranslated region (TEV), a polyadenylation signal of a soybean vegetative storage protein gene \( (3'vspB) \) and the multiple cloning sites including Smal, KpnI and SacI. RB and LB indicate the right and left transferred DNA (T-DNA) borders.

pGPTV-KAN (glucuronidase plant transformation vector) is a 13387 bp plant binary T-DNA vector. The \( \beta \)-glucuronidase \( (uidA) \) reporter gene can be exchanged by any other gene of interest as desired. The plant selectable marker gene neomycin phosphotransferase \( (nptII) \) is located near the T-DNA left border so that the selectable marker and \( uidA \) reporter genes are divergently organized for efficient expression. The plasmid contains unique cloning sites EcoRI, Smal, XbaI, SaI and HindIII. RB and LB indicate the right and left transferred DNA (T-DNA) borders.
amplification of HIV-1 specific env DNA fragments. The PCR reaction conditions were as follows: 4 ng of plasmid template and 100 ng of each of N160F/S160R primers (see section 2.2.4.19e) were used per reaction. PCR mixture consisted of 200 mM Tris-HCl (pH 8.0), 60 mM ammonium sulphate, 20 mM MgCl$_2$, 100 mM KCl, 1% Triton® X-100 and 100 μg/ml nuclease-free BSA, 200 μM each dNTP (0.8 mM total) and 2 units of cloned Pfu DNA polymerase (Stratagene Ltd., Cambridge, UK). A control reaction was set up which lacked any DNA template. All reaction mixtures, including the control, were made up to 40 μl with DEPC (Diethyl procarbamine) treated distilled water (dH$_2$O) and overlaid with one drop (or~20 μl) of paraffin oil to prevent evaporation. This reaction mixture was initially denatured at 94°C for 45 seconds before 2 units of Pfu DNA polymerase was added, followed by thirty cycles of 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 6 minutes, and one extension cycle of 72°C for 10 minutes, on GENEE Thermal Cycler or PCR Express (Hybaid). 8 μl samples of each PCR reaction were analysed on a 1% TAE agarose gel by electrophoresis (see section 2.2.3.2).

4.2.2.2 Ligation of DNA fragments in gel slices

After digestion with the appropriate restriction endonucleases, the treated DNA was subject to electrophoresis in 0.6% (in 1 X TAE buffer) low melting temperature agarose gel (Flowgen, Staffordshire, UK) (see section 2.2.3.2). The desired bands containing DNA were then cut out in the smallest possible volume with a clean razor blade, and the gel slice was placed in a microcentrifuge tube and incubated at the 65°C for 10 minutes to melt the gel. The gel slices containing appropriate DNAs for ligation were combined and the mixture was placed at 37°C for 2 minutes. For each ligation reaction, 1/10 of the total volume of 10 X ligation buffer (500 mM Tris-HCl, pH7.5; 70 mM MgCl$_2$; 10mM DTT), 10mM rATP (pH7.5) and T4 DNA ligase (4u/μl, Stratagene Ltd., Cambridge) were added into the tube separately. After mixing immediately by flicking, the tube containing ligation reaction was incubated at 12°C-16°C for 48 hours. When the ligation reaction was completed, the tube was incubated at 37°C for 10 minutes to remelt the resolidified gel slices. Then the ligated products were then used to transform into DH5α.
competent cells, as described in 2.2.3.10.

4.2.2.3 Deletion of NcoI site using mung bean nuclease

1 μg of recombinant plant plasmids pIBT210.1-env160 (pIBT210.1NS) or pIBT240.1-env160 (pIBT240.1SS) were digested with 0.5 μl (5 Units) restriction endonuclease NcoI (New England Biolabs, UK). 2 μl of 10 X NEBuffer 4 (500 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol (pH 7.9)) was added and the reaction made up to 20 μl with dH2O before the reaction mixture was incubated at 37°C for 2 hours.

To this was then added 10 μl of 10 X mung bean nuclease buffer (500 mM sodium acetate (pH5.0), 30 mM NaCl and 1 mM ZnSO4) and 0.5 μl of mung bean nuclease (MBN) (New England Biolabs) and the mixture made up to 100 μl with dH2O. After mixing well, it was incubated at room temperature for 15 minutes. This was then concentrated to 20 μl in the speed vacuum microcentrifuge at 13000 rpm for 10 minutes at 4°C and loaded on 1% agarose gel, using undigested plasmid in several lanes away as a control. The DNA was electrophoresised at 40-50 mA for 2-3 hours until the digested DNA fragment was well resolved from any undigested plasmid by comparison with undigested plasmid. Finally the digested DNA band was cut out with a clean razor blade under UV light and purified using Prep-A-Gene DNA purification Kit (BIO-RAD) as described in 2.2.3.6b.

About 1/10 (1-2 μl) of this purified digested DNA fragment was used to blunt end ligate in 1 X ligase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 10 mM dithiothreitol, 1 mM ATP, 25 μg/ml BSA) containing 0.5 μl (200 Units) of T4 DNA ligase (New England Biolabs, UK) in a total of volume of 10 μl. After incubating at 16°C for overnight, 2-4 μl of the ligation mixture was used to transform 100 μl of DH5α competent cells as described in 2.2.4.10 and 2.2.4.1. The colonies were screened by digestion with NcoI restriction endonuclease.
4.3 RESULTS

4.3.1 Generation of HIV-1 env160 DNA bearing restriction enzyme site at ends

PCR primer N160FI/S160R (see section 2.2.3.18e) was designed to introduce a NcoI site and a SacI site at the 5' and 3' of HIV-1 env gene respectively. Plasmid pRNBXX containing the whole sequence of HIV-1 env160 sequence was used as PCR template employing either Taq or Pfu DNA polymerase.

A series of reactions were set up to try to obtain optimal production because of additional bases on primer N160FcoIS160R. Different concentrations of template (2 ng, 4 ng, 6 ng, 10 ng and 20 ng), primers (100 ng, 200 ng, 500 ng and 1 μg) and dNTPs (0.4 mM, 0.8 mM and 1 mM) in total 40 μl reaction were analysed. For the PCR reaction condition, different denaturing temperatures (94°C, 95°C and 97°C), different annealing temperatures (50°C, 55°C, 60°C and 65°C) and different PCR cycle number (25, 30 and 35) were also tested. In addition, different total reaction volumes (20 μl, 40 μl, 50 μl and 100 μl) were examined. The experiments showed that the ideal conditions for amplification with this paired primers were DNA template at a concentration of 4 ng; primer concentration at 100 ng and the total dNTPs concentration at 0.8 mM in total volume of 40 μl PCR reaction mixture, and 30 cycles of amplification under 94°C for denature and 55°C for annealing. The results for generation of HIV gp160 DNA bearing enzyme sites at both ends were shown in Figure 4.8. A fragment of 2596 bp was successfully amplified with primer N160FcoIS160R when either Taq or Pfu PCR was used. The result also showed that Pfu PCR amplification gave a cleaner and sharper 2596 bp band than Taq PCR amplification.

4.3.2 Cloning of env160 PCR products into pCR-Script Amp SK (+) vector

In order to sequence the PCR products and facilitate the subsequent cloning of the env160 gene into plant plasmids, the env160 Pfu PCR products were purified and blunt end ligated into pCR-Script Amp SK (+) cloning vector following the manufacturer's
Figure 4.8 Amplification of HIV-1 env160 by PCR with designed N160FcoI/S160R primers

Amplification of HIV-1 env160 gene with designed primer set N160F/S160R resulted in a 2596 bp product when either Taq or Pfu PCR was used. 8 µl of the PCR reaction was subjected to electrophoresis on 1% agarose gel stained with ethidium bromide.

Lane 1: Lamda/HindIII DNA marker
Lane 2 & 3: PCR products with Pfu DNA polymerase
Lane 4: PCR product with Taq DNA polymerase
Lane 5: Negative PCR control without DNA template
Lane 6: pGEM DNA marker
instructions (Stratagene Ltd., Cambridge). Ligation was carried out with different molar ratios of vector to insert DNA fragment (1:40, 1:60, 1:80 and 1:100). Different amounts of ligation reaction mixture (2, 4, 6 and 8 μl) were used to transform into XL-1 Blue MRF' Kan supercompetent cells (Stratagene Ltd., Cambridge), using 1 μl of insert DNA and cloning vector as a control. The products of each transformation mixture were plated out onto three LB/amp plates precoated with 20 μl of 10% (w/v) 5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) for color selection. The colonies were screened for blue/white phenotype, white colonies representing possible transformed cells containing recombinant plasmids. The results showed that 1:40 and 1:60 molar ratio of vector to insert DNA ligation were not successful, while 1:80 and 1:100 molar ratio of vector to insert DNA gave efficient ligation and 4 μl of ligation reaction mixture gave the most efficient transformation.

DNA was prepared by miniprep procedure from 86 of 96 white colonies and screened by Taq PCR with primer set of N160F/S160R. The results showed that 37 out of 86 plasmid DNA minipreps gave the right size (2596 bp) PCR product (Figure 4.9). 22 PCR positive plasmid DNA minipreps were double digested with NcoI and SacI or/and XbaI and SacI to check the size and the directionality of the insert. The predicted main restriction fragment sizes are 2935 bp and 2580 bp when cut with SacI and NcoI with both cloning orientation; and 4799 bp and 716 bp with cloning direction of NcoI at the upstream of SacI site, or 2935 bp, 1906 bp and 716 bp with cloning direction of SacI at the upstream of NcoI site when cut with XbaI and SacI. As shown in Figure 4.10a and b, 21 colonies analysed gave the predicted sized fragments 4799 bp and 716 bp when double digested with XbaI and SacI, demonstrating that env160 had been successfully cloned into pCR-Script SK(+) cloning vector in the orientation of NcoI site at the upstream of SacI site (Figure 4.11). These recombinant plasmids containing HIV-1 env160 (5557 bp) were designated as pBSNS for subsequent cloning HIV-1 env160 into plant plasmids pIBT210.1 and pIBT240.1.
Figure 4.9 *Taq* PCR screening of recombinant plasmids pBSNS containing insert HIV-1 env160

Plasmid DNA minipreps were screened by PCR using designed primer set N160F/S160R. 8 µl of the PCR reaction was subjected to electrophoresis on 1% agarose gel. Positive recombinant plasmid (designated as pBSNS) resulted in a 2596 bp PCR product.

Lane 1: Lambda/HindIII DNA marker  
Lane 2: Positive control plasmid pRNBXX  
Lane 3-20: DNA minipreps from white colonies  
Lane 21: Negative control without DNA template  
Lane 22: pGEM DNA marker
Figure 4.10 Restriction fragment analysis of recombinant plasmid pBSNS

Plasmid DNA minipreps extracted from PCR positive colonies were digested with restriction enzymes and were subjected to electrophoresis alongside undigested plasmid in order to confirm the size and the directionality of the insert and check the completion of the digestion. The predicted main restriction fragment sizes of double digestion with SacI and NcoI are 2935 bp and 2580 bp; and with XbaI and SacI are 4799 bp and 716 bp.

a: Double digestions with both XbaI/SacI and NcoI/SacI
Lane 1: Lamda/HindIII DNA marker
Lanes 2, 5 and 11: Undigested plasmid preparation
Lanes 3, 6 and 13: Digestion with XbaI/SacI
Lanes 4, 7 and 12: Digestion with NcoI/SacI
Lane 8-10: empty (no samples loaded)
Lane 14: Inserted DNA control (2596 bp)
Lane 15: 1Kb DNA ladder

b: Double digestion with XbaI/SacI
Lane 1: Lamda/HindIII DNA marker
Lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18: Undigested plasmid preparation
Lanes 3, 5, 7, 9, 11, 13, 15, 17 and 19: Digestion with XbaI/SacI
Lane 20: 1Kb DNA ladder
4.3.3. Cloning of HIV-1 env160 into plant plasmid pIBT210.1

pIBT210.1 (see section 4.2.1.3) was chosen for the generation of a recombinant plasmid containing CaMV promoter 35S, translation enhancement TEV, HIV-1 env160 and a polyadenylation signal of a soybean vegetative storage protein gene (3'vspB). The plasmid pIBT210.1 contains a multiple cloning site with NcoI, SmaI and SacI restriction endonuclease sites to facilitate the cloning of foreign DNA. The required portion of env160 was generated by Pfu PCR incorporating NcoI and SacI restriction enzyme sites at the 5' and 3' ends, respectively, to enable directional cloning of env160.

Typically 5 X 40 µl of Pfu PCR reactions were set up and the PCR products were pooled before being purified using QIAquick PCR purification kit (QIAGene, Germany) as described in 2.2.4.4. 5 µg of pIBT210.1 and purified PCR product were both cut with NcoI and SacI using 1 X NEBuffer 1 (10 mM Bis Tris Propane-HCl, 10 mM MgCl2, 1 mM DTT (pH7.0 at 25°C)) (New England Biolabs, UK) following the manufacturer's instructions. Restriction enzyme digestion was carried out for 4 hours at 37°C. The digested pIBT210.1 fragment was phenol:chloroform extracted and was ethanol precipitated as described in 2.3.3.5. The DNA was then resuspended in 10 µl of DEPC treated dH2O. The digested and purified PCR sample was loaded on 1% low melting gel. The correctly sized bands (2596 bp) were excised, extracted from the low melting gel using Geneclean II kit (BIO 101 Inc., Stratech Sci.) as described in 2.2.3.6a and resuspended in 6 µl of DEPC treated dH2O. 0.5 µl of each sample was run on 1% agarose gel to estimate the amount of DNA in the sample by comparing band intensities with those of known amounts of DNA markers. Ligation was set up in molar ratio of 1:10 of vector to insert DNA. 3 µl and 6 µl of ligation reaction and 1 µl of vector and insert DNA control were transformed into Epicurian Coli DH5α competent cells respectively and colonies grown on agar plates for 16 hours at 37°C. The result showed that ligation was successful and 3 µl of ligation reaction mixture gave the most efficient transformation. 20 colonies were selected and grown in 3 ml of LB/amp for 16 hours at 37°C for minipreparation. The plasmid DNA minipreps were screened by Taq PCR with
N160F/S160R primer set and resulted in 15 PCR positive (2596 bp as PCR product) (Figure 4.12). 5 out of 15 PCR positive plasmid minipreps were further cut with XbaI which site only located in the insert DNA, NcoI/SacI and NcoI/HindIII, respectively. The recombinant plasmid would give sized fragments 6606 bp from digestion with XbaI, 4026 bp and 2580 bp from double digestion with NcoI and SacI and 3843 bp, 1927 bp and 836 bp from double digestion with NcoI and HindIII. The 5 colonies analysed gave these predicted sized fragments (Figure 4.13a and b), demonstrating that HIV-1 env160 had been successfully cloned into pIBT210.1 in the correct orientation (Figure 4.14). The recombinant plasmid containing HIV-1 env160, which is 6606 bp in size, was designated as pIBT210.1NS and used for subsequent cloning of env160 into plant expression vector pGPTV-KAN.

4.3.4 Cloning of HIV-1 env160 into plant plasmid pIBT240.1

A description of plant plasmid pIBT240.1 is given in the 4.2.1.3 and was chosen for the generation of a recombinant plasmid containing potato tuber-specific promoter patatin, translation enhancement TEV, HIV-1 env160 and a polyadenylation signal of a soybean vegetative storage protein gene (3′vspB ). The plasmid pIBT240.1 contains a multiple cloning site with NcoI, SmaI and SacI restriction endonuclease sites which similar to pIBT210.1 to facilitate the cloning of foreign DNA. The required portion of env160 was generated by Pfu PCR or pBSNS incorporating NcoI and SacI restriction enzyme sites at the 5’ and 3′ ends, respectively, to enable directional cloning of env160.

Initial attempts at inserting env160 into pIBT240.1 employed the directional cloning approach with DNA fragment produced from pBSNS or Pfu PCR products. Typically 5 x 40 μl of Pfu PCR reactions were set up and the PCR products were pooled and purified using QIAquick PCR purification kit (QIAGene, Germany) as described in
Figure 4.12 *Taq* PCR screening of recombinant plasmids pIBT210.1NS containing insert HIV-1 *env160*

Plasmid DNA minipreps were screened by PCR using designed primer set N160F/S160R. 8 µl of the PCR reaction was subjected to electrophoresis on 1% agarose gel. Positive recombinant plasmid (designated as pIBT210.1NS) resulted in a 2596 bp PCR product.

Lane 1: Lambda/HindIII DNA marker  
Lane 2: Positive control plasmid pRNBXX  
Lane 3-12: Plasmid DNA minipreps from 10 colonies  
Lane 13: Negative control without DNA template  
Lane 14: pGEM DNA marker
Figure 4.13 Restriction fragment analysis of recombinant plasmid pIBT210.1NS

Plasmid DNA extracted from PCR positive colonies were digested with restriction enzymes and were subjected to electrophoresis alongside undigested plasmid in order to confirm the DNA band size and completion of digestion. The predicted restriction fragment size with XbaI was 6606 bp; with NcoI and SacI 4026 bp and 2580 bp, and with NcoI and HindIII 3843 bp, 1927 bp and 836 bp.

a: Digestions with XbaI and NcoI/SacI
Lane 1: Lambda/HindIII DNA marker
Lane 2: Insert DNA control (2596 bp)
Lanes 3, 6, 9 and 12: Undigested plasmid DNA
Lanes 4, 7, 10 and 13: Digestion with XbaI
Lane 5, 8, 11 and 14: Double digestion with NcoI and SacI

b: Double digestion with NcoI and HindIII
Lane 1: Lambda/HindIII DNA marker
Lanes 2 and 4: Undigested plasmid DNA
Lanes 3 and 5: Double digestion with NcoI and HindIII
Lane 6: 1Kb DNA ladder
Fig. 4.13a

1  2  3  4  5  6  7  8  9  10  11  12  13  14

Bands
9416 bp
6557 bp
4361 bp
2322 bp

Fig. 4.13b

1  2  3  4  5  6

Bands
3843 bp
1927 bp
836 bp

4072 bp
3054 bp
2036 bp
1636 bp
1018 bp
506/517 bp
2.2.3.4. 5 µg of pIBT240.1, pBSNS and purified PCR product were all cut with Ncol and SacI using 1 X NEBuffer 1 (10 mM Bis Tris Propane-HCl, 10 mM MgCl₂, 1 mM DTT (pH7.0 at 25°C)) (New England Biolabs, UK) following the manufacturer’s instructions. Restriction enzyme digestion was carried out for 4 hours at 37°C. The digested pIBT240.1 and pBSNS fragments were phenol:chloroform extracted and ethanol precipitated described in 2.2.3.5. The DNA was then resuspended in 10 µl of DEPC treated dH₂O. Digested and purified PCR product or pBSNS DNA samples were loaded on 1% low melting gel and the correctly sized bands (2584 bp fragment produced from digestion of purified PCR product and pBSNS) were excised and extracted from the agarose using Geneclean H kit (BIO 101 Inc., Stratech Sci.) as described in 2.2.3.6a before being resuspended in 6-10 µl of DEPC treated dH₂O. 0.5 µl of each sample was run on 1% agarose gel to estimate the amount of DNA in the sample by comparing band intensities with those of known amounts of DNA markers. Ligation of PCR or pBSNS fragment with pIBT240.1 fragment was set up in molar ratios of 1:3, 1:4, 1:5, 1:8 and 1:10 of vector to insert DNA, respectively. 3, 5 and 7 µl of each ligation reaction were transformed into both E. coli DH5α cells respectively and colonies grown on LB/amp plates for 16 hours at 37°C. This approach was repeated three times without successful ligation between env160 and pIBT240.1.

A second approach was taken to attempt to clone env160 into pIBT240.1 involving both blunt and sticky end ligation. 5 µg of pIBT240.1 was initially cut with restriction endonuclease SmaI to produce blunt ends in 1 X NEBuffer 4 (20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT (pH7.9 at 25°C)) (New England Biolabs, UK) at 25°C for 2 hours, then digested with SacI using the same buffer as SmaI at 37°C for 2 hours, while purified PCR product was digested only with SacI at 37°C for 3 hours. Digested DNA samples were loaded and extracted from agarose gel as above. Ligation was set up in molar ratio of 1:8 of vector to insert DNA. 24 colonies were selected and grown in 3 ml of LB/amp for 16 hours at 37°C. 5 µl of
plasmid DNA minipreps were loaded on 1% agarose gel alongside of plasmid pIBT240.1 for size comparison (Figure 4.15). 12 plasmid DNA minipreps from possible positive colonies were subjected to PCR using N160F/S160R primer set for further checking. 9 out of 12 screened recombinant plasmid DNAs gave the right size PCR product (2596 bp). 3 out of 9 PCR positive plasmid DNAs were analysed by restriction enzyme digestion to determine the presence of env160. The predicted restriction fragments would be 8132 bp when digested with a single enzyme either NcoI or SacI, and 5535 bp and 2580 bp when double digested with NcoI and SacI. All 3 plasmid DNA analysed gave the predicted sized fragments (Figure 4.16). One of these was subsequently sequenced. These results showed that the recombinant plasmid was 8129 bp in size and gave the correctly predicted restriction fragments when digested with a single enzyme either NcoI or SacI (Figure 4.17). This recombinant plasmid was designated as pIBT240.1SS using for subsequent cloning of env160 into plant expression vector pGPTV-KAN.

4.3.5 Construction of plant expression vector pIBT110-env160

pIBT110 is a 13150 bp T-DNA binary vector (as described in 4.2.1.3) and contains a CaMV 35S promoter that is plant leaf specific (Figure 4.5). Thus, pIBT110 was initially chosen as a vector to generate a recombinant plasmid containing HIV-1 env160 to establish HIV-1 env160 transgenic plant leaf expression system. pIBT110 contains the multiple cloning sites SmaI and SacI which can be used for inserting env160 gene.

HIV-1 env160 gene for insertion was generated by Pfu PCR as described in 4.3.1 containing SacI restriction enzyme site at the 3' ends. The amplified env160 gene was then ligated into pIBT110 using both blunt and sticky end ligations. Pfu PCR products were digested with SacI and pIBT110 was cut with SmaI and then with SacI as described above. Ligation was then set up in molar ratio of 1:5 of vector to insert DNA. 3 µl of ligation reaction was transformed into DH5α competent cells as described in 2.2.4.10. and colonies grown on agar plates containing 50 µg/ml kanamycin for 16
Figure 4.15 Plasmid DNA size screening of possible recombinant pIBT240.1

5 µl of plasmid DNA minipreps was subject to electrophoresis on 1% agarose gel alongside pIBT240.1 to compare the plasmid sizes. The possible recombinant plasmids containing HIV-1 env160 would be 8129 bp in size resulting in a higher position on agarose gel than that of pIBT 240.1 (5554 bp in size) after electrophoresis.

Lane 1: Lambda/HindIII DNA marker
Lane 2 & 14: Plasmid pIBT240.1
Lane 3-13: Plasmid DNAs from different colonies

Figure 4.16 Restriction fragment analysis of recombinant plasmid pIBT240.1SS

Plasmid DNA extracted from PCR positive colonies was digested with restriction enzymes and was subjected to electrophoresis alongside undigested plasmid in order to confirm the DNA band size and completion of digestion. The predicted restriction fragment size with NcoI or SacI was 8129 bp; and with NcoI and SacI mainly 5535 bp and 2580 bp.

Lane 1: Lambda/HindIII DNA marker
Lane 2: Undigested plasmid pIBT240.1SS
Lane 3: Digestion with NcoI
Lane 4: Digestion with SacI
Lane 5: Double digestion with NcoI and SacI
Lane 6: 1 Kb DNA ladder
Figure 4.11 Diagrammatic representation of recombinant plasmid pBSNS containing env160


Figure 4.14 Diagrammatic representation of recombinant plant plasmid pIBT210.1NS containing env160

Diagram of recombinant plant plasmid pIBT210.1NS (6606 bp) constructed by inserting env160 into plant plasmid pIBT210.1 at NcoI and SacI sites to generate the \textit{Agrobacterium} transform vector containing a cauliflower mosaic virus 35S promoter (35S') which is plant leaves specific, a tobacco etch virus 5' untranslated region (TEV), env160, a polyadenylation signal of a soybean vegetative storage protein gene (3'vspB).

Figure 4.17 Diagrammatic representation of recombinant plant plasmid pIBT240.1SS containing env160

Diagram of recombinant plant plasmid pIBT240.1SS (8129 bp) constructed by inserting env160 into plant plasmid pIBT240.1 at SmaI and SacI sites to generate the \textit{Agrobacterium} transform vector containing a plant tuber-specific expression promoter \textit{patatin}, a tobacco etch virus 5' untranslated region (TEV), env160, a polyadenylation signal of a soybean vegetative storage protein gene (3'vspB).
hours at 37°C. 10 colonies were picked up with a sterile plastic pipette tip and dispersed in 50 μl of DEPC treated dH₂O. 18 μl of each bacterial suspension was subject to a 25 μl *Taq* PCR reaction with hot start using N160FcoI/S160R primer set. The PCR program (as described in 2.2.3.1) was run for 30 cycles with last extension cycle of 10 minutes at 72°C. 15 μl of each PCR reaction was loaded on 1 % agarose gel to check the product. The results showed that all 10 colonies gave the right size band (2596 bp) with 6 giving strong positive (Figure 4.18a). All 10 positive colonies were further separately cultured in 3 ml of LB/amp for 16 hours at 37°C for plasmid DNA minipreps and then were subject to *Taq* PCR for further check. The results showed that 9 out of 10 plasmid DNA minipreps gave the same pattern as seen for colony bacterial suspension with one exception which gave multiple bands on plasmid DNA PCR but a low intensity band of correct size on colony suspension PCR (Figure 4.18b). This demonstrated that PCR screening bacterial suspension is a reliable method for screening colonies. 3 plasmid DNA samples which were colony suspension and plasmid DNA PCR positive were analysed by restriction enzyme to determine the presence of *env160*. The correct recombinant plasmids containing *env160* would give DNA fragments 15728 bp; 13845 bp and 1883 bp; 12423 bp and 3305 bp when cut with single enzyme SacI, XbaI and EcoRI respectively; and 12423 bp, 2738 bp and 567 bp when double digested with EcoRI and SacI. All 3 plasmid DNA samples analysed gave the correctly sized fragments (Figure 4.19). One of them was subsequently subjected to PCR using plant primer set TEV and vsp (see section 2.2.3.18d) and showed the correct sized band (2723 bp) (Figure 4.21b) and was designated as pIBT110-*env160* (Figure 4.20).

**4.3.6 Construction of plant expression vector pIBT140-*env160***

Plasmid pIBT140 is a 14652 bp T-DNA binary vector (see section 4.2.1.3 for details). This plasmid contains the plant tuber-specific promoter *patatin* and thus was initially chosen as a vector to produce a recombinant plasmid containing HIV-1 *env160* which can transform *Agrobacterium* to establish HIV-1 *env160* transgenic plant
Figure 4.18 Taq PCR screening recombinant plasmid pIBT110 containing env160

Colony bacterial suspensions or plasmid minipreps were screened by PCR using designed primer set N160F/S160R. 8 µl of the PCR reaction was subjected to electrophoresis on 1% agarose gel. Positive recombinant plasmid colonies or minipreps resulted in a 2596 bp PCR product.

a. Colony bacterial suspension PCR
10 bacterial colony suspensions gave the right sized band (2596 bp) with 6 strong positive colonies (colony 3, 4, 5, 6, 7 and 10) which showed high intensity bands and 4 weak positive colonies (colony 1, 2, 8 and 9) which showed lower intensity band.

Lane 1: Lamda/HindIII DNA marker
Lane 2: Positive control plasmid pRNBXX
Lane 3-12: Colony 1-10 bacterial suspensions
Lane 13: Negative control without DNA template
Lane 14: pGEM DNA marker

b. Plasmid DNA PCR
9 out of 10 plasmid minipreps from the corresponding 10 colonies gave the same pattern as that of colony bacterial suspension PCR (Figure 4.18a). All 9 samples gave the right sized band (2596 bp) with 6 giving strong positive samples from colony 3, 4, 5, 6, 7 and 10, which showed high intensity bands; and 3 weak positive samples from colony 1, 2 and 9, which showed lower intensity bands. Colony 8 (lane 10) gives multiple bands.

Lane 1: Lamda/HindIII DNA marker
Lane 2: Positive control plasmid pRNBXX
Lane 3-12: Plasmid DNA miniperps 1-10 from the corresponding colony 1-10.
Lane 13: Negative control without DNA template
Lane 14: pGEM DNA marker
Fig. 4.18a

Fig. 4.18b
Figure 4.19 Restriction fragment analysis of recombinant plasmid pIBT110-env160

Plasmid DNA with both colony bacterial suspension and plasmid DNA PCR was digested with restriction enzymes and were subjected to electrophoresis alongside undigested plasmid in order to examine the DNA band sizes and completion of digestion. The predicted restriction fragment sizes with SacI are 15728 bp; with XbaI 13845 bp and 1883 bp; with EcoRI 12423 bp and 3305 bp and with EcoRI and SacI 12423 bp, 2738 bp and 567 bp.

Lane 1: Lamda/HindIII DNA marker
Lane 2: Digestion with SacI
Lane 3: Digestion with XbaI
Lane 4: Digestion with EcoRI
Lane 5: Double digestion with EcoRI and SacI
Lane 6: Undigested plasmid
Lane 7: 1Kb DNA ladder
tuber expression system. Similar to pIBT110, pIBT140 contains the multiple cloning sites SmaI and SacI which can be used for inserting \textit{env160} PCR product. Cloning HIV-1 \textit{env160} into pIBT140 employed the same strategy as inserting \textit{env160} into pIBT110. Inserting \textit{env160} generated by \textit{Pfu} PCR was digested with SacI and ligated to predigested pIBT140 with SmaI and SacI to generate the recombinant plasmid pIBT140-\textit{env160} as described above with a few modifications.

Ligation was set up in molar ratio of 1:6 of vector to insert DNA. Both 3 and 6 μl of ligation reaction were transformed into DH5α competent cells. Colonies were grown on agar plates containing 50 μg/ml kanamycin for 16 hours at 37°C and 28 colonies were picked and dispersed in 50 μl of DEPC treated dH2O. 18 μl of each bacterial suspension was subjected to a 25 μl \textit{Taq} PCR reaction with hot start using N150F/S160R primer set. The results showed that 20 out of 28 colony bacterial suspensions gave the right sized band (2596 bp) with 9 strong positive which showed higher intensity bands than 11 weak positive. 20 plasmid DNA minipreps from the 20 corresponding PCR positive colonies were prepared following the standard alkaline lysis protocol (described in 2.2.3.12a) and subjected to \textit{Taq} PCR as further check. The results showed that while 11 plasmid DNA preparations from the corresponding 11 weak positive colonies failed to show any bands, 9 plasmid DNA preparations from the corresponding 9 PCR strong positive colonies were again PCR positive (Figure 4.21a). 3 of these were subjected to \textit{Taq} PCR using plant primer set TEV/vsp to check the presence of inserting DNA. Primer TEV is located 69 bp upstream of NcoI site, and vsp 55 bp downstream of SacI site in plant binary vector (see section 2.2.3.18d). Thus the correct recombinant plasmids would give a 2723 bp PCR products using TEV/vsp primer set while pIBT110 and pIBT140 would give 145 bp as control. The result showed that all 3 plasmids produced the right sized bands (Figure 4.21b). These 3 plasmid DNAs were subsequently analysed by restriction enzyme digest to determine the presence and orientation of \textit{env160}. The correct recombinant plasmids containing \textit{env160} would give
Figure 4.21 Taq PCR screening recombinant plasmid pIBT 110 containing env160

3 Plasmid DNA minipreps from the corresponding PCR strong positive colonies were further checked by PCR using primer sets N160F/S160R and TEV/vsp. 8 μl of the PCR reaction was subjected to electrophoresis on 1% agarose gel. All plasmid DNAs tested gave the right size 2596 bp PCR products with primer set N160F/S160R and 2723 bp fragments with primer set TEV/vsp (positive control pIBT140 and pIBT110 gave 145 bp bands and the recombinant plasmid pIBT110-env160 gave the same size band (2723 bp) as pIBT-140-env160).

a. PCR using primer set N160F/S160R
Lane 1: Lamda/HindIII DNA marker
Lane 2: Positive control plasmid pRNBXX
Lane 3-5: 3 plasmid DNAs
Lane 6: Negative control without DNA template
Lane 7: pGEM DNA marker

b. PCR using plant primer set TEV/vsp
Lane 1: Lamda/HindIII DNA marker
Lane 2: plasmid pIBT140
Lane 3-5: 3 Plasmid DNAs
Lane 6: plasmid pIBT110
Lane 7: pIBT110-env160
Lane 8: Negative control without DNA template
Lane 9: pGEM DNA marker
Fig. 4.21a

Fig. 4.21b
3 plasmid DNAs from both colony bacterial suspension and plasmid miniprep PCR positive colonies were digested with restriction enzymes and were subjected to electrophoresis alongside undigested plasmid in order to examine the DNA band size and completion of digestion. The predicted restriction fragment sizes with SacI are 17230 bp; with EcoRI 13925 bp and 3305 bp; and with SacI and SalI 12311 bp and 4919bp.

Lane 1: 1Kb DNA ladder
Lane 2-4: Undigestion plasmid DNA
Lane 5-7: Digestion with SacI
Lane 8-10: Double digestion with SacI and SalI
Lane 11-13: Digestion with EcoRI
Lane 14: Lamda/HindIII DNA marker
Fig. 4.22

17230 bp (band)
5090 bp
4072 bp
3054 bp
2036 bp

23130 bp (M)
13925 bp
12311 bp
4919 bp
3305 bp
Figure 4.20 Diagrammatic representation of recombinant plant vector pIBT110SS containing env160

Diagram of recombinant plant vector pIBT110SS (15728 bp) constructed by inserting env160 into plant T-DNA vector pIBT110 at SmaI and SacI sites in order to introduce env160 into Agrobacterium and then generate transgenic env160 leaf expression system. This recombinant plasmid contains the selection marker gene neomycin phosphotransferase (nptII) which is located near the right border of T-DNA, the cauliflower mosaic virus 35S promoter (35S'), a tobacco etch virus 5' untranslated region (TEV) and a polyadenylation signal of a soybean vegetative storage protein gene (3'vspB). RB and LB indicate the right and left transferred DNA (T-DNA) borders.

Figure 4.23 Diagrammatic representation of recombinant plant vector pIBT140SS containing env160

Diagram of recombinant plant vector pIBT140SS (17230 bp) constructed by inserting env160 into plant T-DNA vector pIBT140 at SmaI and SacI sites in order to introduce env160 into Agrobacterium and then generate transgenic env160 tuber expression system. This recombinant plasmid contains the selection marker gene neomycin phosphotransferase (nptII) which is located near the right border of T-DNA, plant tuber specific promoter patatin, a tobacco etch virus 5' untranslated region (TEV) and a polyadenylation signal of a soybean vegetative storage protein gene (3'vspB). RB and LB indicate the right and left transferred DNA (T-DNA) borders.
fragments 17230 bp; 13925 bp and 3305 bp when cut with single enzyme SacI and EcoRI, respectively; and 12311 bp and 4919 bp when double digested with SauI and SacI. All 3 plasmid DNA samples analysed gave the correctly sized restriction fragments (Figure 4.22) and were designated as pIBT 140-env160 (Figure 4.23).

4.3.7 Construction of plant expression vector pGPTV-KAN-210.1S-env160 and pGPTV-KAN-240.1S-env160

Plant plasmid pGPTV-KAN (glucuronidase plant transformation vector) is a 13387 bp plant binary vector. As described in 4.2.1.4, plasmid pGPTV-KAN has a novel trait in that the β-glucuronidase (uidA) reporter gene located near the right border and the plant selectable marker gene nptII, which confer kanamycin resistance to plants and located near the T-DNA left border, are divergently organised for efficient expression. The plasmid contains unique cloning sites Smal, XbaI, SauI and HindIII upstream of the uidA gene and EcoRI downstream of the promoter for uidA (Pnos) to allow the exchange by any other gene of interest as desired (Figure 4.7).

4.3.7.1 Construction of plant expression vector pGPTV-KAN-210.1NS-env160

To construct a plant expression vector for efficient expression of env160 in plant leaves, recombinant plasmid pIBT210.1NS containing CaMV promoter 35S, translation enhancement TEV, HIV-1 env160 and a polyadenylation signal of a soybean vegetative storage protein gene (3'vspB ) was chosen to generate a recombinant plant binary vector pGPTV-KAN-210.1NS-env160.

The required portion of 35S promoter, 5' TEV, env160 and vsp3' gene produced from plasmid pIBT210.1NS were designed to insert into pGPTV-KAN at EcoRI and HindIII sites. Considering that there is one HindIII site inside the env160 gene, the generation of pGPTV-KAN-210.1NS-env160 initially involved cloning the insert DNA fragments into pGPTV-KAN at Smal and EcoRI sites. Typically, 5 µg of pIBT210.1NS was digested with PstI in 1 X NEBuffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl2,
1 mM DTT (pH 7.9 at 25°C)) at 37°C for 2 hours and then 15 units of T4 DNA polymerase, 50 μg/ml of BSA and 100 μM of each dNTP were added. This was incubated at 12°C for 30 minutes to remove 3’ overhangs and thus generate a blunt end. The digested plasmid was extracted with phenol:chloroform and ethanol precipitated as described in 2.2.3.5. It was then resuspended in 10 μl of DEPC treated dH₂O and digested with EcoRI in 1 X NEBuffer EcoRI (100 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl, 0.025% Triton X-100 (pH 7.5 at 25°C)) for 2 hours. 5 μg of pGPTV-KAN was digested with SmaI in 1 X NEBuffer EcoRI for 2 hours at 25°C and then cut with EcoRI for 2 hours at 37°C. All digested DNA fragments were loaded on 0.6% low melting gels to isolate the required band (3965 bp from pINT210.1NS and 11126 bp from pGPTV-KAN). Then the required DNA fragments were extracted from the agarose gel using GeneClean II kit as described in 2.2.4.6. The ligation was set up at both 1:3 and 1:5 molar ratio of vector to insert DNA. Then 3-5 μl of ligation reaction was transformed into DH5α competent cells. This experiment was repeated for 3 times without successful ligation.

The second attempt involved 3 fragments ligation, that is, both 35S promoter and TEV gene fragment produced by digestion of pIBT210.1 at HindIII and NcoI sites, and env160 and vsp3’ fragment produced from digestion of pIBT210.1NS at NcoI and EcoRI, were cloned into pGPTV-KAN at EcoRI and HindIII sites in one ligation reaction. Initially, the cloning was attempted by gel slice ligation, thus the step of extraction digestion fragments from agarose gel was removed to keep the intact sticky end and increase the efficiency of ligation reaction. Generally, 2 μg of pIBT210.1 was digested with NcoI and HindIII in 1 X NEBuffer2 (10 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT (pH 7.9 at 25°C)) for 3 hours at 37°C. 2 μg of pIBT210.1NS was digested with NcoI and EcoRI in 1 X NEBuffer EcoRI (100 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl, 0.025% Triton X-100 (pH 7.5 at 25°C)) for 3 hours, and 2 μg of pGPTV-KAN was digested with EcoRI and HindIII in 1 X NEBuffer EcoRI (100 mM
Tris-HCl, 10 mM MgCl₂, 50 mM NaCl, 0.025% Triton X-100 (pH 7.5 at 25°C)) for 3 hours. All digested samples were loaded on 0.7% low melting gels and run for 3 hours until the required bands were well separated on the gel. Then the required bands were excised from the agarose gel in as smaller slices as possible. The gel slices containing DNA were melted at 65°C for 10 minutes and the melting mixture containing appropriate DNAs were mixed and the ligation were set up as described in 4.2.2.2. The ligation mixture was incubated for 48 hours at 12-16°C. When the ligation reaction was completed, the tube was incubated at 37°C for 10 minutes to remelt the resolidified gel slices and diluted at 1:30, to avoid gel resolidifying when performing the transformation. 10 µl of diluted ligated product was transformed into MAX efficiency DH5α competent cells (GIBCO BRL) as described in 2.2.3.10. Clones were grown on agar plates containing 50 µg/ml of kanamycin. This experiment was repeated three times without successful ligation.

Subsequently the 3 fragment ligation was performed with purified DNA sections. The digestion procedures of pIBT210.1 with HindIII and NcoI; pIBT210.1NS with NcoI and EcoRI and pGPTV-KAN with EcoRI and HindIII were performed as above. All digested DNA fragments were loaded on 0.7% low melting or routine agarose gel and run for 4-5 hours to isolate the required bands (836 bp from pIBT210.1; 3141 bp from pINT210.1NS and 11126 bp from pGPTV-KAN). Then the required DNA bands from pIBT210.1, pIBT210.1NS and pGPTV-KAN were cut using sharp and clean blades. The DNA fragments produced from both pIBT210.1 and pIBT210.1NS were extracted from the agarose gel using Geneclean II kit as described in 2.2.3.6a, while the pGPTV-KAN vector was extracted and purified using Prep-A Gene DNA purification kit (BIO-RAD) following as described in 2.2.3.6b. The ligation was set up at 1:15-1:20 molar ratio of pGPTV-KAN fragment to insert DNA from pIBT210.1 and 1:4-1:5 molar ratio of pGPTV-KAN vector to insert DNA from pIBT210.1NS and performed at 12-16°C for 24 or 48 hours, respectively. 3-5 µl of ligation reaction was transformed into DH5α competent cells. This experiment was repeated for 3 times without successful ligation.
Subsequently, to reduce shearing DNA fragments when extracting from agarose gel, all the digested DNA fragments were extracted and purified using Prep-A Gene DNA purification kit as described in 2.2.3.6b. The DNAs were resuspended in 10 μl of dH2O. To increase the ligation efficiency, the ligation reaction of the same molar ration as before was incubated at 16°C for 72 hours. 5 μl of ligation reaction was transformed into DH5α competent cells by heat shock protocol as described in 2.2.3.10, while 1 μl of ligation reaction was transformed into DH5α competent cells by electroporation as described in 2.2.4.1. After incubating on agar plates containing 50 μg/ml of kanamycin at 37°C for 16 hours, electroporation transformed DH5α cells produced more than 100 colonies. Although heat shock transformed cells produced only about 50 colonies they were generally larger that those from electroporation transformed colonies. 6 colonies from heat shock transformed and 5 colonies from electroporation transformed colonies were picked and subjected to PCR screening with N160F and S160R primers. 9 out of 11 colonies showed the right sized band (2596 bp), 5 from heat shock transformed colonies and 4 from electroporation transformed colonies. 4 out of 9 PCR positive colony bacterial suspensions were further cultured in 3 ml of LB containing kanamycin for 16 hours at 37°C to prepare plasmid DNA. 4 plasmid DNA minipreps were further confirmed by PCR using plant primer set TEV and vsp and digested with EcoRI and HindIII restriction enzyme. The results showed that all DNA samples analysed gave the predicted PCR products (2704 bp) (Figure 4.24) and restriction fragments 11126 bp, 2763 bp and 1214 bp when cut with EcoRI and HindIII which demonstrated that the 35S promoter, TEV, env160 and vsp 3' genes were successfully cloned into pGPTV-KAN. 2 colonies containing the recombinant plasmids were further cultured in 2 x 6 ml and then 200 ml of LB containing 50 μg/ml of kanamycin for midiprep stock of plasmid DNAs. The plasmid DNA midipreps were cut with EcoRI and HindIII and gave the correctly sized restriction fragments as above (Figure 4.25). This recombinant plasmid containing the insert DNA fragments were used for transformation of Agrobacterium and named as pGPTV-KAN-210.1NS-env160 (Figure 4.26).
Figure 4.24 *Taq* PCR examination of recombinant plasmids pGPTV-KAN-210.1NS-envl60 and pGPTV-KAN-240.1NS-envl60

Plasmid DNAs were examined by PCR using plant primer set TEV and vsp. 8 μl of the PCR reaction was subjected to electrophoresis on 1% agarose gel. All the plasmid DNA tested (4 from pGPTV-KAN-210.1NS-envl60 and 4 from pGPTV-KAN-240.1NS-envl60) showed the correct 2704 bp PCR products. Control plasmid pIBT240.1 gave a 143 bp band.

Lane 1: Lambda/HindIII DNA marker  
Lane 2: Positive control plasmid pIBT240.1  
Lane 3-6: Recombinant plasmid pGPTV-KAN-210.1NS-env160 minipreps  
Lane 7-10: Recombinant plasmid pGPTV-KAN-240.1NS-env160 minipreps  
Lane 11: Negative control without DNA template  
Lane 12: pGEM DNA marker

Figure 4.25 Restriction fragment analysis of recombinant plasmids pGPTV-KAN-210.1NS-env160 and pGPTV-KAN-240.1NS-env160

Plasmid DNA midipreps were digested with restriction enzymes and were subjected to electrophoresis to examine the DNA band sizes. All DNA samples analysed gave the predicted restriction fragment sizes with pGPTV-KAN-210.1NS-env160 11126 bp, 2763 bp and 1214 bp, and pGPTV-KAN-240.1NS-env160 11126 bp, 4266 bp and 1214 bp when cut with EcoRI and HindIII.

Lane 1: 1Kb DNA ladder  
Lane 2-3: Recombinant plasmid pGPTV-KAN-210.1NS-env160 DNA digested with EcoRI and HindIII  
Lane 4-5: Recombinant plasmid pGPTV-KAN-240.1NS-env160 DNA digested with EcoRI and HindIII
4.3.7.2 Construction of plant expression vector pGPTV-KAN-240.1NS-env160

To efficiently express HIV-1 *env*160 in plant tuber system, a plant expression vector containing tuber-specific promoter a *patatin*, translation enhancement TEV, HIV-1 *env*160 and a polyadenylation signal of a soybean vegetative storage protein gene (3′vspB) was designed to construct plant expression vector pGPTV-KAN-240.1NS-env160.

The initial attempt at cloning *patatin*, TEV, *env*160 and 3′vspB was by inserting the required genes produced from pIBT240.1SS into pGPTV-KAN at EcoRI and SalI sites. At first, the cloning was attempted by gel slice ligation to keep the intact sticky end and facilitate the ligation efficiency. 2 µg of pIBT240.1SS and 1 µg of pGPTV-KAN was digested with EcoRI and SalI in 1 X NEBuffer EcoRI (100 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl2, 0.025% Triton X-100 (pH 7.5 at 25°C)) for 3 hours at 37°C, separately. Both digested pIBT240.1SS and pGPTV-KAN fragments were loaded on 0.7% low melting gel and run for 3-4 hours until the required bands (5476 bp from pIBT240.1SS and 11144 from pGPTV-KAN) were well resolved on the gel. The required bands were cut from the agarose gel and melted at 65°C for 10 minutes. The melting mixture containing appropriate DNAs were mixed and the ligation were set up as described in 4.2.2.2 and carried out for 48 hours at 12-16°C. When the ligation reaction was completed, the tube was incubated at 37°C for 10 minutes to remelt the resolid gel slices and diluted at 1:30 to avoid resolidifying the gel during performing transformation. 10 µl of diluted ligated product were transformed into MAX efficiency DH5α competent cells (GIBCO BRL) as described in 2.2.3.10. Clones were grown on agar plates containing 50 µg/ml of kanamycin. This experiment was repeated 3 times without successful ligation.

Then the cloning was attempted using purified fragments ligation. 5 µg of pIBT240.1SS and pGPTV-KAN was digested and loaded on a 0.7% low melting gel as before. The required DNA fragments were extracted from agarose gel using Geneclean II kit or
Prep-A Gene DNA purification kit (BIO-RAD) following the manufacturer's instructions. The ligation was set up at molar ratios of 1:3, 1:4, and 1:5. 3 and 5 μl of each ligation reaction was transformed into DH5α competent cells and colonies were grown on agar plates as above. This experiment was repeated for 3 times but failed to ligate the desired DNA fragments.

The second approach of cloning the desired genes into pGPTV-KAN involved a 3 fragment ligation, that is, both *patatin* promoter and TEV fragments produced by digestion of pIBT240.1 at HindIII and NcoI sites, and *envl60* and vsp3' fragment produced from digestion of pIBT210.1NS at NcoI and EcoRI, were cloned into pGPTV-KAN at EcoRI and HindIII sites in one ligation reaction. Briefly, 2 μg of pIBT240.1 was digested with NcoI and HindIII in 1 X NEBuffer2 (10 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT (pH 7.9 at 25°C)) for 3 hours at 37°C. 2 μg of pIBT210.1NS was digested with NcoI and EcoRI in 1 X NEBuffer EcoRI (100 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl, 0.025% Triton X-100 (pH 7.5 at 25°C)) for 3 hours, and 2 μg of pGPTV-KAN was digested with EcoRI and HindIII in 1 X NEBuffer EcoRI (100 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl, 0.025% Triton X-100 (pH 7.5 at 25°C)) for 3-4 hours. All digested samples were loaded on 0.7% low melting gel and run for 3-4 hours until the required bands were well resolved on the gel. The required bands (2339 bp from pIBT240.1; 3141 bp from pIBT210.1NS and 11126 bp from pGPTV-KAN) were cut and extracted from the agarose gel using Prep-A Gene DNA purification kit (BIO-RAD) as described in 2.2.3.6b. The DNAs were resuspended in 10 μl of dH₂O. The ligation was set up at 1:5 and 1:6 molar ratio of pGPTV-KAN fragment to insert DNA from pIBT210.1 and 1:4-1:5 molar ratio of pGPTV-KAN vector to insert DNA from pIBT210.1NS. All reactions were performed at 12-16°C for 72 hours. 5 μl of each ligation reaction was transformed into DH5α competent cells by heat shock protocol as described in 2.2.3.10, while 1 μl of each ligation reaction was also transformed into DH5α competent cells by electroporation as described in 2.2.4.1. After incubating on agar plates containing 50 μg/ml of kanamycin at 37°C for 16 hours,
electroporation transformed DH5α cells produced more than 100 colonies while heat shock transformed cells produced only about 40 colonies but with more bigger colonies than that of electroporation transformed ones. 4 colonies from each method transformed colonies were picked and subjected to PCR screening using primer set N160F and S160R. 7 out of 8 colonies showed the right sized band (2596 bp) with 4 from heat shock transformed and 3 from electroporation transformed colonies. 4 out of 7 PCR positive colony bacterial suspensions were further cultured in 3 ml of LB containing kanamycin for 16 hours at 37°C to prepare plasmid DNA. 4 plasmid DNA minipreps were further confirmed by PCR using plant primer set TEV and vsp and digested with EcoRI and HindIII. The results showed that all DNA samples analysed gave the right sized PCR products (2704 bp) (Figure 4.24) and restriction fragments 11126 bp, 4266 bp and 1214 bp when cut with EcoRI and HindIII This demonstrated that the patatin promoter, TEV, env160 and 3vspB genes had been successfully cloned into pGPTV-KAN. 2 colonies containing the recombinant plasmids were further cultured in 2 x 6 ml and then 200 ml of LB containing 50 µg/ml of kanamycin for midiprep stock of plasmid DNAs. The plasmid DNA midipreps were examined with restriction digestion with EcoRI and HindIII and produced the right restriction fragments as above (Figure 4.25). This recombinant plasmid containing the insert DNA fragments was used for transformation of Agrobacterium and named as pGPTV-KAN-240.1NS-env160 (Figure 4.27).

4.3.7.3 Deletion of NcoI site in pIBT210.1NS and pIBT240.1SS
The NcoI restriction site located upstream of the env160 gene in the constructed expression vectors pGPTV-KAN-210.1NS-env160 and pGPTV-KAN-240.1NS-env160 contains three adjacent nucleotides ATG which could be used as start codon by plant translation system and result in breaking the reading frame of env160. It is therefore decided to delete this site using mung bean nuclease (MBN), which is capable of degrading single-stranded extensions from the ends of DNA.
Diagram of recombinant plant vector pGPTV-KAN-210.1NS-env160 (15103 bp) constructed by inserting DNA fragment containing the cauliflower mosaic virus 35S promoter (35S), env160, a tobacco etch virus 5' untranslated region (TEV) and a polyadenylation signal of a soybean vegetative storage protein gene (3'vspB) produced from pIBT210.1 and pIBT210.1NS into plant binary vector pGPTV-KAN (glucuronidase plant transformation vector) at EcoRI and HindIII sites to introduce env160 into Agrobacterium and then generate transgenic env160 leaf expression system. This recombinant plasmid contains the plant selection gene neomycin phosphotransferase (nptII) which is located near the left border of T-DNA. RB and LB indicate the right and left transferred DNA (T-DNA) borders.

Diagram of recombinant plant vector pGPTV-KAN-240.1NS-env160 (16606 bp) constructed by inserting DNA fragment containing the plant tuber specific promoter patatin, env160, a tobacco etch virus 5' untranslated region (TEV) and a polyadenylation signal of a soybean vegetative storage protein gene (3'vspB) produced from pIBT240.1 and pIBT210.1NS into plant binary vector pGPTV-KAN (glucuronidase plant transformation vector) at EcoRI and HindIII sites to introduce env160 into Agrobacterium and then generate transgenic env160 tuber expression system. This recombinant plasmid contains the plant selection gene neomycin phosphotransferase (nptII) which is located near the left border of T-DNA. RB and LB indicate the right and left transferred DNA (T-DNA) borders.
The deletion of NcoI sites resulted in discharging of 4 nucleotides for pIBT210.1NS and 18 nucleotides for pIBT240.1SS (Figure 4.28a and b). The NcoI deleted recombinant plasmids pIBT210.1NS and pIBT240.1SS were designated as pIBT210.1S and pIBT240.1S, respectively. The predicted size of pIBT210.1S and pIBT240.1S would be 6602 bp and 8111 bp.

Deletion of NcoI site was performed as described in 4.2.2.3. Briefly, 1 μg of recombinant plant plasmids pIBT210.1NS and pIBT240.1SS were digested with 0.5 μl (5 Units) of NcoI in 1 X NEBuffer 4 (50 mM potassium acetate, 20mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT (pH 7.9 at 25°C)) (New England Biolabs, UK) at 37°C for 2 hours, followed by blunt-ending with 5 units mung bean nuclease (MBN) (New England Biolabs) at room temperature for 15 minutes. The reaction mixtures were loaded on 1% agarose gel with undigested plasmid as a control and run for 2-3 hours until the digested DNA fragments was well resolved from any undigested plasmids. Then the digested and blunt-ending DNA band was cut out and purified with Prep-A-Gene DNA purification Kit (BIO-RAD) as described in 2.2.3.6b. 2 μl of each of DNA resuspension was used to blunt end ligate in 1 X ligase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 10 mM dithiothreitol, 1 mM ATP, 25 μg/ml BSA) using 0.5 μl (200 Units) of T4 DNA ligase at 16°C for overnight. 2-4 μl of each of the ligation mixtures were transformed into DH5α competent cells by both heat shock and electroporation methods as described in 2.2.3.10 and 2.2.3.1, and the colonies grown on agar plates containing 100 μg/ml ampicillin. 7 and 5 plasmid DNA minipreps from pIBT210.1S and pIBT240.1S colonies respectively were screened by digestion with NcoI restriction endonuclease and confirmed by digestion with HindIII. NcoI deleted recombinant plasmids would give no digestion pattern when cut with NcoI enzyme. pIBT210.1S would be expected to give 2759 bp and 3843 bp and pIBT240.1S 4262 bp and 3849 bp when cut with HindIII. 4 out of 7 NcoI deleted pIBT210.1NS and 4 out of 5 NcoI deleted pIBT240.1SS plasmid DNA minipreps gave the predicted digestion pattern (Figure 4.29a, b and c). The sequences of pIBT210.1S and pIBT240.1S plasmid
Figure 4.28 Deletion of NcoI sites on recombinant plasmids pIBT210.1NS and pIBT240.1SS

Deletion of NcoI was achieved by mung bean nuclease (MBN) which removes 5' extensions produced by digestion with NcoI restriction enzyme. Then the blunt ended linearised plasmid DNAs were circularised using T4 DNA ligase. There are one and two NcoI sites on recombinant plasmid pIBT210.1NS and pIBT240.1SS, respectively, thus deletion of NcoI sites resulted in the loss of 4 nucleotides for pIBT210.1NS and 18 nucleotides for pIBT240.1SS. The bold nucleotides indicate the target sequence of restriction endonuclease NcoI.

a. Deletion of NcoI site on recombinant plasmid pIBT210.1NS

pIBT210.1NS

\[
\begin{align*}
5'\text{---ATAGCCATGGGAATGA---3'} \\
3'\text{---TATCGGTACC TTACT ---5'}
\end{align*}
\]

cut with NcoI

\[
\begin{align*}
5'\text{---ATAGC CATGGAATGA---3'} \\
3'\text{---TATCGGTAC CTT ACT---5'}
\end{align*}
\]

add MBN (5' extensions were removed and 4 nucleotides were discharged)

\[
\begin{align*}
5'\text{---ATAGC GAATGA---3'} \\
3'\text{---TATCG CT TACT---5'}
\end{align*}
\]

add T4 DNA ligase

\[
\begin{align*}
5'\text{---ATAGCGAATGA---3'} \\
3'\text{---TATCGC TTACT ---5'}
\end{align*}
\]
Figure 4.28 (continued)

b. Deletion of NcoI sites on recombinant plasmid pIBT240.1SS

pIBT240.1SS

\[
\begin{align*}
5'--&GCCATGGATCCCCC TCCATGGA--3' \\
3'--&CGGTACCTAGGGGAGGTACCT--5'
\end{align*}
\]

cut with NcoI

\[
\begin{align*}
5'--&GC \quad CATGGATCC CCCTC \quad CATGGA--3' \\
3'--&CGGTAC \quad CTAGGGGAGGTAC \quad CT--5'
\end{align*}
\]

add MBN (5' extensions were removed and 18 nucleotides were lost)

\[
\begin{align*}
5'--&GC \quad GA--3' \\
3'--&CG \quad CT--5'
\end{align*}
\]

add T4 DNA ligase

\[
\begin{align*}
5'--&GCGA--3' \\
3'--&CGCT--5'
\end{align*}
\]
Figure 4.29 Restriction digestion to demonstrate deletion of NcoI sites on pIBT210.1NS and pIBT240.1SS

Plasmid DNA minipreps from pIBT210.1S and pIBT240.1S colonies were digested with NcoI and then loaded on a 1% agarose gel alongside with undigested plasmid DNAs to examine the deletion of NcoI sites. The correct recombinant plasmids with no NcoI sites would give no digestion pattern, which is the same as the corresponding undigested plasmid DNA. 4 of 7 NcoI deleted pIBT210.1NS colonies (colony 2, 4, 6 and 7) (a) and 4 out of 5 NcoI deleted pIBT240.1SS colonies (colony 2, 3, 4 and 5) (b) gave the correct pattern.

a. Plasmid DNA minipreps from NcoI deleted pIBT210.1NS colonies
Lane 1: Lambda/HindIII DNA marker
Lane 2, 4, 6, 8, 10 & 14: Undigested plasmid DNAs from the colony 1-5 and 7.
Lane 3, 5, 7, 9, 11, 12 and 13: Plasmid DNAs from the corresponding colony 1-7 digested with NcoI.

b. Plasmid DNA minipreps from NcoI deleted pIBT240.1SS colonies
Lane 1: Lambda/HindIII DNA marker
Lane 2, 4, 6, 8 and 10: Undigested plasmid DNAs from the colony 1-5.
Lane 3, 5, 7, 9 and 11: Plasmid DNAs from the corresponding colony 1-5 digested with NcoI.

c. Restriction digestion of pIBT210.1S and pIBT240.1S with HindIII
The NcoI deleted recombinant plasmids pIBT210.1S and pIBT240.1S were analysed by digestion with restriction enzyme HindIII. All DNA samples analysed (4 of each pIBT210.1S and pIBT240.1S) gave the correctly sized fragments, which are 3843 bp and 2759 bp for pIBT210.1S and 4262 bp and 3849 bp for pIBT240.1S.
Lane 1: Lambda/HindIII DNA marker; Lane 18: 1Kb DNA ladder
Lane 2, 4, 6 & 8: Undigested plasmid DNAs of pIBT210.1S
Lane 3, 5, 7 & 9: Plasmid DNAs of pIBT210.1S digested with HindIII
Lane 10, 12, 14 & 16: Undigested plasmid DNAs of pIBT240.1S
Lane 11, 13, 15 & 17: Plasmid DNAs of pIBT240.1S digested with HindIII
were ascertained on an ABI PRISM 310 Genetic Analyser (Perkin-Elmer) as described at 2.2.3.17c using primer TEV to verify the deletion of NcoI sites. The results demonstrated that NcoI site in both pIBT210.1S and pIBT240.1S had been deleted and the MBN blunted ends had been successfully ligated as desired (Figure 4.43 and Figure 4.44). These two colonies were chosen for subsequent cloning the required genes into plant binary vector pGPTV-KAN.

4.3.7.4 Construction of plant expression vector pGPTV-KAN-210.1S-env160

To construct the desired plant expression vector for efficient expression of env160 in plant leaf system, NcoI site deleted recombinant plasmid pIBT210.1S containing CaMV promoter 35S, translation enhancement TEV, HIV-1 env160 and a polyadenylation signal of a soybean vegetative storage protein gene (3'vspB) was chosen to generate a recombinant plant binary vector pGPTV-KAN-210.1S-env160.

The required portion of 35S promoter, 5' TEV, env160 and vsp3' gene produced from plasmid pIBT210.1S were designed to be inserted into pGPTV-KAN at EcoRI and HindIII sites. Considering that there is one HindIII site inside the env160 gene, partial digestion of pIBT210.1S with HindIII restriction enzyme was employed. Typically, 1 µg of pIBT210.1S was cut with 20 units (1 µl) EcoRI and different concentrations of HindIII (10, 5, 2.5, 1.25, 1, 0.75, 0.5 and 0.25 units) in a total volume of 40 µl of 1 X NEBuffer EcoRI (100 mM Tris-HCl, 10 mM MgCl2, 50 mM NaCl, 0.025% Triton X-100 (pH 7.5 at 25°C)). After incubating at 37°C for 2 hours, all digestion reaction mixtures were loaded on 1% agarose gel and run for 3-4 hours until required bands were well resolved. The result showed that all 10, 5 and 2.5 units of HindIII resulted in complete digestion (2759 bp, 2629 bp and 1214 bp), while 1.25, 1, 0.75 and 0.5 units of HindIII resulted in partially digestion (3973 bp and 2629 bp or 5388 bp and 1214 bp), and 0.25 unit of HindIII led to no digestion. (6602 bp) (Figure 4.30a and b). All the required bands (3973 bp) produced by partial digestion in different lanes were cut and mixed and extracted from the agarose gel using Prep-A Gene DNA purification kit as
Figure 4.30 partial digestion of recombinant plasmid pIBT210.1S with restriction endonuclease HindIII

1 µg of plasmid pIBT210.1S was cut with 15 units and different concentration of 10, 5, 2.5, 1.25, 1, 0.75, 0.5 and 0.25 units of HindIII in a total of 40 µl of 1 X NEBuffer EcoRI (100 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, 0.025% Triton X-100 (pH 7.5 at 25°C)). 10, 5 and 2.5 units; 1.25, 1, 0.75 and 0.5 units; and 0.25 units of HindIII resulted in completely digestion; partial digestion and undigestion of analysed pIBT210.S, respectively. 2759 bp, 2629 bp and 1214 bp; 3973 bp and 2629 or 5388 bp and 1214 bp; and 6602 bp restriction fragments were generated by complete digestion; partial digestion and without any digestion, respectively.

a. Digestion of pIBT210.1S with EcoRI and different concentration of 10, 5, 2.5 and 1.25 units of HindIII

Lane 1: Lambda/HindIII DNA marker
Lane 2: Digestion of pIBT210.1S with EcoRI and 10 units of HindIII
Lane 3: Digestion of pIBT210.1S with EcoRI and 5 units of HindIII
Lane 4: Digestion of pIBT210.1S with EcoRI and 2.5 units of HindIII
Lane 5: Digestion of pIBT210.1S with EcoRI and 1.25 units of HindIII

b. Digestion of pIBT210.1S with EcoRI and different concentration of 2.5, 1, 0.75, 0.5 and 0.25 units of HindIII

Lane 1: Digestion of pIBT210.1S with EcoRI and 2.5 units of HindIII
Lane 2: Digestion of pIBT210.1S with EcoRI and 1 unit of HindIII
Lane 3: Digestion of pIBT210.1S with EcoRI and 0.75 units of HindIII
Lane 4: Digestion of pIBT210.1S with EcoRI and 0.5 units of HindIII
Lane 5: Digestion of pIBT210.1S with EcoRI and 0.25 units of HindIII
Lane 6: 1Kb DNA ladder
described in 2.2.3.6b. The DNA pellet was resuspended in 10 μl of dH₂O and used for ligation with pGPTV-KAN vector digested with EcoRI and HindIII. 1 μg of pGPTV-KAN was cut with EcoRI and HindIII as before and the required fragment (11126 bp) purified using Prep-A Gene DNA purification kit. The ligation reaction was set up at molar ratio of 1:3 of vector to insert DNA fragment and 5 and 10 μl of ligation reaction was transformed into DH5α competent cells. Colonies were grown on agar plates containing 50 μg/ml kanamycin. This experiment was repeated 3 times until the ligation was successful. Colonies were screened by PCR, as above, using plant primer set TEV and vsp. 20 out of 24 colonies showed the right sized PCR products (2700 bp) including 15 strong and 5 weak positive colonies (Figure 4.31). 6 plasmid DNA minipreps were prepared from 6 out of 15 strong PCR positive colonies and analysed by restriction digestion to examine the presence of the required inserted DNA. The correctly recombinant plasmids would give 11126 bp, 2759 bp and 1214 bp when cut with EcoRI and HindIII. All samples analysed gave the correct sized fragments (Figure 4.32) and one of them was further cultured for midiprep stock. This recombined plasmid was designated as pGPTV-KAN-210.1S-env160 (Figure 4.33) and used for subsequent Agrobacterium transformation and transgenic plant generation.

4.3.7.5 Construction of plant expression vector pGPTV-KAN-240.1S-env160

To construct the desired plant expression vector for efficient expression of env160 in plant tuber system, NcoI site deleted recombinant plasmid pIBT240.1S containing plant tuber-specific promoter patatin, translation enhancement TEV, HIV-1 env160 and a polyadenylation signal of a soybean vegetative storage protein gene (3'vspB) was chosen to generate a recombinant plant binary vector pGF1TV-KAN-240.1S-env160. The attempt to clone the required portion of patatin promoter, 5' TEV, env160 and 3'vspB genes into pGPTV-KAN involved partial digestion of pIBT240.1S with EcoRI and HindIII because there is one HindIII site inside the env160 gene. Briefly, 1 μg of
Figure 4.31 Taq PCR screening recombinant plasmid pGPTV-KAN containing CaMV 35S promoter and env160

Bacterial cell suspensions were subjected to PCR using plant primer set TEV and vsp. 8 µl of the PCR reaction was loaded on 1% agarose gel and run for 1 hour at 80 mA. The correctly recombined plasmid colonies resulted in a 2700 bp PCR product. Strong positive results were obtained for colonies 2-8, 12, 13, 15, 17 and 18 and weak positive results for colonies 14 and 16.

Lane 1: Positive control plasmid pIBT210.1S  
Lane 2-19: Colony 1-19 bacterial suspensions  
Lane 20: Negative control (without DNA template)  
Lane 21: pGEM DNA marker

Figure 4.32 Restriction fragment analysis of recombinant plasmid pGPTV-KAN-210.1S-env160

Six plasmid DNAs prepared from strong PCR positive colonies were cut with restriction enzymes and were subjected to electrophoresis alongside undigested plasmid in order to examine the DNA band sizes and extent of digestion. The predicted restriction fragment sizes were 11126 bp, 2759 bp and 1214 bp when cut with EcoRI and HindIII. All samples analysed gave the correct sized restriction fragments.

Lane 1: 1 Kb DNA ladder  
Lane 2, 4, 6, 8, 10 and 12: Undigested plasmid DNAs  
Lane 3, 5, 7, 9, 11 and 13: Double digestion with EcoRI and HindIII
pIBT240.1S was cut with 20 units (1 μl) EcoRI and different concentrations of HindIII (10, 5, 2.5, 1.25, 0.5, and 0.25 units) in a total volume of 40 μl of 1 X NEBuffer EcoRI (100 mM Tris-HCl, 10 mM MgCl2, 50 mM NaCl, 0.025% Triton X-100 (pH 7.5 at 25°C)) at 37°C for 2 hours. All digestion reactions were run on a 1% agarose gel. 10, 5, and 2.5 units of HindIII resulted in completely digestion (4262 bp, 2635 bp and 1214 bp), while 1.25 and 1 units of HindIII resulted in partial digestion (5476 bp and 2635 bp or 6897 bp and 1214 bp); and 0.5 and 0.25 unit of HindIII resulted in no digestion (8111 bp). The required bands (5476 bp) were cut and extracted from the agarose gel using Prep-A Gene DNA purification kit. The DNA pellet was resuspended in 10 μl of dH2O and used for ligation with EcoRI and HindIII digested pGPTV-KAN vector. 1 μg of pGPTV-KAN was cut with EcoRI and HindIII as before and the required fragment (11126 bp) was also purified using Prep-A Gene DNA purification kit. The ligation reaction was set up at molar ratio of 1:3 of vector to insert DNA fragment. Then 5 or 1 μl of ligation reaction was transformed into DH5α competent cells by heat shock or electroporation method, and colonies were grown on agar plates containing 50 μg/ml kanamycin. 49 and more than 100 colonies were grown from heat shock and electroporation transformed cells, respectively. 4 colonies from each method of transformation were screened by PCR using plant primer set TEV and vsp. All 8 colonies gave the right size PCR products (2700 bp) (Figure 4.34). 4 plasmid DNA minipreps were prepared from 4 out of 8 PCR positive colonies and analysed by restriction digestion to examine the presence of the required insert DNA. The correctly recombinant plasmids would give 11126 bp, 4262 bp and 1214 bp when cut with EcoRI and HindIII. All samples analysed gave the correct sized fragments (Figure 4.35) and one of them was further cultured for midiprep stock and designated as pGPTV-KAN-240.1S-env160 (Figure 4.36) for subsequent Agrobacterium transformation.

4.3.8 Sequencing of cloned env160 in recombinant plasmids
Figure 4.34 *Taq* PCR screening recombinant plasmid pGPTV-KAN containing *patatin* promoter and *env160* gene

8 bacterial cell suspensions were subjected to PCR using plant primer set TEV and vsp. 8 μl of the PCR reaction was loaded on 1% agarose gel and run for 1 hours at 80 mA electric current. All samples tested gave the right sized PCR products (2700 bp).

Lane 1: Lambda/HindIII DNA marker
Lane 2: Positive control plasmid pIBT240.1S
Lane 3-10: Colony 1-8 bacterial suspensions

Figure 4.35 Restriction fragment analysis of recombinant plasmid pGPTV-KAN-240.1S-env160

4 plasmid DNA minipreps prepared from PCR positive colonies were cut with EcoRI and HindIII. All the digests were loaded on 0.7% agarose gel alongside undigested plasmid to examine the DNA band sizes and completion of digestion. The predicted restriction fragment sizes were 11126 bp, 4262 bp and 1214 bp and all 4 samples analysed gave the correct sized restriction fragments.

Lane 1, 3, 5 and 7: Undigested plasmid
Lane 2, 4, 6 and 8: Plasmid DNAs double digested with EcoRI and HindIII
Lane 9: 1 Kb DNA ladder
Diagrammatic representation of recombinant plant vector pGPTV-KAN-210.1S-env160 (15099 bp) constructed by inserting a DNA fragment containing the cauliflower mosaic virus 35S promoter (35S), env160, a tobacco etch virus 5' untranslated region (TEV) and a polyadenylation signal of a soybean vegetative storage protein gene (3'vspB) produced by partial digestion of recombinant plasmid pIBT210.1S into plant binary vector pGPTV-KAN (glucuronidase plant transformation vector) at EcoRI and BndHI sites to introduce env160 into Agrobacterium and then generate transgenic env160 leaf expression system. This recombinant plasmid contains the plant selection gene neomycin phosphotransferase (nptII) which is located near the left border of T-DNA. RB and LB indicate the right and left transferred DNA (T-DNA) borders.

Diagrammatic representation of recombinant plant vector pGPTV-KAN-240.1S-env160 (16602 bp) constructed by inserting a DNA fragment containing the plant tuber specific promoter patatin, env160, a tobacco etch virus 5' untranslated region (TEV) and a polyadenylation signal of a soybean vegetative storage protein gene (3'vspB) produced by partial digestion of recombinant plasmid pIBT240.1S into plant binary vector pGPTV-KAN (glucuronidase plant transformation vector) at EcoRI and HindIII sites to introduce env160 into Agrobacterium and then generate transgenic env160 tuber expression system. This recombinant plasmid contains the plant selection gene neomycin phosphotransferase (nptII) which is located near the left border of T-DNA. RB and LB indicate the right and left transferred DNA (T-DNA) borders.
Fig. 4.33

Fig. 4.36
The cloned HIV-1 env160 genes in different recombinant plasmids including pBSNS, pIBT210.1NS, pIBT240.1SS, pIBT110SS and pIBT140SS were produced by *Pfu* PCR amplification using pRNBXX containing the whole env160 sequence of HXB2 as DNA template. The primer set N160F and S160R was designed to amplify the env160 gene in *Pfu* PCR reaction and introduced restriction enzymes NcoI and SacI sites at 5' and 3' ends respectively to facilitate the construction of plant recombinant expression vector containing HIV-1 env160. As PCR amplification may introduce base changes and a single PCR reaction may also, by chance, amplify one variant preferentially from another, the inserting env160 genes of each above recombinant plasmids were partially sequenced using ABI 373 DNA sequencer as described in 2.2.3.17. Different primers were used when sequencing the insert env160 and junction regions, primers TEV and N160F for the upstream junction and the 5' end regions; primer 306 and 634 for V3 and V4 regions and S160R and vsp for the 3' end and downstream junction regions (for details of primers see section 2.2.3.18). Approximately 1125 bp out of 2571 bp of env160 in each of recombinant plasmids and the junction regions were sequenced. The results showed sequences obtained from different recombinant plasmids using the same set of primer were all the same and sequences obtained using primer 634 were completely overlapped with that obtained using primer 306; sequences obtained using primers N160F and S160R were partially overlapped with that using primers TEV and vsp, respectively. All portions of sequences were checked against the published sequences of HXB2. No major deletions or mutations were observed in the analysed env160 or junction sequences (Figure 4.37, 38, 39, 40, 41 and 42). The amino acid sequences were read and compared to that of HXB2 Env160 polypeptide sequence. No deletions or mutations were found in all sequence analysed. Therefore these clones were used for further expression work.

4.4 DISCUSSION AND CONCLUSION

To generate transgenic plants expressing HIV-1 env160 protein, plant expression
Figure 4.37 Sequence of cloned env160 in recombinant plasmids using primer N160F

The cloned HIV-1 env160 genes in different recombinant plant plasmids including pBSNS, pIBT210.1NS, pIBT240.1SS, pIBT110SS and pIBT140SS were sequenced using ABI 373 DNA sequencer and both nucleotide and polypeptide sequences obtained were checked and compared with that of published HXB2 sequences. All sequences obtained from different recombinant plasmids using primer N160F were the same as that of HXB2 at position of 5800-6115.
Figure 4.38 Sequence of cloned env160 in recombinant plasmids using primer S160R

The cloned HIV-1 env160 genes in different recombinant plant plasmids including pBSNS, pIBT210.1NS, pIBT240.1SS, pIBT110SS and pIBT140SS were sequenced using ABI 373 DNA sequencer and both nucleotide and polypeptide sequences obtained were checked and compared with that of published HXB2 sequences. All sequences obtained from different recombinant plasmids as above using primer S160R were the same as that of HXB2 at position of 7991-8341.
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**Figure 4.39** Sequence of cloned *env160* in recombinant plasmids using primer 306

The cloned HIV-1 *env160* genes in different recombinant plasmids including pBSNS, pIBT210.1NS, pIBT240.1SS, pIBT110SS and pIBT140SS were sequenced using ABI 373 DNA sequencer and both nucleotide and polypeptide sequences obtained were checked and compared with that of published HXB2 sequences. All sequences obtained from different recombinant plasmids as above using primer 306 were completely the same and align with that of HXB2 at position of 6606-7013 with no mutations and deletions.
Figure 4.40 Sequence of cloned env160 in recombinant plasmid pIBT210.1NS using primer TEV

The HIV-1 env160 gene was inserted into plant plasmid pIBT210.1 at NcoI and SacI sites and generated recombinant plant plasmid pIBT210.1NS. The inserting junction and 5' end of cloned env160 region in pIBT210.1NS was sequenced using ABI 373 DNA sequencer and the result was compared with that of pIBT210.1, primer NcoI and 5' region of HXB2. The sequence obtained aligns with that of pIBT210.1 at positon 780-836, oligonucleotides CATGGA which were introduced by the designed primer N160F and HXB2 at position 5785 (start codon of env160 gene in HXB2 genome)-6014. The NcoI site introduced by designed primer N160F is underlined.
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Figure 4.41 Sequence of cloned env160 in recombinant plasmid pIBT240.1SS using primer TEV

The HIV-1 env160 gene was inserted into plant plasmid pIBT240.1 at Smal and SacI sites and generated recombinant plant plasmid pIBT240.1SS. The inserting junction and 5’ end of cloned env160 region in pIBT240.1NS was sequenced using ABI 373 DNA sequencer and the result was compared with that of pIBT240.1, primer N160F and 5’ region of HXB2. The sequence obtained aligns with that of pIBT240.1 at position 2283-2350, oligonucleotides CTCCATGGA which was introduced by designed primer N160F and HXB2 at position 5785 (start codon of env160 gene in HXB2 genome)-6014 without any deletion or mutation contained. The NcoI site introduced by designed primer N160F is underlined.
The cloned HIV-1 env160 genes in different recombinant plasmids including pIBT210.1NS, pIBT240.1SS, pIBT110SS and pIBT140SS were sequenced using an ABI 373 DNA sequencer and both nucleotide and polypeptide sequences obtained were checked and compared with that of published HXB2 sequences. All sequences obtained from different recombinant plasmids using primer vsp were the same as that of HXB2 at position of 8078-8355, oligonucleotides GAGAGCT which were introduced by primer S160F at pIBT210.1 position 860-891 which is the same as pIBT240.1 at position 2363-2394. The SacI site introduced by designed primer S160R is underlined.
Deletion of restriction endonuclease NcoI site on recombinant plasmid pIBT210.1S was verified by sequencing on ABI PRISM 310 Genetic Analyser using primer TEV. The sequence obtained was checked and compared with that of pIBT210.1, primer N160R and HXB2 and aligns with pIBT210.1 at position 772-836, two junction nucleotides GA which introduced by primer NcoI and HXB2 at position of 5785 (the start codon of env160 in HXB2 genome)-6146. The arrow indicates the location of restriction enzyme NcoI target sequence prior to deletion.
Deletion of restriction endonuclease NcoI site on recombinant plasmid pIBT240.1S was verified by sequencing on ABI PRISM 310 Genetic Analyser using primer TEV. The sequence obtained was checked and compared with that of pIBT240.1, primer N160R and HXB2 and aligns with pIBT240.1 at position 2275-2339, two junction nucleotides GA which were introduced by primer N160R at HXB2 position of 5785 (the start codon of env160 in HXB2 genome)-6137. The arrow indicates the location of two sites of NcoI endonuclease and the sequence between them prior to deletion.
Figure 4.45 Representative diagram showing generation of plant expression vectors containing plant leaf specific promoter CaMV 35S or plant tuber specific promoter patatin, HIV-1 env160 gene, a translation enhancer element TEV and a transcription processing mediating sequence 3'vspB.
Nco I → PCR amplification → Sac I

gp160

NcoI deletion → Tobacco transformation
NT-1 cell transformation

NcoI deletion → Potato transformation
vectors containing plant leaf specific promoter CaMV 35S or plant tuber specific promoter patatin, HIV-1 env160 gene, a translation enhancer element TEV and a transcription processing mediating sequence 3'vspB were constructed. The construction procedure was concluded as shown in schematic diagram (Figure 4.45).

4.4.1 PCR amplification and cloning of HIV-1 env160

In order to clone into plant vector, HIV-1 env160 was initially amplified by PCR using plasmid pRNBXX (see section 4.2.1.2) as DNA template. Sequences from the 5' and 3' ends of env160 in pRNBXX were chosen for the complementary sense and antisense PCR primer pair, respectively. Restriction enzyme target sequences were attempted to introduce at 5' and 3' ends of env160 to make the cloning easier. Primer set N160F and S160R (detail see section 2.2.4.19e) was designed to introduce NcoI and SacI at 5' and 3' ends of env160, respectively. The whole sequence of HIV-1 (2596 bp) was successfully amplified using primer set pNF and pSR. As Taq polymerase has no editing function there is always a possibility that PCR amplification will introduce base changes. It is reported that the estimates for the error rate introduced by Taq vary from 1 in 300 to 1 in 9000 for the single base pair substitutions and 1 in 41000 for frameshift mutations (Meyerhans et al., 1989; Perrin and Gilliand, 1990; Tindall and Kunkel, 1988). Pfu DNA polymerase, a proofreading DNA polymerase isolated from Pyrococcus furiosus, is devoid of terminal deoxynucleotidyltransferase (TdT) activity which is characterized by the addition of nontemplate-directed nucleotide(s) at the 3' end of PCR-generated fragments and generates blunt-ended PCR products exclusively. Therefore, Pfu DNA polymerase was the choice for use in cloned env160 PCR amplification to increase the specificity and fidelity of PCR products. In exploring the optimal condition of PCR amplification, the results show that template and primer concentrations of 4-6 ng and 100 ng respectively in a total volume of 40 µl PCR reaction mixture appeared to be most effective amplification. For the PCR reaction conditions, most product was found when the denaturing temperature was 94°C; the annealing temperature was 55°C and 30 PCR cycles (Figure 4.8).
In order to sequence the PCR products and facilitate the subsequent cloning of the env16 gene into plant plasmids, the env16 Pfu PCR fragment was firstly cloned into a cloning plasmid. As Pfu DNA polymerase possesses the ability to remove extended bases by its 3'-5' exonuclease activity to generate the inserts ensures the high-fidelity, blunt-ended PCR products and thus Pfu PCR amplified env16 products works ideally with the pCR-Script Amp SK (+) cloning vector. The results showed that 1:80-1:100 ligation molar ratio of vector to insert DNA gave the efficient ligation and 4 μl of ligation reaction mixture gave the most efficient transformation. 37 out of 86 colonies (43%) gave the right size (2596 bp) products when subjected to PCR screening (Figure 4.9) and 21 out 22 PCR positive colonies gave the predicted sized fragments when double digested with XbaI and SacI, demonstrated that env16 had been successfully cloned into pCR-Script SK(+) cloning vector. Furthermore, all 21 colonies containing recombinant plasmid showed the same cloning orientation with NcoI site upstream of SacI site (Figure 4.11). The transformation protocol allows one hour of growth of competent cells in LB to express the ampicillin resistant phenotype prior to plating for single colonies on selective agar but this period is likely to be insufficient to allow more than one cell division and therefore the 21 colonies are unlikely to have been the result of a single ligation or transformation event. Several factors such as the insert sequence, heat pulse length of transformation, the sensitivity and preference of the competent cells might involved into the orientation of insert DNA of recombinant plasmids, however, no clear explanation could be put forward.

4.4.2 Construction of recombinant plant binary vector containing HIV-1 env16

Progress in the development of transgenic plants can be attributed to the exploitation of Agrobacterium transformation mechanism resulted in the design of modified, non-tumourigenic Agrobacterium Ti plasmids for the engineering of transgenic plants. Binary vectors, which were developed came after the discovery that the transferred DNA (T-DNA) region remain functional when separated from the resident Ti plasmid onto two separate replicons in Agrobacterium (de Framond et al., 1983; Hoekema et al., 1983), are now the most commonly used vector system for creating and regenerate
transgenic plants. These vectors contain a number of unique restriction endonuclease sites which can be used for inserting the foreign genes of interest and plant selectable marker(s) between the T-DNA borders and a cloning vehicle capable of replicating in *E. coli* and *Agrobacterium*.

Three plant binary vectors pGPTV-KAN, pIBT110 and pIBT140 were used in constructing the plant vector containing HIV-1 *env160* for expression of HIV-1 *Env160* in transgenic plants. All of them contain the left and right T-DNA borders (LB, RB) which denote the limits of the DNA that is integrated into the plant genomic DNA via *Agrobacterium tumefaciens*-mediated transformation and the neomycin phosphotransferase (*nptII*) for selection of plant transformants using kanamycin. However, the plant select marker gene (*nptII*) was located near right border in pGPTV-KAN but left border in both pIBT110 and pIBT140. It was reported that T-DNA is transferred from right border to left border to plant cells by *Agrobacterium* mediated transformation (Zambryski, 1992). Therefore, the identification of drug-resistant, plant transformants containing complete T-DNA insertions is facilitated by using plant vector containing selectable marker gene, *nptII* located close to the left T-DNA border. The position of *nptII* located near the right border of T-DNA increases the likelihood of obtaining plants carrying the selectable marker gene, but not the gene of interest, which results in obtaining the kanamycin-resistant plant cells harbouring truncated T-DNA insertions. Thus, pGPTV-KAN was the optimal plant vector used for constructs containing HIV-1 *env160*. As the interested plant leaf and tuber promoter CaMV 35S and *patatin* are not constructed in pGPTV-KAN but in pIBT110 and pIBT140 respectively, cloning of HIV-1 *env160* into pGPTV-KAN to generate the constructs used for *Agrobacterium* transformation and subsequent production of transgenic plants had to be achieved in two stages. Furthermore, as the transformation of *Agrobacterium* and plant cells or plantlets had to be performed in the USA and the time was limited, cloning of HIV-1 *env160* into pIBT110 and pIBT140 was also attempted as a back-up constructs in case the cloning of *env160* into pGPTV-KAN might not be achieved.
4.4.2.1 Cloning of envl60 into plant vector pIBT110 and pIBT140

To make constructs containing envl60 and 35S or patatin promoters using plant vector pIBT110 and pIBT140, the required envl60 portions produced by Pfu PCR were inserted into pIBT110 and pIBT140 at Smal and SacI sites and successfully generated the desired recombinant plasmids pIBT110-envl60 and pIBT140-envl60 (see section 4.3.5 and 4.3.6). The colonies containing the recombinant plasmids were screened using bacterial suspension PCR using primer set N160F/S160R or lately TEV and vsp. For screening of pIBT110-env colonies, 10 out of 10 colonies gave the right sized PCR products (2596 bp) with 6 strong positive and 4 weak positive. The results of subsequent plasmid minipreps PCR confirmation demonstrated that all 6 colonies with strong bacterial suspensions PCR positive remain plasmid DNA PCR positive but 3 out of 4 weak positive remain positive showed plasmid DNA PCR positive (Figure 4.18a and b). For screening of pIBT140-env160 colonies, 20 out of 28 colony bacterial suspensions gave the right sized band (2596 bp) with 9 strong positive and 11 weak positive. 20 plasmid DNA minipreps from 20 corresponding PCR positive colonies were subjected to Taq PCR for a further check. The results showed that 11 plasmid DNA preparations from the corresponding 11 weak positive colonies failed to show any band, while 9 plasmid DNA preparations from the corresponding 9 PCR strong positive colonies remained PCR positive (Figure 4.21a). Colony screening PCR is a faster, effective and painless screening method compared to the traditional plasmid miniprep screening although some false positive colonies could be introduced. However, the false positive could be avoided by only considering the strong positive colonies which showed the strong intensity predicted size band as the colonies containing recombinant plasmids.

4.4.2.2 Cloning of envl60 into plant vector pGPTV-KAN

As mentioned above, cloning of HIV-1 envl60 into pGPTV-KAN to generate the constructs used for Agrobacterium transformation and subsequent production of transgenic plants had to be achieved in two stages. HIV-1 envl60 produced by Pfu PCR amplification or digestion of recombinant plasmid pBSNS with NcoI and SacI was
attempted to be initially cloned into plant plasmid pIBT210.1 and pIBT240.1 to generate the initial stage recombinant plasmids containing CaMV promoter 35S, translation enhancement gene TEV, env160 and a polyadenylation signal of a soybean vegetative storage protein gene (3'vspB); and plant tuber-specific promoter patatin, env160, TEV and 3'vspB genes. Then the required portion containing env160, 35S or patatin promoter TEV and 3'vspB genes was produced from the above initial stage recombinant plasmids and cloned into pGPTV-KAN at EcoRI and HindIII sites.

HIV-1 env160 gene produced by Pfu PCR reaction were successfully cloned into pIBT210.1 at NcoI and SacI sites and generated the recombinant plasmid pIBT210.1NS containing CaMV promoter 35S, env160, TEV and 3'vspB genes and pIBT240.1SS containing plant tuber promoter patatin, env160, TEV and 3'vspB genes. The insert env160 fragments produced by either Pfu PCR reaction or digestion of pBSNS with NcoI and SacI could not be successfully cloned into pIBT240.1 vector at NcoI and SacI sites, even by varying the ligation molar ratio (1:3, 1:4, 1:5, 1:8 and 1:10) of vector to insert. This unsuccessful ligation might have occurred because the sticky ends of the insert or the vector DNA fragments produced after restriction enzyme digestion were broken due to shearing on more than one silica particle when extracted from the low melting gel using GenecleanII kit. Then the alternative approach was used by cloning of Pfu env160 PCR products into pIBT240.1 at Smal and SacI sites involving one blunt end ligation and the recombinant plasmid pIBT240.1SS containing plant tuber promoter patatin, env160, TEV and 3'vspB genes was successfully generated.

When performing plant transformation using the above recombinant pGPTV-KAN plasmids containing env160 and 35S or patatin promoter, the NcoI site located at the upstream of env160 gene in the constructed contains three adjacent nucleotides ATG which may be, by chance, used as start codon by plant translation system and result in breaking the reading frame of env160. To ensure the efficiency of expression of env160 in transgenic plants, NcoI site at the upstream of env160 in recombinant plant vectors was deleted. As there are other three NcoI sites in the constructed plant vectors pGPTV-
KAN-210.1NS-env160 and pGPTV-KAN-240.1NS-env160 (Figure 4.26 and Figure 4.27), the deletion work had to move back to the first stage recombinant plasmids pIBT210.1NS and pIBT240.1SS. The deletion of NcoI site was performed using mung bean nuclease (MBN), which is capable of removing 3' and 5' extensions from the ends of DNA fragments. NcoI sites were successfully deleted when incubating the NcoI digested plasmid DNA with MBN at room temperature for 15 minutes. These results were verified by sequencing two NcoI deleted recombinant plasmids pINT210.1S and pIBT240.1S (Figure 4.28a and b). The deletion of NcoI resulted in discharging of 4 nucleotides for pIBT210.1NS and 18 nucleotides for pIBT240.1S (Figure 4.28a and b).

The NcoI site deleted recombinant plasmids pIBT210.1NNS and pIBT240.1S were designated as pIBT210.1S and pIBT240.1S respectively and used for generating desired plant constructs containing 35S or patatin, env160, TEV and 3'vspB genes to transform Agrobacterium and plant cells. The portions of required DNA fragments produced from both pIBT210.1S and pIBT240.1S were designed for cloning into optimal plant binary vector pGPTV-KAN at EcoRI and HindIII sites. As there is one HindIII restriction site located inside env160, partial digestions of pIBT210.1S and pIBT240.1S with EcoRI and HindIII were employed to generate the required DNA fragments containing env160 (3973 bp for pIBT210.1S and 5476 bp for pIBT240.1). The digestion patterns varied between each experiment even with the same reaction conditions. The reason for this discordance is not clear. Perhaps the efficiency and activity of restriction enzyme slightly varied or the incubation time was slightly different. The required sized DNA fragments produced by partial digestion of pIBT210.1S or pIBT240.1S with HindIII and EcoRI were successfully ligated with EcoRI and HindIII cut pGPTV-KAN vector.

4.4.3 Sequencing of cloned env160 in recombinant plasmids

As the cloned HIV-1 env160 genes were produced by Pfu PCR amplification using pRNBXX containing the whole env160 sequence of HXB2 as DNA template, the base changes may be introduced. Furthermore, a single PCR reaction may also by chance amplify one variant preferentially from another. The inserting env160 genes of
recombinant plasmids including pBSNS, pIBT210.1NS, pIBT240.1SS, pIBT110SS and pIBT140SS were partially sequenced as described in 2.2.4.18 using 3 primer sets TEV/vsp, N160F/S160R and 634/306. Primer sets TEV/vsp and N160F/S160R were used for sequencing 5' and 3' end of env160 and adjacent junction regions, respectively; primer set 306/634 were used for sequencing V3-V4 region of env160. Approximately 1125 bp out of 2571 bp of env160 in each of recombinant plasmids were analysed. The results were checked against the published sequences of HXB2 and pIBT210.1 or pIBT240.1 and the amino acid sequences were read and compared to that of HXB2 env160 polypeptide sequence. No deletions or mutations were observed in both analysed env160 or junction regions DNA and amino acid sequences (Figure 4.37, 38, 39, 40, 41 and 42) when using the above 3 primer sets TEV/vsp, N160F/S160R and 634/306. Therefore these desired recombinant plant vectors containing HIV-1 env160 (pGPTV-KAN-210.1S-env160 and pGPTV-KAN-240.1S-env160) were used for further Agrobacterium transformation.
Chapter 5

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5.1 INTRODUCTION

Plant cells exhibit a variety of traits that distinguish them from animal cells, such as the presence of a large central vacuole and a cell wall. However, perhaps the most significant factor that distinguishes plant cells from animal cells is that many varieties of full-grown adult plants can regenerate from individual plant cells and that an adult plant can be said to have been cloned from a single cell of a parent plant (White, 1954). Plants that can be cloned with relative ease include tomatoes, potatoes, carrots, petunias, and cabbage, to name only a few. The ability to grow a whole plant from a single cell means that researchers can engage in the genetic manipulation of the cell, let the cell develop into a mature plant, and examine the genetic manipulation effects within a relatively short period of time. Based on this significant characteristic of plant cells, together with the refinement of gene cloning, tissue culture and plant transformation, a springboard was produced from which unprecedented applications could evolve. One such application is edible vaccines which should be cheaper and simpler to be produced, be more heat stable than vaccines made in the conventional way, and more applicable to use in the low-funded health care systems of developing countries.

*Agrobacterium*-mediated transformation is most commonly used for the production of edible vaccines. *Agrobacterium* is a plant pathogen that during the process of infection transfers a segment of its DNA (T-DNA) to its host plant cells. By taking advantage of this process, a gene of interest, inserted into a plant expression cassette, can be transferred into plant genomes through a process similar to conjugation. During tissue culture, transformed plant cells are positively selected on medium containing the appropriate antibiotics and regenerated into transgenic plants. In nature, *Agrobacterium tumefaciens* infects only dicotyledons or "dicots", plants which have two embryonic leaves, including such plants as tobacco, potatoes and soybeans (Peters, 1993).

Considerable progress has been made in development of edible vaccines since the expression of a vaccinogen in tobacco plants was firstly reported in 1990 (Curtiss and
Norwalk virus capsid protein expressed in transgenic tobacco was able to induce both serum IgG and secretory IgA specific for rNV in mice dosed by gavage (Mason et al., 1996). Respiratory syncytial virus (RSV) fusion (F) protein expressed in transgenic tomato plants has been shown to induce the development of both serum and mucosal RSV-F specific antibodies in mice orally dosed by ripe transgenic tomato fruits (Sandhu et al., 2000). Hepatitis B surface antigen (HbsAg) was successfully expressed in transformed tobacco plants (Mason et al., 1992) and have been demonstrated to be immunogenic when administered parenterally to mice (Thanavala et al., 1995). Recently HbsAg was successfully expressed in edible (potato) plants (Richter et al., 2000). Kong et al. (2001) have demonstrated that edible plant-based rHBsAg is an oral immunogen in mice. In this study, transgenic potato plants were grown to maturity in soil and harvested to collect tubers, which contained on average 8.35 μg of HBsAg per gram of fresh tuber. Mice were then fed with peeled potato pieces once per week for 3 consecutive weeks. Each mouse received 5 g of tuber (containing an average of 42 μg of HBsAg per dose) per feed. Anti-HBsAg antibodies were detected in mice fed with HBsAg-transgenic potatoes. Furthermore, the strong secondary response seen after boosting with rHBsAg represents a true memory response, generated as a result of the mice being fed HBsAg transgenic potatoes. These results demonstrated that plant-derived HBsAg, delivered as food, is orally immunogenic in mice and can elicit a primary antibody response. These support the concept that a food source containing a foreign antigen given by mucosal routes can induce the development of immune response to a pathogen. Thus the feasibility of using transgenic plants as an expression and delivery system for an edible vaccine against human diseases is verified. The transgenic plant edible vaccines may represent a potential new generation of novel vaccine production and delivery systems with distinct characteristics including inexpensive production, unnecessary preparation or purification and easy transportation and storage.

The use of transgenic plants as vaccine production and delivery systems to protect
animals from a number of infectious diseases has also been explored. The structural protein VP1 of foot-and-mouth disease virus (FMDV) (Carrillo et al., 1998), The major structural protein VP60 of rabbit hemorrhagic disease virus (RHDV) has been successfully produced in transgenic potato plants (Castanon et al., 1999). Recently, transgenic potato plants were created for expression of the N-terminal domain of the glycoprotein S (N-gS) from transmissible gastroenteritis coronavirus (TGEV), which carries the major antigenic sites of the protein. Mice were fed with potato tubers expressing TGEV N-gS (0.02-0.07% of total soluble tuber protein) three times per week with 2g of transgenic potato tuber for a two months period. Antibodies specific for N-gS protein were developed in mice orally fed with potato tubers expressing N-gS proteins (Gómez et al., 2000). These studies showed that the protection of animals against a viral disease by feeding with a transgenic plant expressed antigens was possible, and thus using a food plant to produce and deliver an edible vaccine is feasible.

Studies on the development of mucosal vaccines against HIV-1 have been shown to induce both mucosal and systemic immune responses to HIV (Lehner et al., 1992; Thibodeau et al., 1992; Mestecky et al., 1994; Morrow et al., 1994; Bond et al., 1995; Muster et al., 1995; VanCott et al., 1998; Staats et al., 1996), which raised the possibility that application of HIV antigens on mucosal surfaces may be a fruitful approach to vaccination against AIDS. To investigate the feasibility of production of HIV edible vaccines, the recombinant expression vectors (see Chapter 4) were introduced into transgenic tobacco by the Agrobacterium-mediated plant transformation system. The transformation efficiency of the HIV-1 gp160 gene to the transgenic tobacco plantlets was tested by PCR. Expression of HIV-1 gp160 gene by the transgenic tobacco was demonstrated at mRNA level with RT-PCR. The specificity and expression level of HIV-1 gp160 proteins were analysed by ELISA.
5.2 MATERIALS AND METHODS

5.2.1 Materials

5.2.1.1 Plant materials

Nicotiana tabacum "sumsun" was maintained as an axenic plant culture in hormone-free MS medium. Leaves of about 1-2 cm in length were used for transformation.

5.2.1.2 Agrobacterium

Agrobacterium tumefaciens strain LBA4404 harbouring Ti plasmid pAL4404 (Hoekema et al., 1983) was kept at -70°C until use.

5.2.2 Methods

5.2.2.1 Production of transgenic tobacco plantlets

Recombinant plant binary vector pGPTV-KAN-210.1S-env160 (see section 4.3.7.4) containing the HIV-1 gp160 expression cassettes were used for tobacco (Nicotiana tabacum) "sumsun" plant transformations using Agrobacterium-mediated methods according to Horsch et al. (1988) with modifications. Briefly, the vector was transferred to Agrobacterium tumefaciens strain LBA4404 by electroporation (see section 2.2.3.1). Tobacco was transformed by cocultivating leaf discs (Horsch et al., 1988) with Agrobacterium strain LBA4404 transformed with transfer vector pGPTV-KAN-210.1S-env160. The explants were transferred to regeneration medium containing 500 μg/ml carbenicillin to kill the bacteria and 300 μg/ml of kanamycin to inhibit untransformed plant cells. After generating on transformed callus, shoots were rooted in selecting medium containing 500 μg/ml carbenicillin and 100 μg/ml of kanamycin (details see section 2.2.4.2a). After 3-6 weeks, leaves from transgenic tobacco plantlets were used for analysing gp160 protein expression.
5.2.2.2 PCR analysis of HIV-1 env160 gene in transgenic tobacco plantlets

Transgenic tobacco plantlet leaf DNA extracts were prepared as described in 2.2.4.4, and the HIV-1 gp160 gene in leaf genomes was detected by PCR (protocol see section 2.2.3.1) using 3 sets of paired primers 402/403, 306/634 and 518/256 (see section 2.2.3.18). Plasmid pGPTV-KAN-210.1S-env160 which had been used to transform tobacco plant was used as positive control and untransformed tobacco leaf DNA extracts were used as negative control. Each sample was tested 2-3 times.

5.2.2.3 Transcription analyzing of HIV-1 env160 in transgenic tobacco plantlets

Total RNA from leaves of tobacco plantlets transformed with recombinant binary vector pGPTV-KAN-210.1S-env160 was isolated as described previously in 2.2.4.5. RT-PCR was performed (as described in 2.2.3.15) to analyse the transcription of HIV-1 gp160 gene in transgenic tobacco plantlets leaf genome. RNA extracted from the serum of an HIV-1 seropositive patient (kindly given by Dr David Yirrell, Centre for HIV research, Edinburgh University) was used for positive control and RNA from untransformed wild type tobacco "sumsun" as negative control.

5.2.2.4 Analysis of the expression of HIV-1 gp160 in transgenic tobacco plantlets

Proteins from transgenic tobacco plantlet leaves were extracted by grinding with liquid nitrogen in Eppendorf tubes, extracted in extraction buffer (see section 2.2.4.6), followed by homogenising with a pestle. After centrifuging at 13,000 rpm for 5 minutes at 4°C, the supernatants were collected and aliquoted. The total protein concentration was measured using protein assay kit II (Bio-Rad) as described in 2.2.3.16. The expression of gp160 was assayed with HIV-1 gp160/120 ELISA (see section 2.2.2.2). Untransformed wild type tobacco "sumsun" plantlet leaves were used as a negative control. Each sample was tested in duplicate. The final concentration of protein was taken as the mean of the readings obtained in 2-3 independent assays. The HIV-1 gp160 protein expression level (ng/mg total soluble protein) in each of transformants was calculated as follows:
(protein concentration in transformant - background concentration in control) x 2

total protein concentration of transformant

Percentage of the total soluble leaf protein in each of transformants was calculated as follows:

\[
\text{expression level of gp160 protein (ng/mg total protein) } \times 10^6 \times 100\%
\]

5.3 RESULTS

5.3.1 Generation of transgenic tobacco plantlets

The recombinant binary vector pGPTV-KAN-210.1S-env160 (see chapter 4) containing HIV-1 gp160 gene was transferred to Agrobacterium tumefaciens strain LBA4404 by electroporation. After incubation at 30°C for 48 hours, the transformants were picked up and verified by both PCR using plant primer set TEV and vsp and restriction digestion to examine the presence of required inserted HIV-1 gp160 gene. The colonies that showed the right sized PCR products (2700 bp) and restriction fragments (11126 bp, 2759 bp and 1214 bp when cut with EcoRI and HindIII) (Figure 5.1 and Figure 5.2) were used to transform tobacco (Nicotiana tabacum "sunsun") by the Agrobacterium-mediated method (Horsch et al. 1988). Approximately 250 shoots were generated from transformed calli selected on medium containing 500 µg/ml carbenicillin and 300 µg/ml of kanamycin. 205 shoots were cut from the calli and rooted in rooting medium containing 500 µg/ml carbenicillin and 100 µg/ml of kanamycin. Approximately 100 plantlets were rooted and selected. The transgenic plantlets resistant to the selective medium did not show any morphological difference compared to non-transgenic tobacco plantlets (Figure 5.3a, b, c, d).

5.3.2 PCR analysis of kanamycin-resistant tobacco plantlets

Total genomic DNA from leaf tissues of regenerated kanamycin-resistant plantlets was used for PCR analysis to verify the integration of HIV-1 env160 genes in the host genome. 3 paired primers 402/403, 306/634 and 256/518 (see section 2.2.3.18)
Figure 5.1 Taq PCR screening *Agrobacterium* transformants transformed with recombinant plasmid pGPTV-KAN-210.1S-env160 containing CaMV 35S promoter and *env160*

*Agrobacterium* transformants DNA minipreps were subjected to PCR using plant primer set TEV and Vsp (see section 2.2.3.18d). 8 µl of the PCR reaction was loaded on 1% agarose gel and run for 1 hour at 80 mA electric current. The correctly transformed colonies resulted in a 2700 bp PCR product.

Lane 1: 1Kb DNA ladder  
Lane 2: Positive control plasmid GPTV-KAN-210.1S-env160  
Lane 3-7: *Agrobacterium* transformants 1-5 DNA minipreps  
Lane 8: Negative control without DNA template

Figure 5.2 Restriction fragment analysis of *Agrobacterium* transformants transformed with recombinant plasmid pGPTV-KAN-210.1S-env160 containing CaMV 35S promoter and *env160*

5 *Agrobacterium* transformants DNA minipreps were cut with restriction enzymes and were subjected to electrophoresis alongside undigested plasmid. The predicted restriction fragment sizes are 11126 bp, 2759 bp and 1214 bp when cut with EcoRI and HindIII. All samples analysed gave the correct sized restriction fragments.

Lane 1: 1 Kb DNA ladder  
Lane 2-6: Double digestion with EcoRI and HindIII
Figure 5.3 Development of transgenic tobacco (*Nicotiana tabacum* "sumsun") plantlets

Tobacco (*Nicotiana tabacum* "sumsun") explants are infected with *A. tumefaciens* strain LBA4404 carrying the recombinant expression vector pGPTV-KAN-210.1S-env160 and cultured on selecting medium containing 500 µg/ml carbenicillin and 300 µg/ml of kanamycin. During the next 4 weeks, the transformed cells grew into calli and differentiate into shoots via organogenesis. Between 4 and 6 weeks, the shoots develop enough to remove them from the transformed callus to inducing root in selecting medium containing 500 µg/ml carbenicillin and 100 µg/ml of kanamycin. After 3-6 weeks, rooted plantlets would grow enough for analysing gp160 protein expression in leaves.

a. Calli were formed around the wounded edge of the explant after transformation with *A. tumefaciens* strain LBA4404 carrying the vector pGPTV-KAN-210.1S-env160.

b. Shoots were generated during 4-6 weeks after transformation.

c. Plantlets were developed in 3-6 weeks after the shoots were removed from the transformed callus and rooted in selecting medium.

d. Untransformed tobacco (*Nicotiana tabacum* "sumsun") plantlets.
annealing to V1/V2, V3/V4 and gp41 regions of HIV-1 env160 gene respectively were used for PCR reactions and specifically amplified 298bp, 456bp and 451bp sized fragments of the env160 gene, respectively. DNA extracts were prepared from 30 kanamycin-resistant plantlet leaves which were selected randomly to detect the presence of the HIV-1 gp160 gene in the transgenic plantlets. PCR analysis showed the presence of all specific V1/V2, V3/V4 and gp41 region products of the expected sizes when amplified with 3 paired primers respectively although some nonspecific bands also appeared in 29 out of 30 tested transformants. 1 out of 30 samples (No 5) showed correctly sized V1/V2 and V3/V4 region PCR products produced with paired primers 402/403 and 306/634 in the absence of gp41 PCR product amplified with primer 518 and 256. No specific V1/V2, V3/V4 and gp41 region PCR products was detected in untransformed tobacco plantlet leaves (Figure 5.4a, b and c and Table 5.2).

5.3.3 Transcriptional analysis of transformants
To investigate the transcription of integrated env160 gene in transgenic tobacco plantlets, the presence of gene fusion mRNA was analysed. Total cellular RNAs were extracted from 17 plantlet leaves positive in all V1/V2, V3/V4 and gp41-specific PCR reactions and further tested by RT-PCR as described in section 2.2.3.15. HIV-1 gp41 primer 254 and oligo (dT)$_{15}$ were used as outer paired primers in the primary PCR and paired HIV-1 gp41 primers 518 and 256 as the inner paired primers used in the secondary PCR which resulted a 451bp DNA fragment product. The result showed that 14 out of 17 transformed plantlets genome conjugated with HIV-1 env160 genes showed specific transcription of env160 genes and 3 out of 17 plant showed negative reaction in RT-PCR. To rule out the possibility of contaminant DNA sequence in the RNA leaf extracts, the leaf RNA preparations were treated with RNase free DNase (RQ1, Promega) before cDNA synthesis. Furthermore, the purified RNA was treated with RNase (Boehringer Mannheim) and subjected to the primary and secondary PCRs. No amplified DNA fragments were detectable under these conditions in 14 RT-PCR positive leaf RNA extracts from the transgenic plantlets. This demonstrated that cDNA was specifically synthesized from mRNA transcribed from integrated env160 gene and
the positive RT-PCR reactions were RNA dependent. No correctly sized PCR product was detected in untransformed wild-type tobacco "sumsun" plantlets (Figure 5.5a and b).

5.3.4 HIV-1 gp160 protein expression in transgenic tobacco plantlets

5.3.4.1 gp160/120 enzyme-linked immunosorbent assay (ELISA)
To investigate the HIV-1 gp160 protein expression in transgenic tobacco "sumsun" plantlets, a specific ELISA was developed to detect the gp160 protein in the leaf protein extracts from transgenic plantlets.

The initial attempt to establish gp160/120 ELISA involved a sandwich ELISA approach. Briefly, ELISA plate or immunosorbant strips were coated with sheep polyclonal antibody to HIV-1 gp120 (D7324, Aalto BioReagents Ltd, Ireland) at room temperature overnight. The plates were then washed three times with TBST (50 mM Tris-HCl, pH7.6; 145 mM NaCl and 0.05% Tween 20) and blocked with 5% milk in TBST for 1 hour at 37°C. Then, doubling dilutions of recombinant gp160 protein (AIDS Reagent Project, MRC, UK) and 2 x diluent of plant leaf extracts to be tested were added and incubated for 2 hours at 37°C. After being washing with TBST, a monoclonal antibody to HIV-1 gp160/gp41 (AIDS Reagent Project, MRC, UK) was added and the plate was incubated at 37°C for 1 hour. After washing three times and incubating with horseradish peroxidase conjugated sheep anti-mouse IgG for 1 hour at 37°C, the plate was washed as before once more. The reaction was developed by the addition of TMB (3,3',5'-Tetramethylbenzidine) liquid substrate system and stopped by addition of 1N H2SO4 10-20 minutes later. The absorbance was read at 450nm. Different coating antibody concentration of 2, 4, 6 and 8 μg/ml; different concentration of monoclonal antibody to HIV-1 gp160/gp41 at 1:25, 1:100, 1:400 and 1:1600; and different concentration of horseradish peroxidase conjugated sheep anti-mouse IgG antibody at1:500, 1:1000, 1:2000, 1:4000, 1:8000 and 1:1600 were tested in combination. After a
Figure 5.4 PCR analysis of DNA extracts from transgenic plantlets

Total genomic DNA from leaf tissues of transgenic tobacco "sumsun" plantlets were extracted as described in 2.2.4.4. The presence of HIV-1 env160 gene in the transgenic plantlets were detected by PCR using 3 paired primers 402/403, 306/634 and 518/256 annealing at V1/V2, V3/V4 and gp4l regions of env160, respectively. PCR reactions were performed as described in 2.2.3.1. 12 µl of each of PCR reactions was loaded on 1% TAE agarose gel and run for 1-2 hours at 80 mA. The correctly integrated transgenic plantlets leaf DNA extracts resulted in a 298bp, 456bp and 451bp PCR product when using paired primers 402/403, 306/634 and 518/256, respectively. Plasmid pGPTV-KAN-210.1S-env160 containing the whole gene of HIV-1 env160 was used as a positive control whereas untransformed tobacco "sumsun" plantlet leaf DNA as a negative control. No specific V1/V2, V3/V4 and gp4l region PCR products were detected in untransformed tobacco plantlet leaf DNA extracts.

M1: 1Kb DNA ladder
M2: pGEM DNA marker
P: Plasmid pGPTV-KAN-210.1S-env160
N1: Untransformed tobacco "sumsun" plantlet
N2: Negative control without DNA template
Lane 1-30: Transgenic tobacco "sumsun" plantlet 5, 6, 24, 27, 30, 34, 46, 52, 56, 63, 64, 69, 74, 87, 89, 97, 100, 102, 106, 107, 109, 110, 113, 114, 116, 119, 120, 122, 126 and 146.

a. PCR analysis with V1/V2 primers 402 and 403
b. PCR analysis with V3/V4 primers 306 and 634
c. PCR analysis with gp4l primers 518 and 256
Figure 5.5 HIV-1 env160 transcription in transformed tobacco "sumsun" plantlets analysed by RT-PCR

17 transformed tobacco plantlets conjugated with env160 genes were analysed for the presence of gene fusion mRNA by RT-PCR as described in 2.2.3.15. Samples were treated with or without RNase to assess the RNA specificity of the reaction. The positive reaction resulted a 451bp PCR products. 14 out of 17 transgenic plantlets tested (24, 30, 46, 63, 74, 87, 97, 100, 106, 107, 116, 119, 120 and 126) showed specific transcription of env160 genes in RNase un-treated RNA samples with no amplified DNA fragments detectable in the corresponding RNase treated RNA samples and untransformed wild type tobacco "sumsun" plantlet leaf RNA extracts.

The upper picture shows the RNA samples without RNase-treated and the lower picture showed the corresponding RNase treated RNA samples.

a. Transgenic plantlet 24, 30, 46, 63, 64, 69, 74, 87 and 97 RT-PCR analysis
lane1: 1Kb DNA ladder
lane2: Positive control HIV-1 RNA
Lane3-11: Transformed tobacco plantlet 24, 30, 46, 63, 64, 69, 74, 87 and 97
Lane12: Untransformed tobacco "sumsun" plantlet
Lane13: Negative control without DNA template
Lane14: pGEM DNA marker

b. Transgenic plantlet 100, 106, 107, 116, 119, 120, 122 and 126 RT-PCR analysis
lane1: 1Kb DNA ladder
lane2: Positive control HIV-1 RNA
Lane3-10: Transformed tobacco plantlet 100, 106, 107, 116, 119, 120, 122 and126
Lane11: Untransformed tobacco "sumsun" plantlet
Lane12: Negative control without DNA template
Lane13: pGEM DNA marker
Fig. 5.5a

RNase-

Band 451 bp

RNase+

Fig. 5.5b

RNase -

Band 451 bp

RNase +
number of attempts, this method was abandoned due to its limited sensitivity and specificity (data not shown).

The subsequent approach involved the application and modification of a gp160/120 ELISA system developed by Moore and Jarrett (1988). As described in 2.2.2.2, the ELISA plates or strips were coated with sheep polyclonal antibody to HIV-1 gp120 (Aalto BioReagents Ltd, Ireland) and blocked with 2% BSA in TBST. After incubating with the twofold dilutions of recombinant gp160 protein (D7434; AIDS Reagent Project, MRC, UK) which was used as standard and 1:2 diluted leaf protein extracts, anti gp120 monoclonal antibody (AIDS Reagent Project, MRC, UK) was added to the plates. Then the detecting antibodies biotinylated F(ab')2 goat anti-human IgG (Zymed, San Francisco) and extravidin alkaline phosphatase were subsequently added to the plate. pNPP substrate (Sigma) system was used as indicating system. The optical density (OD) was read at 405nm. Combinations of different concentrations of coating antibody (2, 4, 6 and 8 μg/ml) and monoclonal antibody to HIV-1 gp120 (0.5, 1.0, 2.0 and 4 μg/ml) were investigated. The results showed that when the coating antibody was used at a working concentration of 4 μg/ml and the secondary antibody monoclonal antibody used at a working concentration of 1 μg/ml with both detecting antibody and extravidin alkaline phosphatase (Zymed, San Francisco) at a working concentration of 1:1000 dilution, the optimal sensitivity and specificity was achieved. The positive control, recombinant HIV-1 gp160 (AIDS Reagent Project, MRC, UK), was used as a twofold diluted series to give gp160 protein levels of 0-25 ng/ml per assay, the absorbance at 405nm after color development gave a linear relationship in this range (Figure 5.6). This ELISA system was used for subsequently investigation of HIV-1 gp160 protein expression in transgenic tobacco "sumsun" plantlets.

5.3.4.2 HIV-1 gp160 protein expression in transgenic tobacco plantlets
The presence of HIV-1 gp160 protein in the Agrobacterium tumefaciens strain LBA4404 containing recombinant expression vector pGPTV-KAN-210.1S-env160
Figure 5.6 Representative ELISA standard curve

The gp160/120 ELISA was performed as described in 2.2.2.2. The positive control recombinant HIV-1 gp160 (AIDS Reagent Project, MRC, UK) was twofold diluted to give gp160 protein levels of 0-25 ng/ml per assay, and the absorbance at 405nm after color development gave a linear relationship in this range.
transformed tobacco plantlets was investigated using the gp160/120 ELISA as described above. The amount of protein in each of the kanamycin-resistant transformants was determined by the Bio-Rad protein assay as described in 2.2.3.16. The gp160 expression level in the kanamycin-resistant transformants was calculated as described in 5.2.2.4. Leaf protein samples were prepared from 96 transgenic tobacco planlets as described in 2.2.4.6. 84 out of 96 tested leaf protein samples showed the presence of HIV-1 gp160 protein, the expression level ranging from 0.03 to 15.88 ng/mg soluble protein (Table 5.1). Extracts from 12 out of 96 transformants tested and untransformed wild type tobacco "sumsun" plantlets showed no detectable levels of gp160 protein (Figure 5.7). The percentage of the total soluble protein corresponding to gp160 protein accumulated in the leaves of tobacco transformants was up to 0.0016% of the total soluble leaf protein (Figure 5.8). The mean level of gp160 in transgenic tobacco "sumsun" plantlets was 4.12 ng per mg total soluble protein. The mean level of gp160 accumulation in the leaves of pGPTV-KAN-210.1S-env160 transformed tobacco plantlets was 0.0004% of the total soluble leaf protein.

Among the 84 transgenic tobacco plantlets which were positive in ELISA, the level of gp160 protein expression was variable. 46 plantlets showed low levels of gp160 protein expression, ranging from 0.01 to 4 ng/mg total soluble protein. 33 plantlets showed relative middle levels of gp160 protein expression, ranging from 4 to 12 ng/mg total soluble protein. 5 plantlets showed relative high levels of protein expression, ranging from 12 to 16 ng/mg total soluble protein (Figure 5.9).

5.4 DISCUSSION
Plant based oral vaccines offer a new approach to vaccination strategies. The major advantages of such edible vaccines are the low production cost and the ease of administration. The concept of vaccine production using transgenic plants was first
The gp160/120 ELISA was performed as described in 2.2.2.2 and the expression level of gp160 in transgenic tobacco "sumsun" plantlets was calculated as described in 5.2.2.4. The figure shows the mean of the expression level of gp160 in each transformant obtained in two independent assays. An untransformed tobacco plantlet was used as the negative control (sample No 1).
Sample No

gp160/total soluble protein (ng/mg)

Sample No

gp160/total soluble protein (ng/mg)

Sample No

gp160/total soluble protein (ng/mg)
Figure 5.8 Accumulation of gp160 in transgenic tobacco plantlets leaves

The figure shows gp160 expression percentage of soluble leaf protein in transgenic tobacco plantlets which was calculated as described in 5.2.2.4. An untransformed tobacco plantlet was used as the negative control (sample No 1).
### Table 5.1 Expression levels of gp160 in transgenic tobacco plantlets

<table>
<thead>
<tr>
<th>gp160/total soluble protein (ng/mg)</th>
<th>Percentage of total soluble protein (%)</th>
<th>Transgenic tobacco plantlets</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>0.01-1.00</td>
<td>0.000001-0.0001</td>
<td>20</td>
</tr>
<tr>
<td>1.01-2.00</td>
<td>0.0001-0.0002</td>
<td>9</td>
</tr>
<tr>
<td>2.01-3.00</td>
<td>0.0002-0.0003</td>
<td>14</td>
</tr>
<tr>
<td>3.01-4.00</td>
<td>0.0003-0.0004</td>
<td>3</td>
</tr>
<tr>
<td>4.01-5.00</td>
<td>0.0004-0.0005</td>
<td>6</td>
</tr>
<tr>
<td>5.01-6.00</td>
<td>0.0005-0.0006</td>
<td>5</td>
</tr>
<tr>
<td>6.01-7.00</td>
<td>0.0006-0.0007</td>
<td>2</td>
</tr>
<tr>
<td>7.01-8.00</td>
<td>0.0007-0.0008</td>
<td>4</td>
</tr>
<tr>
<td>8.01-9.00</td>
<td>0.0008-0.0009</td>
<td>2</td>
</tr>
<tr>
<td>9.01-10.00</td>
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</tr>
<tr>
<td>10.01-11.00</td>
<td>0.001-0.0011</td>
<td>4</td>
</tr>
<tr>
<td>11.01-12.00</td>
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</tr>
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<td>0.0012-0.0013</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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<td>0.0015-0.0016</td>
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</tr>
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</table>
Figure 5.9 Distribution of expression level of gp160 protein in transgenic tobacco plantlets

The figure shows the distribution of expression level of protein of gp160 in the transgenic tobacco plantlets.
Table 5.2 PCR, RT-PCR and ELISA analysis of leaf extracts from transgenic tobacco plantlets

<table>
<thead>
<tr>
<th>Transgenic plantlets</th>
<th>PCR Primers</th>
<th>RT-PCR</th>
<th>ELISA (gp160 ng/mg total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>402/403</td>
<td>306/634</td>
<td>518/256</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>-</td>
<td>ND#</td>
</tr>
<tr>
<td>6</td>
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<tr>
<td>122</td>
<td>+</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>126</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>146</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Control¹</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Untransformed plantlet leaves

¹ Not done

$ Represent positive reaction

@ Represent negative reaction.
described by Mason et al. in 1992, and since more authors have described antibody response to parentally or orally administration of plant-derived antigens (Haq et al., 1995; Thanvala et al., 1995; Mason et al., 1995; 1996; 1998; Arakawa et al., 1998; Carrillo et al., 1998; Ma et al., 1998; Tracket et al., 1998; Castanon et al., 1999; Tuboly et al., 2000).

Transformation of tobacco by cocultivating leaf discs with the appropriate Agrobacterium strain is the paradigm for Agrobacterium-mediated transformation of plant tissues and subsequent selection and regeneration of transgenic plants. This system possesses the unique advantage of allowing efficient gene transfer, selection and regeneration to be coupled together in a simple process. Together with the fact that tobacco is naturally susceptible to infection with A. tumefaciens, and also responds exceedingly well in culture (Horsch et al., 1988). Tobacco plant is considered as the ideal experimental system for the initial establishment of transgenic plant system.

In this chapter, we have reported the production of a transgenic tobacco plantlet system expressing the envelope glycoprotein, gp160 of HIV-1.

5.4.1 DNA analysis of kanamycin-resistant tobacco plantlets

The recombinant binary vector pGPTV-KAN-210.1S-env16O (see chapter 4), containing CaMV promoter 35S, translation enhancement TEV, HIV-1 env160 and a polyadenylation signal of a soybean vegetative storage protein gene 3’vspB, was used to transform Agrobacterium tumefaciens strain LBA4404 harbouring Ti plasmid pAL4404 (Hoekema et al., 1983). The HIV-1 env160 gene was then subsequently transferred to tobacco (Nicotiana tabacum "sumsun") genome by the Agrobacterium-mediated transformation (Horsch et al. (1988). 30 out of 96 rooted kanamycin-resistant tobacco plantlets were selected for the detection of the presence of the integrated gp160 gene by PCR using primers based the inserted genes (Figure 5.4). The result showed the presence of all HIV region products of the expected size, suggesting that the HIV env160 genes were efficiently integrated into the plant host genomes, which is similar to other studies on animal viruses showing that foot-and-mouth disease virus VP1 protein
(Carrillo, et al. 1998) and porcine transmissible gastroenteritis virus S gene (Tuboly et al., 1999) were efficiently inserted into the plant host genome.

1 (No 5) out of 30 samples tested showed correctly sized V1/V2 and V3/V4 region PCR products produced with paired primers 402/403 and 306/634 but with the absence of gp41 PCR product amplified with primer 518 and 256 (Table 5.2). The reason for this incomplete gp160 gene amplification by PCR is unclear, but unlikely due to the truncated T-DNA insertion as the gp41 region gene is close to the right border of recombinant binary vector pGPTV-KAN-210.1S-env160 and it is reported that the transfer of T-DNA to plant cells by Agrobacterium-mediated transformation is thought to start at the right border (Zambryski 1992).

5.4.2 Transcriptional analysis of Transgenic tobacco plantlets
To investigate the transcription of integrated gp160 gene in transgenic tobacco plantlets, 17 transgenic tobacco plantlets positive in all V1/V2, V3/V4 and gp41 specific PCR reactions were further analysed by reverse transcription (RT)-PCR to verify the mRNA of env160. 14 out of 17 tested plantlets showed specific transcription of env160 gene. No amplified DNA fragments were detectable when RNA preparation treated with RNase in tested 14 leaf RNA extracts, which demonstrated that cDNA were specifically synthesized from mRNA transcribed from integrated env160 gene and assessing that the positive RT-PCR reactions are RNA dependant. This result is consistent with other studies (Gomez et al., 1998).

The above results demonstrated the presence and proper processing of mRNA of gp160 gene in the leaves of tested transgenic tobacco plantlets, and that there was no inherent transcriptional limitation to the expression of gp160 protein in the transgenic tobacco plantlet leaves, which is similar to other reports (Mason et al., 1992; 1996).

5.4.3 HIV-1 gp160 protein expression in transgenic tobacco plantlets
The presence of HIV-1 gp160 protein in the transgenic tobacco plantlet leaves was investigated by a gp160/120 ELISA using monoclonal antibody to HIV-1 gp120.
protein extracts from kanamycin-resistant tobacco "sumsun" plantlets were analysed for the expression of gp160 protein. Because the site of insertion of the transferred DNA into the cellular chromosomal DNA is random, different levels of protein expression in independent transformants were expected. Results showed that 84 out of 96 tested plants were expressing gp160 protein at levels ranging from 0.03 to 15.88 ng/mg soluble protein, and 12 out of them showed no detectable gp160 protein (Table 5.1). The gp160 protein accumulated in the leaves of tobacco transformants ranged from 0.000003 to 0.002% of the total soluble leaf protein (Figure 5.8).

TEV translation enhancer sequence was fused to CaMV 35S leaf promoter to enhance env160 gene translation in transgenic plantlets while constructing the recombinant expression vector (see Chapter 4). It has been reported that TEV 5' UTR appeared to enhance the translation of foreign gene, such that it can increase the expression level of HbsAg or norwalk virus capsid protein (NVCP) in transgenic tobacco grown plant leaves up to 0.01% and 0.23%, respectively (Mason et al., 1992; 1996). However, most transformants obtained in our research expressed low levels of gp160 protein in tobacco plantlet leaves (Table 5.1), which is similar to E. coli LT-B expression level in transgenic tobacco plant leaves (Haq et al., 1995). This inconsistency is perhaps due to the response of the plant tissue to HIV env160 being different from that of HBsAg genes and NVCP genes, or might be because the tested leaf extracts were from the different growth stage of the plantlets and plants which could affect the amount of the foreign protein accumulated in tobacco leaves. Further study is needed to clarify this point.

In comparison to the results obtained from PCR, RT-PCR and ELISA in the 30 transgenic tobacco plantlets tested (Table 5.2), 5 transformants showed the presence of env160 genes but the absence of the expression of gp160 protein. The reason for the absence of expression of the integrated foreign genes is unclear, and could be due to the site of integration of the T-DNA into the plant nuclear chromosomal DNA which is random. Positional effects can have substantial effects on the level of transcription and hence translation in independent transformants.
It was found that the expression level of foreign protein was correlated to the abundance of mRNA detected in each of the transformants (Mason et al. 1992; 1996). Our results, however, observed that 3 transformants were both PCR and RT-PCR positive but without detectable expression of gp160 protein; and in contrast, 3 transformants were negative in RT-PCR reaction but expression of gp160 protein in tobacco plantlet leaves occurred (Table 5.2). The reason for this discrepancy is unclear, it is possibly due to the short or incomplete translation of the foreign genes or the amount expressed in the leaves tested was not detectable using the current method, or might be because gp160 protein in transgenic tobacco plantlet leaves degraded before harvest as suggested by some reports (Fang et al., 1989; Benfey et al., 1990), and also RNA may have been degraded during shipping from USA to UK. However the correlation between transcription level of foreign gene and expression level of foreign protein in transgenic plant should be further investigated.

5.4.4 Conclusion
This study demonstrated that HIV-1 env160 gene can be integrated into the plant host genome and gp160 protein can be expressed in transgenic tobacco plantlets, which indicates the feasibility of expression of HIV-1 env160 in plants for possible use as oral vaccines.
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Expression Of HIV-1 gp160 Protein In Transgenic Potato And NT-1 Cell Line
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6.1 INTRODUCTION

As described before (Chapter 5), transgenic plants represent an inexpensive and easily delivered system for the production of animal and human vaccines. The use of transgenic potato as a potential edible vaccine delivery system was initially described by Haq et al. (1995). Transgenic potato plants were made with the use of genes encoding the binding subunit of *Escherichia coli* heat-labile enterotoxin (LT-B). Mice that consumed transgenic potato samples developed serum IgG and mucosal IgA that were specific for LT-B, whereas mice fed with the control (non-transformed) tubers developed no LT-B-specific antibodies in either serum or mucosal samples. It was further demonstrated, by Mason et al. (1998), that mice fed with transgenic potatoes expressing LT-B had higher levels of serum and mucosal anti-LT-B than those gavaged with bacterial LT-B. This verified the feasibility of using transgenic potatoes as expression and delivery systems for oral vaccines against human diseases. Subsequently, the expression of norwalk virus capsid protein in transgenic potato has been shown to induce specific serum antibodies when fed to mice (Mason et al., 1996). Also cholera toxin B subunit (CTB) pentamer was synthesised in transgenic potato plants which, when fed to mice, induced mucosal and systemic anti-CTB antibodies at levels sufficient to generate protective immunity against the biological effects of CTB (Arakawa et al., 1997).

Transgenic potatoes have also been shown to be potential vaccine production and delivery systems against animal virus diseases. The major structural protein VP60 of rabbit hemorrhagic disease virus (RHDV) has been successfully produced in transgenic potato plants. Two rabbits inoculated with leaf extracts from such plants showed high anti-VP60 antibody titers and were fully protected against the hemorrhagic disease (Castanon et al., 1999). Transgenic potato plants were created for expression of the N-terminal domain of the glycoprotein S (N-gS) from transmissible gastroenteritis coronavirus (TGEV), which carries the major antigenic sites of the protein. Mice fed or immunised intraperitoneally with potato tuber extracts expressing N-gS developed serum antibodies specific for gS protein and serum IgG specific for TGEV, respectively (Gómez et al., 2000).
The first human clinical trials for a transgenic, plant-derived antigen was approved by US food and drug administration and performed in 1997 (Tacket et al., 1998). Transgenic potatoes constitutively expressing a synthetic bacterial diarrhea vaccinogen (LT-B) were orally delivered to human volunteers in Phase I/II clinical trials. Serum and faecal samples were collected prior to and at multiple time points after ingestion of the transgenic potato. The results showed that a significant rise in both IgG anti-LT-B antibodies in 10 of 11 test participants and IgA anti-LT-B antibodies in 6 of 11 test volunteers. No LT-B specific antibodies were detected in the control participants. Furthermore, a 4-fold rise in sIgA was detected in 5 of 10 volunteers who ingested transgenic potatoes. These results demonstrated that transgenic potato plants-derived recombinant LT-B was protected against digestion and proved capable of inducing an immune response in humans.

In this chapter, HIV-1 env160 containing in the recombinant expression vector pGPTV-KAN-210.1S-env160 (see section 4.3.7.4) was transformed to NT-1 tobacco cell line by Agrobacterium-mediated plant transformation system. As an edible vaccine, injection is not the aim; feeding is. Therefore, in this chapter, recombinant expression vector pGPTV-KAN-240.1S-env160 containing plant tuber specific promoter patatin and HIV-1 env160 genes (see section 4.3.7.5) was used to transform potato cultivar "FL1607". The expression level of HIV-1 gp160 protein in transgenic potato and NT-1 cell transformants was analysed by gp160/120 ELISA, respectively.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Both plant materials and Agrobacterium strain LBA4404 were kindly given by Dr Hugh Mason (Boyce Tompson Institute for Plant Research, Cornell University, USA) and his co-workers.

6.2.1.1 Plant materials

a. Potato cultivar "FL1607"
Potato cultivar (*Solanum tuberosum*, variety "Frito-Lay (FL) 1607") shoots were maintained by serial propagation as an axenic plant culture in hormone-free CM medium (see Appendix I) at 25°C, 16 hours days and 22°C, 8 hour nights.

b. **NT (Nicotiana tabacum)-1 cell line**

NT (*Nicotiana tabacum*)-1 cell line was cultured in 40 ml of NT-1 media (see Appendix I) and maintained at 26°C in dark with shaking. The cell culture was diluted in 1:40 in volume and fed with fresh medium every week.

### 6.2.1.2 Agrobacterium

*Agrobacterium tumefaciens* strain LBA4404 harbouring Ti plasmid pAL4404 (Hoekema *et al.*, 1983) was kept at -70°C until use.

### 6.2.2 Methods

#### 6.2.2.1 Production of transgenic potato microtubers

Recombinant plant binary vector pGPTV-KAN-240.1S-\textit{env160} (see section 4.3.7.5) containing the HIV-1 gp160 expression cassettes was used for potato "FL1607" plant transformations by the *Agrobacterium*-mediated methods (Wenzler *et al.*, 1989b). Briefly, the vector containing HIV-1 \textit{env160} gene was transferred to *Agrobacterium tumefaciens* strain LBA4404 by the electroporation (see section 2.2.4.1). Potato "FL1607" leaf strips were transformed by cocultivation with *Agrobacterium* strain LBA4404 carrying expression vector pGPTV-KAN-210.1S-\textit{env160}. The explants were transferred to regeneration medium containing 500 \textmu g/ml carbenicillin, to kill excess bacteria and 50 \textmu g/ml of kanamycin to inhibit growth of untransformed plant cells. Shoots were regenerated and rooted in CM selecting medium containing 50 \textmu g/ml kanamycin. When the rooted plantlets were about 5-6 cm high, the stem including one node was cut from the plantlet and placed upright on the petri plates containing tuberization media (see Appendix I) and incubated at 18°C in the dark for 4-6 months until the microtuber was formed and grown up to 3-6 mm in diameter (see section 2.2.4.2b). The microtubers were then harvested for analysis of HIV-1 gp160 protein...
6.2.2.2 Production of transgenic NT-1 cells
Recombinant plant binary vector pGPTV-KAN-210.1S-env16O (see section 4.3.7.4) containing the HIV-1 gp160 expression cassettes was used for NT (Nicotiana tabacum)-1 cell transformations by the Agrobacterium-mediated method. Briefly, the vector was transferred to Agrobacterium tumefaciens strain LBA4404 by the electroporation method as before (see section 2.2.4.1). Acetosyringone (3', 5'-Dimethoxy-4'-hydroxy-acetophenone, Aldrich Chem. Co.) treated NT-1 cells were mixed with different concentrations of 0 µl, 50 µl, 75 µl, 100 µl and 200 µl of bacterial suspension. After washing with NT-1 medium containing 500 µg/ml carbenicillin (to get rid of the excess bacteria), the cells were resuspended and plated on selective NT-1 medium containing 100 µg/ml carbenicillin to kill the excess bacteria and 300 µg/ml of kanamycin to suppress the growth of untransformed cells. The plates were incubated at 26°C for 3-4 weeks to allow the transformants to generate (details see section 2.2.4.3). The colonies, which grew large enough (about 50-100 mm in diameter) in 4-5 weeks, were collected and weighed before analysis of gp160 protein expression.

6.2.2.3 Analysing the expression of HIV-1 gp160 protein in transgenic potato microtubers and NT-1 transformants
Proteins from transgenic potato microtubers or NT-1 cell transformants were extracted in ice cold extraction buffer (see sections 2.2.4.7 and 2.2.4.8), and homogenised with a pestle or glass homogeniser. After centrifuging at 13,000rpm for 5 minutes at 4°C, the supernatant was collected and aliquoted. The total protein concentration was measured using protein assay kit II (Bio-Rad) as described in 2.2.3.16. The expression of gp160 in transgenic potato microtuber and NT-1 transformant extracts was assayed using a HIV-1 gp160/120 ELISA (details see section 2.2.2.2), untransformed wild type potato microtuber and NT-1 cell protein extracts were used as control samples, respectively. Each sample was tested in duplicate and the test repeated twice. Extracts from transgenic potato microtubers and NT-1 transformants were diluted 1:10 and 1:2, respectively, with antigen dilution buffer containing 0.1% Empigen (Calbiochem) before added to the
sheep polyclonal antibody to HIV-1 gp120 (D7324, Aalto BioReagents Ltd, Dublin, Ireland) coated ELISA plates (see section 2.2.2.2b). The final concentration of protein was taken as the mean of the readings obtained in 2 independent assays. The HIV-1 gp160 protein expression level (ng/mg total soluble protein) in each of transformants was calculated as follows:

\[
\frac{(\text{protein concentration in transformant} - \text{background concentration in control}) \times \text{dilution factor}}{\text{total protein concentration of transformant}}
\]

The percentage of HIV-1 gp160 protein expression in the total soluble potato tuber or NT-1 cell protein in each of transformants was calculated as follows:

\[
\text{expression level of gp160 protein (ng/mg total protein)} \times 10^6 \times 100\%
\]

Expression of gp160 protein in per gram NT-1 cell transformants (ng/g) was calculated as follows:

\[
(\text{protein concentration in transformant} - \text{background concentration in control}) \times 2 \times 2
\]
\[(\text{ml/g, extraction buffer/transformant})
\]

6.3 RESULTS

6.3.1 Generation of transgenic potato microtubers and NT-1 transformants

6.3.1.1 Generation of transgenic potato microtubers

Expression vector pGPTV-KAN-240.1S-env160 containing HIV-1 env160 (see section 4.3.7.5) was used to transform Agrobacterium tumefaciens strain LBA4404 by electroporation (see section 2.2.4.1). After incubation at 30°C for 48 hours, the transformants were picked up and verified by both PCR using plant primer set TEV and vsp and restriction digestion to confirm the presence of required inserting HIV-1 env160 gene. The colonies showed the right sized PCR products (2700 bp) and restriction
fragments (11126 bp, 4262 bp and 1214 bp when cut with EcoRI and HindIII) (Figure 6.1 and Figure 6.2) and were thus used to transform potato leaf strips by co-cultivation (Wenzler et al., 1989b). After 3-6 weeks, kanamycin-resistant shoots were regenerated from transformed calli selected on LC2 c/k (see Appendix I) (Figure 6.3a) at a frequency of 20%. Leaf explants usually gave rise to one to three shoots (Figure 6.3b). Approximately 60 shoots were cut from the calli and rooted in CM medium (see Appendix I) containing 50 μg/ml kanamycin. Approximately 40 plantlets were rooted and transferred to tuberization media (see Appendix I) for microtuber regeneration (Figure 6.3c). 32 transgenic potato microtubers were regenerated on tuber induction medium (Figure 6.3d). All kanamycin-resistant rooted potato "FL1607" plantlets showed no morphological difference compared to non-transgenic potato "FL1607" plantlets. However, some microtubers from the kanamycin-resistant potato "FL1607" plantlets showed slower microtuber growth rates compared to those from untransformed potato "FL1607" plantlets (data not shown).

6.3.1.2 Generation of transgenic NT-1 cell transformants
The recombinant binary vector pGPTV-KAN-210.1S-env160 containing HIV-1 gp160 gene was constructed (see section 4.3.7.4) and transferred to Agrobacterium tumefaciens strain LBA4404 by the electroporation method (see section 2.2.4.1). The presence of required HIV-1 envl60 gene in Agrobacterium transformants was verified by both PCR using paired plant primer set TEV and vsp and restriction digestion. The colonies that showed the right sized PCR products (2700 bp) and restriction fragments (11126 bp, 2759 bp and 1214 bp when cut with EcoRI and HindIII) (Figure 5.1 and Figure 5.2) were used to transform tobacco NT (Nicotiana tabacum)-1 cells by the Agrobacterium-mediated method (see section 2.2.4.3). Different volumes (0 μl, 50 μl, 75 μl, 100 μl and 200 μl) of bacterial culture were mixed with 4 ml of NT-1 cell suspensions, respectively. After incubated at 26°C in dark for 3-4 weeks on selecting medium containing 300 μg/ml of kanamycin, kanamycin-resistant transformants began to appear. Only those cultures containing 50 μl, 75 μl, 100 μl of Agrobacterium culture inoculated NT-1 cells generated kanamycin-resistant transformants. There was no apparent difference in
Figure 6.1 *Taq* PCR screening *Agrobacterium* transformants carrying recombinant plasmid pGPTV-KAN-240.1S-env160

Recombinant binary vector pGPTV-KAN-240.1S-env160 containing *patatin* promotor and *env160* was used to transform *Agrobacterium tumefaciens* strain LBA4404 by electroporation method (see section 2.2.4.1). Transformant DNA minipreps were subjected to PCR using plant primer set TEV and vsp. 8 μl of the PCR reaction was loaded on 1% agarose gel and run for 1 hour at 80 mA electric current. The correctly transformed colonies resulted in a 2700 bp PCR product.

Lane 1: 1Kb DNA ladder
Lane 2: Positive control plasmid GPTV-KAN-240.1S-env160
Lane 3-5: *Agrobacterium* transformants 1-3 DNA minipreps
Lane 8: pGEM DNA marker

Figure 6.2 Restriction fragment analysis of *Agrobacterium* transformants carrying recombinant plasmid pGPTV-KAN-240.1S-env160

3 *Agrobacterium* transformants DNA minipreps were cut with restriction enzymes and were subjected to electrophoresis. The predicted restriction fragment sizes are 11126 bp, 4262 bp and 1214 bp when cut with EcoRI and HindIII. All samples analysed gave the correct sized restriction fragments.

Lane 1-3: Double digestion with EcoRI and HindIII
Lane 4: 1 Kb DNA ladder.
Figure 6.3 Regeneration of transgenic potato (*Solanum tuberosum*, variety "Frito-Lay (FL) 1607") microtubers

Potato (*Solanum tuberosum*, variety "Frito-Lay (FL) 1607") leaf strips were infected with *A. tumefaciens* strain LBA4404 carrying the vector pGPTV-KAN-240.1S-env160 and cultured on selecting medium containing 500 µg/ml carbenicillin and 50 µg/ml of kanamycin. During the next 3 weeks, the transformed cells grew into callus and differentiated into shoots via organogenesis. Between 3 and 6 weeks, the shoots developed enough to remove them from the transformed callus to induce rooting in selecting medium containing 50 µg/ml of kanamycin. After 3-6 weeks, rooted plantlets had grown enough for microtuber induction on tuberization media (see Appendix I). After 4-6 months culture at 18°C in dark, microtubers grew large enough for the analysis of gp160 protein expression.

a. Calli were formed around the wounded edges of the leaf strips after transformation with *A. tumefaciens* strain LBA4404 carrying the vector pGPTV-KAN-240.1S-env160

b. Shoots were generated during 3-6 weeks after transformation. Leaf explants usually gave rise to one to three shoots.

c. Plantlets were developed in 3-6 weeks after the shoots were removed from the transformed callus and rooting induced on selecting medium

d1. Transgenic potato "FL1607" microtubers would grew enough in 4-6 months for analysis of gp160 protein expression.

d2. Untransformed potato "FL1607" microtubers.
transformant formation between these bacterial culture concentrations. NT-1 cell
cultures inoculated with 200 \mu l of bacteria showed no or very few kanamycin-resistant
transformants (data not shown). No kanamycin-resistant colonies were generated from
untransformed control NT-1 culture plates (Figure 6.4a, b, c and d).

6.3.2 HIV-1 gp160 protein expression in transgenic potato microtubers and NT-1
cell transformants
The presence of HIV-1 gp160 protein in transgenic potato microtubers and NT-1 cell
transformants conjugated with HIV-1 gp160 genes was investigated by the gp160/120
ELISA (see sections 2.2.2.2 and 5.3.4.1), respectively. The amount of protein in each of
the kanamycin-resistant transformants was determined by the Bio-Rad protein assay as
described in 2.2.3.16. The gp160 protein expression level in the kanamycin-resistant
transformant was calculated as described in 6.2.2.3.

6.3.2.1 HIV-1 gp160 protein expression in transgenic potato microtubers
Following Agrobacterium-mediated potato leaf transformation with recombinant binary
vector pGPTV-KAN-240.1S-env160 containing tuber-specific patatin promotor and
HIV-1 env160 genes, 32 transgenic potato "FL1607" microtubers were generated. The
expression level of gpi60 protein in microtuber extracts was analysed as described in
6.2.2.3. Results showed that 8 out of 32 tested microtuber extracts showed the
expression of gp160 protein, ranging up to 14.92 ng/mg total soluble protein. 24 out of
32 tested microtuber extracts and that from untransformed wild type potato "FL1607"
microtubers showed no detectable levels of gp160 protein (Figure 6.5). The percentage
of the total soluble protein corresponding to gp160 protein accumulated in the potato
microtuber tissues was up to about 0.002% of the total soluble tuber protein (Figure
6.6). The mean level of gp160 protein in 8 transgenic potato "FL1607" microtubers was
7.4 ng per mg of total soluble protein. The mean level of gp160 protein accumulation in
8 transgenic potato "FL1607" microtuber tissues was 0.00074% of the total soluble tuber
protein.

Among the 8 transgenic potato "FL1607" microtuber extracts which were positive in
Tobacco NT (*Nicotiana tabacum*)-1 cells were inoculated with *A. tumefaciens* strain LBA4404 carrying the recombinant expression vector pGPTV-KAN-210.1S-env160 and cultured on selecting medium containing 100 µg/ml carbenicillin and 300 µg/ml of kanamycin. During the next 3-4 weeks, the transformed cells grew into colonies and these grew enough for analysis of gp160 protein expression in 4-5 more weeks. No kanamycin-resistant colonies appeared in untransformed NT-1 cell culture.

a. Kanamycin-resistant colonies appeared in 3-4 weeks after NT-1 cells were inoculated with *A. tumefaciens* strain LBA4404 carrying the recombinant expression vector pGPTV-KAN-210.1S-env160.

b. Kanamycin-resistant NT-1 cell transformants grew bigger on selecting medium containing 300 µg/ml kanamycin.

c. Kanamycin-resistant NT-1 cell colony grew up enough for analysing gp160 protein expression in transformants in 4-5 weeks since they first appeared.

d. Untransformed NT-1 cell culture.
ELISA, the level of gp160 protein expression was variable. 3 samples showed lower level of gp160 protein expression, ranging from 1- 5 ng/mg total soluble protein; 4 samples showed an relative intermediate level of gp160 protein expression, ranging from 5 to 10 ng/mg total soluble protein and only 1 sample showed a relatively higher level of gp160 protein expression, of about 15 ng/mg total soluble protein (Table 6.1).

<table>
<thead>
<tr>
<th>gp160/total soluble protein (ng/mg)</th>
<th>Percentage of total soluble protein (%)</th>
<th>Transgenic potato transformants</th>
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<tr>
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6.3.2.2 HIV-1 gp160 protein expression in transgenic NT-1 cell transformants

Recombinant binary vector pGPTV-KAN-210.1S-env160 was introduced into tobacco NT (Nicotiana tabacum)-1 cells by Agrobacterium-mediated method (see section 2.2.4.3). 32 kanamycin-resistant transformants were generated and extracted for analysis of the expression of gp160 protein in NT-1 cell transformants. Results showed that 6 out of 32 kanamycin-resistant transformant extracts expressed HIV-1 gp160 protein with the maximum expression level of 2.76 ng/mg total soluble protein and 0.872 ng/g transformed cells (Table 6.2). 26 out of them and extracts from untransformed NT-1 cells showed no detectable gp160 protein (Figure 6.7). The percentage of the total soluble protein corresponding to gp160 protein accumulated in the NT-1 cell transformants was up to about 0.0003% (Figure 6.8). The mean level of gp160 expression in transgenic NT-1 cell transformants was 0.92 ng/mg total soluble protein and 0.4 ng/g transformed cells. The mean level of gp160 accumulation in the transgenic NT-1 cell transformants was about 0.0001% of the total soluble cell protein.

All of 6 transgenic NT-1 cell transformants showed low expression level of gp160
Figure 6.5 Analysis of gp160 protein in potato "FL1607" microtubers from kanamycin-resistant transformants by gp160/120 ELISA

The gp160/120 ELISA was performed as described in 2.2.2.2 and the expression level of gp160 protein in potato "FL1607" microtubers from kanamycin-resistant transformants was calculated as described in 6.2.2.3. The figure shows the mean of the expression level of gp160 in each microtuber extract obtained in two independent assays. The untransformed potato "FL1607" microtuber extract was used as the negative control (sample No 1).

Figure 6.6 Accumulation of gp160 protein in potato "FL1607" microtubers from kanamycin-resistant transformants

The figure shows gp160 protein expression as a percentage of the total soluble microtuber protein. The wild type potato "FL1607" microtuber extracts was used as the negative control (sample No 1).
Fig. 6.5

gp160/total soluble protein (ng/mg)

Sample No

Fig. 6.6

Percentage of total soluble protein (%)

Sample No
Figure 6.7 Analysis of gp160 protein expression level in kanamycin-resistant NT-1 cell transformants by gp160/120 ELISA

The gp160/120 ELISA was performed as described in 2.2.2.2 and the expression level of gp160 in NT (Nicotiana tabacum)-1 cell transformant was calculated as described in 6.2.2.3. The figure shows the mean of the expression level of gp160 in each transformant obtained in two independent assays. The untransformed NT-1 extracts was used as the negative control (sample No 1).

Figure 6.8 Accumulation of gp160 protein in kanamycin-resistant NT-1 cell transformants

The figure shows gp160 protein expression percentage of the total soluble NT (Nicotiana tabacum)-1 cell protein of kanamycin-resistant NT-1 cell transformant, which was calculated as described in 6.2.2.3. The untransformed NT-1 cell extract was used as the negative control (sample No 1).
Table 6.2 Expression levels of gp160 protein in each transgenic NT-1 cell transformant

<table>
<thead>
<tr>
<th>NT-1 transformants</th>
<th>Transformants weights (g)</th>
<th>gp160/total soluble protein (ng/mg)</th>
<th>Percentage of total soluble protein (%)</th>
<th>gp160/transformant (ng/g)</th>
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Table 6.3 Expression levels of gp160 protein in transgenic NT-1 cell transformants

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<th>gp160/total soluble protein (ng/mg)</th>
<th>Percentage of total soluble protein (%)</th>
<th>Transgenic NT-1 transformants</th>
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<td>0</td>
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protein. 4 out of 6 transgenic NT-1 cell transformants showed gp160 protein expression level ranging from 0.01 to 1 ng/mg total soluble protein; 1 out of 6 showed gp160 protein expression level of 1.23 ng/mg total soluble protein and 1 out of 6 showed gp160 protein expression level of 2.76 ng/mg total soluble protein (Table 6.3).

### 6.4 DISCUSSION AND CONCLUSION

Plant-based expression of candidate vaccines provide unique traits that include low production cost, easy transportation and storage and will thus be especially applicable to developing countries where they are most needed. Vaccine-producing plant species that grow in these countries and the plant tissues containing the recombinant antigen that can be directly consumed will be the essential factors to achieve this. The feasibility of expressing hepatitis B surface antigen in transgenic tobacco plants was initially demonstrated by Mason et al. in 1992. The advantage of using a transgenic tobacco system as the initial test system, was described before (5.4). However, tobacco tissues are rich in toxic alkaloids that prevent direct animal -feeding studies. Other plant species such as transgenic lettuce expressing a hepatitis antigen (Ehsani et al., 1997); transgenic tomato expressing rabies virus glycoprotein (McGarvey et al., 1995) and transgenic potato expressing a bacterial diarrhea antigen (Haq et al., 1995), norwalk virus capsid antigen (Mason et al., 1996) and a cholera antigen (Arakawa et al., 1997) were subsequently used to show the potential of "edible vaccine" production and delivery systems. Encouraged by the success of the above studies, we decided to experiment with transgenic potato system and tobacco NT-1 cell line which have characteristics of being easily manipulated in the laboratory and the shortest transformants regeneration period for expressing HIV-1 gp160 protein. Part of this work was carried out in collaboration with Dr Hugh Mason at the Boyce Tompson Institute for Plant Research, Cornell University, USA.

Kanamycin-resistant shoots were regenerated from transformed calli at a frequency of 20%, which is lower than reported from Castanon et al. (1999) where regenerants appeared on selecting medium at a frequency of 55%. This difference is perhaps due to
different transforming efficiencies between potato cultivar species "FL1607" and "Desirée", and foreign genes of HIV-1 env160 and rabbit hemorrhagic disease virus VP60 gene carried by Agrobacterium tumefaciens strain LBA4404.

Kanamycin-resistant potato "FL1607" plantlets showed no morphological difference compared to non-transgenic potato "FL1607" plantlets, however, microtubers expressing gp160 protein from the corresponding kanamycin-resistant transformants presented reduced growth rates compared to control microtubers from untransformed potato "FL1607" plantlets. This result is similar to a report from Mason et al. (1998) that plants expressing higher levels of E. coli heat-labile enterotoxin B subunit (LT-B) showed stunting of shoot growth and poor tuber yield. It is likely that, similar to LT-B, HIV-1 gp160 protein expression in potato tuber cells might affect tuber growth pattern by an unclear mechanism. It is possible that insertion of the transferred DNA into the plant cellular chromosomal DNA (which is random), might have negative effects on the growth of plant cells.

The presence of HIV-1 gp160 protein in potato "FL1607" microtubers from kanamycin-resistant transformants and tobacco NT-1 cell transformants was analysed by the gp160/120 ELISA using a monoclonal antibody to HIV-1 gp120. 32 of each potato microtuber lines from kanamycin-resistant "FL1607" plantlets and NT-1 cell transformants were obtained, respectively. Because the site of insertion of the transferred DNA into the plant cellular chromosomal DNA is random, and position can have substantial effects on the level of expression of foreign protein in independent transformants, different expression levels of HIV-1 gp160 protein in each of potato microtubers and NT-1 transformants tested were expected.

Different volumes (0 µl, 50 µl, 75 µl, 100 µl and 200 µl) of bacterial culture were used to inoculate with NT-1 cell suspensions. NT-1 cell cultures inoculated with 200 µl of bacteria showed fewer kanamycin-resistant transformants than those NT-1 cell cultures inoculated with other concentration of bacterial cultures. The fewer transformants at a
higher concentration of bacterial culture inoculation is probably because the higher concentration of bacterial culture is toxic to NT-1 cells and inhibits the cell growth.

The results demonstrated the feasibility of expressing the HIV-1 gp160 protein in transgenic potato microtubers and tobacco NT-1 cells. Both transgenic potato "FL1607" microtubers and tobacco NT-1 cell transformants showed lower expression levels of HIV-1 gp160 protein (up to about 0.0015% and 0.0003% of total soluble protein in transgenic potato "FL1607" microtubers and NT-1 cell transformants, respectively.) than those obtained for expressing the N-terminal domain of the glycoprotein S (N-gS) of transmissible gastroenteritis coronavirus (TEGV) in transgenic potato "desirée" tubers which gave a maximum of 0.07% of total tuber protein (Gómez et al., 2000); expressing the cholera toxin B subunit (CTB) pentamer in transgenic potato "Bintje" (both microtuber and leaf tissues up to 0.3% of total soluble protein) (Arakawa et al., 1997) and expression for E. coli enterotoxin (LT-B) in transgenic potato "FL1607" microtubers which gave a maximum of 0.01% of total soluble protein (Haq et al., 1995). This inconsistency is perhaps due to the different response of the potato tuber tissues to HIV env160 and other foreign genes such as above N-gS, CTB and LT-B genes, or might be because the different potato variety and growth stages could affect the accumulated amounts of foreign proteins in tested potato tuber tissues. However, the mechanism and subcellular localization of HIV-1 gp160 protein in plant cells need to be investigated. Thus an enhanced plant expression sequence could be designed and new recombinant binary vectors which could result in higher levels of HIV-1 gp160 protein expression in transgenic plant cells and tissues could be constructed.

In conclusion, results presented in this chapter have demonstrated the feasibility of expressing HIV-1 gp 160 protein in transgenic potato "FL1607" microtubers and tobacco NT-1 cell transformants, which indicates the possibility of using transgenic potato plants as potential HIV-1 env160 edible vaccine production and delivery system.
Chapter 7

General Discussion
HIV-1 is mainly transmitted by heterosexual contact (Overbaugh et al., 1999), therefore initial exposure to HIV occurs most frequently at the genital mucosa. This emphasises the need to understand the nature of HIV-specific mucosal immune responses, an approach that could lead to the generation of protection against HIV infection.

It is clear that human IgA displays a unique heterogeneity in its molecular forms. There are known to be two subclasses of IgA; IgA1 and IgA2. Serum IgA is predominantly monomeric IgA\(_1\). The IgA in external secretions is called secretory IgA (sIgA) and is produced locally at mucosal surfaces (see section 1.3.2). In this study, the presence of both IgG and IgA antibodies against HIV-1 viral antigens gp160/120, p65, p55, p51, gp41, p31, p24 and p17 was demonstrated in sera of HIV infected individuals using Western blot assay. Interestingly, the specific sIgA antibodies to HIV-1 viral antigens gp160, p65, p55, p51, gp41, p31, p24 and p17 were also detected in one of three serum samples. No clear explanation can be put forward for the presence of sIgA in serum, although it is possible that it could be the result of transudation of sIgA. This could have been locally synthesised in genital mucosa and reached the serum by tissue damage in a similar way to the increased level of sIgA detected in serum during lactation which is thought to be the result of a backflow of IgA produced in the mammary glands (Halsey et al., 1982).

HIV infects susceptible cells such as Langerhans cells, macrophages, T cells and epithelial cells in genito-urinary tract (Miller et al., 1992b), leading to a local mucosal immune response (Bélec et al., 1989a; Cao et al., 1990; Kutteh et al., 1994; Bélec et al., 1995a). This concept is further supported by the finding of sIgA against HIV-1 gp160, p65, gp41, p31 and p24 was present in urine samples and sIgA to p24 in a vaginal swab sample from HIV-1 infected individuals in this study. These support the suggestion that female and male genital tracts can produce a strong, specific mucosal response to HIV (Bélec et al., 1995a) and could therefore play an efficient role in limiting the virus infectivity on mucosal surface by neutralizing HIV-1.
In most pathogen-infected individuals, both specific IgG and IgA are present in genital tract (Artenstein et al., 1997). IgA antibody is the predominant immunoglobulin on the mucosal surface (Mestecky et al., 1987). However, it is unlikely to be true for HIV infection. The results of this study showed that IgG is the predominant isotype in urine and vaginal swab samples where IgG antibodies against gp160/120, p55, p65/51, p41, p31, p24 and p17 were present. The HIV specific IgG antibody pattern in paired urine and vaginal swabs samples was not similar to that in the corresponding serum samples. Thus it is unlikely that the source of these HIV-1-specific IgG antibodies is through serum transudation as suggested by some reports (Wolf et al., 1992; Black et al., 1997). It has been demonstrated that IgG could be produced locally (Morgan et al., 1980; Heinen et al., 2000). Therefore it is assumed that the predominant IgG antibody to HIV-1 present in urine and vaginal swab samples in the present study was locally produced. This finding is similar to the previous reports that IgG antibodies to HIV-1 appeared to be largely synthesised in situ within the genital tract of both genders (Bélec et al., 1995b; Hocini et al., 1997) and IgG excretion was increased in HIV-1 infected women, whereas the IgA secretion tended to decrease, suggesting a possible enhanced local IgG synthesis (Bélec et al., 1995c; Lu et al., 1993). This hypothesis was also supported by the observations in an animal models that nonclassical mucosal antibodies of the IgG isotype were found to be the predominant antibody in the saliva, rectal swabs, vaginal washes, semen and urethral washes in HIV-1 infected chimpanzees (Israel and Marx, 1995), and specific SIV IgG antibodies were found to be the predominant immunoglobulin in vaginal swab samples of chronically infected rhesus macaques (Miller et al., 1992a). Thus it is possible that anti-HIV IgG is the predominant antibody response on mucosal surface in humans. The reason for this phenomenon is unclear. It has reported that the secretion rates of total IgA, IgA1 and IgA2 were markedly reduced in women with AIDS when compared with those at early stage of HIV infection, indicating impairment of IgA class-specific immunoglobulin production in the cervical/vaginal mucosa during HIV infection (Bélec et al., 1995a). Similar selective reductions in salivary IgA and in intestinal IgA-producing plasma cells have also reported (Kotlre et al., 1987; Jackson et al. 1990; Sun et al., 1990; Müller et al., 1991;
Janoff et al., 1994). Since CD4\(^+\) helper T (Th) cells are essential to both induce and maintain appropriate IgA responses in mucosa-associated tissue and since HIV-1 infection causes a loss of mucosal CD4\(^+\) lymphocytes (McGhee et al., 1989), it is conceivable that the number of CD4\(^+\) lymphocytes and subsequently IgA-bearing plasma cells within the cervical/vaginal mucosa of HIV-1 infected women may be reduced.

There is increasing evidence that susceptibility to HIV-1 in humans is variable and that some individuals are able to resist infection despite multiple and repeated exposure to HIV (HEPS) (Rowland-Jones & McMichael 1995a; Shearer et al., 1996). This condition is likely to depend on the features of both the host and the virus (Shearer et al., 1996). In some individuals the resistance to HIV infection has a genetic basis. It has been reported that a mutant form of the HIV co-receptor, CCR5, on the surface of monocyte/macrophages is present in such individuals (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996). However, this is not the story for all the HEPS population. There are some reports which supported the idea that HEPS may acquire resistance to infection through the induction of CD8\(^+\) class I MHC-restricted CTL and CD4\(^+\) T-helper responses. Several studies have demonstrated the presence of HIV specific T cell responses, in the form of T cell proliferation and interleukin 2 (IL-2) production induced by HIV peptides. In addition the generation in vitro of HIV specific cytotoxic T lymphocytes (CTLs) has been shown in these individuals (Detels et al., 1994; Shearer et al., 1996; Paxton 1996) suggesting that the cellular immune responses may contribute to protection from HIV infection in some highly exposed, persistently seronegative (HEPS) persons.

Increasing evidence (see section 1.4.3) now suggests that mucosal immune responses may be involved in protection from HIV transmission in some HEPS individuals (Shearer et al., 1996; Burastero et al., 1996). Analysis of immune activity in HEPS is invaluable in order to understand fully the mechanism for protective activity against HIV infection. However, data concerning the contribution of mucosal immunity to
protection against HIV-1 infection in HEPS population are still limited.

In exploring the mucosal immunity to HIV-1 in HEPS, it was observed that specific IgG antibodies against HIV-1 gp160, gp120 and p51 were detected in a couple of urine but not the corresponding serum samples, which indicated that these IgG antibodies were locally produced and IgG were the predominant isotype in these specimens. This finding is consistent with the antibody production pattern in DNA vaccine vaginally immunised mice that higher levels of vaginal IgG were induced than that of vaginal IgA, suggesting that IgG producing B cells within the vaginal mucosa may be preferentially stimulated by HIV-1 envelope antigen (Wang et al., 1997). A similar result was also observed in a macaque model in which targeted iliac lymph nodes were immunised with a subunit SIV envelope and core vaccine (Lehner et al., 1996). Together with IgA antibody-secreting cells to p27, IgG antibody -secreting cells to p27 and gp120 were also increased in the iliac lymph nodes of the immunised macaques. However, this observation is not similar to previous studies on HEPS population where HIV-specific IgA but not IgG was present in urine and vaginal wash samples from HIV-exposed seronegative individuals (Mazzoli et al., 1997) and in the genital tract of HIV-1 resistant sex workers (Kaul et al., 1999). The reason for this discrepancy remains unclear. However, it should be considered that both HIV-1 specific IgG and IgA may be produced locally and contribute to the protection from HIV infection observed in HEPS populations.

In exploring HIV-1 specific sIgA in HEPS individuals, there was no anti-HIV sIgA present in all urine samples tested, however, a weak sIgA band to HIV viral p51 was detected in one of nine serum samples. The reason for absence of sIgA in urine samples is unclear but might relate to the low concentration of secretory antibody present in urine. It is clear that sIgA is secreted at mucosal sites (Rudzik et al., 1975; Brandtzaeg et al., 1989), and therefore, it is possible that this HIV-1 specific sIgA antibody found in serum samples could have been produced locally in genital mucosa and transported back to serum from the mucosal production site by an unknown mechanism. Further
study is required to clarify this point.

Overall, all these findings are in keeping with the view that mucosal immunity might be important in protection against HIV transmission. HIV-specific antibody with neutralising activity in secretions on the mucosal surface is critical for the prevention of infection in individuals exposed to HIV, although the types of anti-HIV mucosal immune responses required are still not fully understood.

It is well known that sexual transmission of HIV occurs mainly through mucosal surface at the genital tracts or rectum. Therefore, elicitation of immune response at mucosal surface is crucial in preventing virus transmission by blocking or neutralising virus at this site. Most HIV vaccine candidates, however, have been designed to elicit circulating antibodies. These vaccines, given by systemic route, may not induce mucosal immune responses (Funkhouser et al., 1993; Perry et al., 1993). Therefore, mucosal HIV vaccines, administered by mucosal routes (oral, rectal, intranasal and intravaginal) to induce both mucosal and systemic immune responses to HIV antigens are under development, although at present most are still restricted to animal models. It has been demonstrated that oral and intranasal immunisations can effectively induce a mucosal immune response upon re-exposure to antigen (Lehner et al., 1992; Thibodeau et al., 1992; Morrow et al., 1994; Bond et al., 1995; Mestecky et al., 1994; Muster et al., 1995; VanCott et al., 1998), raising the possibility that application of HIV antigens to mucosal surfaces may be a fruitful approach to vaccination against AIDS. However, these vaccine candidates still depend on costly protein purification, storage and transportation. If less expensive vaccines were available, they would be in immediate demand worldwide. Recently, it has been demonstrated that transgenic plants represent an inexpensive and easily delivered system for the production of animal and human vaccines.

One significant factor that distinguishes plant cells from animal cells is that full-grown adult plants can regenerate from individual plant cells and that an adult plant can be said
to have been cloned from a single cell of a parent plant (White, 1954; Krikorian and Berqua, 1969). This means that the effects of genetic manipulation in plant cells can be examined within a relatively short period of time.

*Agrobacterium*-mediated transformation (Horsch *et al.*, 1988) is most commonly used for generation of transgenic plants expressing foreign gene(s). *Agrobacterium* (a plant pathogen) can transfer a segment of its DNA (T-DNA) into its host plant cells during the process of infection. It is known that the transferred DNA (T-DNA) region remain functional when separated from the resident Ti plasmid onto two separate replicons in *Agrobacterium* (de Framond *et al.*, 1983; Hoekema *et al.*, 1983). Therefore a gene of interest constructed into a plant expression cassette can be transferred into plant genomes through a process similar to conjugation. Based on these findings, plant binary vectors were constructed and became the most commonly used vector system for creating and regenerate transgenic plants. These vectors contain a number of unique restriction endonuclease sites which can be used for inserting the foreign genes of interest and plant selectable marker(s) between the T-DNA borders and a cloning vehicle capable of replicating in *E. coli* and *Agrobacterium*. The recombinant plasmids containing the foreign gene of interest are transformed into *E. coli*, and subsequently mobilised into an *Agrobacterium* strain harbouring a Ti plasmid (either wild-type, or a "disarmed" Ti plasmid in which the T-DNA is deleted). During tissue culture, transformed plant cells are positively selected on medium containing the appropriate antibiotics and regenerated into transgenic plants.

It is clear that the HIV gp160 plays a pivotal role in the early events of virus attachment and entry into the target cell. Neutralising antibodies against HIV found in the sera of infected individuals are predominantly directed against this glycoprotein (Matthews *et al.*, 1986), and antibodies to HIV gp160 have been detected in genital secretions, urine samples, tears, milk, nasal washing, intestinal fluid, cervicular fluid and saliva (Bélec *et al.*, 1989a; O'shea *et al.*, 1990; Sun 1990; Thongcharoen *et al.*, 1992; Wolff *et al.*, 1992; Constantine *et al.*, 1994; Janoff *et al.*, 1994; Mestecky *et al.*, 1994), suggesting that
gp160 is an antigenic target during immune responses to HIV infection. The presence of neutralising antibodies might inhibit the transmission of HIV infection in vivo, therefore, in this study, tobacco and potato plants were engineered to express a gene for HIV-1 glycoprotein gp160.

Initially, two different vectors were constructed for either plant leaf or microtuber expression systems. A plant binary vector pGPTV-KAN was used in constructing of plant vector containing HIV-1 env160. pGPTV-KAN contains the left and right T-DNA borders (LB, RB) which denote the limits of the DNA that is integrated into the plant genomic DNA via Agrobacterium tumefaciens-mediated transformation. The predominant trait of this vector is that the plant selectable marker gene neomycin phospho-transferase (nptII) which confers kanamycin-resistance to the plant was located near left border. It is known that T-DNA is transferred from right border to left border to plant cells by Agrobacterium mediated transformation (Zambryski, 1992). Therefore, the identification of drug-resistant, plant transformants containing complete T-DNA insertions is facilitated by using plant vector containing selectable marker gene, nptII located close to the left T-DNA border. If nptII is located near the right border of T-DNA, it increases the likelihood of obtaining plants carrying the selectable marker gene, but not necessarily all the genes of interest since kanamycin-resistant plant cells can harbour truncated T-DNA insertions. Thus, pGPTV-KAN was the optimal plant vector used for constructs containing HIV-1 env160. However, pGPTV-KAN does not contain the plant leaf promoter CaMV 35S or tuber promoter patatin. Therefore construction of recombinant pGPTV-KAN containing HIV-1 env160 had to be achieved in two stages. The cloned gp160 encoding sequence was initially cloned into plant plasmid pIBT210.1 and pIBT240.1 to generate the recombinant constructs containing plant leaf specific promoter CaMV 35S, and plant tuber-specific promoter patatin, respectively. Then the required portion containing env160, 35S or patatin promoter, translation enhancement element TEV 5' UTR and mRNA 3' end processing sequence 3'vspB genes was subsequently cloned into pGPTV-KAN.
Three expression systems were attempted. Tobacco NT-1 cell line was chosen as it possesses characteristics of being easily manipulated in the laboratory and the shortest transformants regeneration period for expressing HIV-1 gp160 protein. It is well known that tobacco transformation by cocultivating leaf discs with the appropriate Agrobacterium strain is the paradigm for Agrobacterium-mediated transformation of plant tissues and subsequent selection and regeneration of transgenic plants. This system has the unique advantage of allowing efficient gene transfer, selection and regeneration to be coupled together in a simple process. Furthermore, tobacco is naturally susceptible to infection with A. tumefaciens, and also responds exceedingly well in culture (Horsch et al., 1988). Therefore the tobacco plant is considered the ideal experimental system for the initial establishment of transgenic plant systems. However, tobacco tissues are rich in toxic alkaloids that prevent direct feeding to animals as well as humans. Once it was established that there was no inherent limitation to the expression or aggregation of HIV gp160 gene in tobacco, other plants were then transformed. As an edible vaccine, injection is not the aim; feeding is. That is why the potato microtuber expression system was tried. For tobacco "sumsun" and NT-1 cells, the recombinant binary vectors pGPTV-KAN-210.1S-env160 containing HIV-1 env160 and leaf promoter CaMV 35S genes was used, whereas for potato "FL1607", expression vector pGPTV-KAN-240.1S-env160 containing env160 and tuber promoter patatin genes was used.

The presence of HIV-1 env160 V1/V2, V3/V4 and gp41 regions was detected in the selected transformed tobacco plantlets suggesting that HIV env160 had been efficiently integrated into the plant host genome. These results are similar to others (Carrillo, et al., 1998; Tuboly et al., 1999), who reported that most of the transgenic tobacco plants were positive for the transgene by PCR. In exploring the presence of the integrated env160 gene in transgenic plants, it was found that one tested tobacco transformant genome had V1/V2 and V3/V4 regions but lacked gp41 region of env160. The reason for this is unclear. It is unlikely due to the T-DNA insertion being truncated as the gp41 region of env160 is close to the right border of recombinant binary vector pGPTV-KAN-210.1S-
env160 and the transfer of T-DNA to plant cells by *Agrobacterium*-mediated transformation is thought to start at the right border (Zambryski, 1992).

In exploring the transcription and translation of integrated env160 in transgenic plantlets, we have demonstrated the presence and proper processing and translation of mRNA of env160 gene in the leaves of tested transgenic tobacco plantlets. Most of tested transgenic plantlets containing env160 gene showed specific transcription of env160 gene, and hence detectable expression of HIV-1 gp160 protein. In comparison the results of integration, transcription and translation of env160 in tested tobacco transformants, a small portion of transformants showed the presence of env160 genes but the absent of the expression of gp160 protein. The reason for the absence of expression of the integrated foreign genes is unclear, and could be due to the site of integration of the T-DNA into the plant nuclear chromosomal DNA which is random and positional effects can have substantial effects on the level of transcription and hence translation in independent transformants. Moreover, three transformants showed both integration and transcription of env160 but undetectable expression of gp160 protein (Table 5.2). This observation was different from some reports that the expression level of foreign protein correlated with the abundance of mRNA detected in each of the transformants (Mason *et al.*, 1992; 1996). The reason for this discrepancy is unclear. The absence of detectable gp160 protein in those transformants might be due to the short or incomplete translation of mRNA or the amount expressed in the leaves tested was not detectable using the current method; or might be because the gp160 protein in transgenic tobacco plantlet leaves degraded before harvest as suggested by other reports (Fang *et al.*, 1989; Benfey *et al.*, 1990).

HIV-1 gp160 was expressed in transgenic tobacco plantlets, potato microtubers and tobacco NT-1 cells. The presence of HIV-1 gp160 protein in the plants harboring and expressing the foreign genes was investigated by a gp160/120 ELISA using a monoclonal antibody to HIV-1 gp120. Because the site of insertion of the transferred DNA into the cellular chromosomal DNA is random, protein expression was variable.
between transformants. The amount of HIV-1 gp160 protein expressed in transgenic potatoes was similar to those found in tobacco plantlets. TEV 5' UTR leader sequence in the binary vector used for transforming the tobacco plants can enhance the translation of foreign gene (Mason et al., 1992; 1996). However, in the present study, a low yield of recombinant gp160 in transgenic plants occurred. The selected transgenic tobacco plantlets leaves and potato microtubers showed expression levels of HIV gp160 lower than those found expressing hepatitis B surface antigen (Mason et al., 1992) or norwalk virus capsid protein (NVCP) (Mason et al., 1996). Similar lower yields of recombinant protein in transgenic tobacco leaves also occurred to *E. coli* gene encoding LT-B when they were introduced into plant cells (Haq et al., 1995).

The reason for a low yield of recombinant protein in transgenic plant leaves is unclear. It has been reported that the CaMV 35S promoter, while constitutive, is most active in rapidly growing tissues but very low in mature tobacco leaves (Benfey et al., 1990; Fang et al., 1989). Therefore, the protein of interest may be degraded before harvest. This hypothesis may be in part supported by the observation that the expression level of foreign protein was not correlated to the presence of mRNA detected in a few transformants as described above. To increase expression of HIV gp160 in tobacco leaves, it is necessary to investigate inducible promoter systems with which wasteful synthesis-degradation cycles of foreign protein during normal growth and development will be avoided.

It had been reported that the level of expression of gp160 in heterologous systems including *E. Coli* (Crowl et al., 1985a; 1985b), yeast (Barr et al., 1987) mammalian cells (Chakrabarti et al., 1986, Dewar et al., 1989) and insect cells (Hu et al., 1987) appears to be very low. Examination of the HIV gp160 protein sequence reveals that it contains a signal sequence which has an unusually long hydrophobic domain preceded by a highly charged region (Douglas et al., 1997). Replacement of the signal sequence of HIV-1 Env protein with those of herpes simple virus glycoprotein increase efficiently expression of gp160 in CHO cells and in insect cells (Lasky, 1986; Berman et al., 1988;
Culp et al., 1991). These results support the hypothesis that the signal sequence of gp160 may be responsible for the poor expression in plants. Therefore, replacement of the signal sequence of HIV-1 gp160 protein with plant-derived leader sequence and signal peptides may be another strategy to increase the expression of HIV gp160 in plants. This strategy is further supported by reports that the increase of expression of bacterial antigen could be promoted by a plant cell localisation signal polypeptide SEKDEL fused to the antigenic protein. The microsomal retention signal SEKDEL (Munro et al., 1987) causes compartmentation of the fusion protein, facilitating oligomerisation (Hirst et al., 1987). Use of this signal has been shown to be effective in producing high level of bacterial antigens in tobacco leaves and potato microtubers (Haq et al., 1995; Arakawa et al., 1998). However, there is limited direct evidence regarding the role of signal sequence of HIV-1 in biosynthesis of the protein and how it might influence expression. Further study is required to clarify this point.

The expression of HIV gp160 in potatoes may be increased by using a plant-optimised coding sequence, which has shown to increase 5 to 40 fold the expression of bacterial genes in potatoes (Mason et al., 1998). Taken together, to obtain the high level of protein accumulation necessary to make plants efficient vaccine production systems, sophisticated gene manipulations are required. However, there is no theoretical limitation to the use of plant cells for generation of antigen-encoded DNA sequences from human pathogens.

The work presented in this thesis demonstrates the feasibility of expressing the immunogenic HIV gp 160 in plants. I have demonstrated by ELISA that the HIV gp160 expressed in transgenic plants retained immunoreactivity with mAb to human derived HIV gp120, indicating that the immunodominant epitopes presented in this protein may be authentic to native gp160. However, further study is required to examine properties of gp160 produced in plants. The N-glycosylation in higher organisms is conserved but differs slightly in details (Faye et al., 1993). The processing of the N-linked glycans occurs along the secretory pathway as the glycoprotein moves from the endoplasmic
reticulum through the Golgi apparatus to its final destination. Plants however, do not introduce sialic acids on its glycoprotein and synthesise N-glycans having carbohydrate motifs that are not found in animal cells (Faye et al., 1993). Thus the complex glycans of plants are often smaller than those of animals in part due to the difference above, i.e. no heterologous system is able to generate mammalian glycan exactly. It has been shown that only some of the properties of HIV gp160 depend on its glycosylation. For example, the lack of complex-type glycans in HIV gp120 expressed in insect cells or in gp120 isolated cells treated with trimming inhibitors seems to have no effect on major envelope glycoproteins functions like CD4 binding and infectivity (Montefiori et al., 1988; Morikawa et al., 1990). However, induction of and the sensitivity towards neutralising antibodies (Bolmstedt et al., 1992; Benjouad et al., 1994; Sawyer et al., 1994) or the host cell tropism of the virus (Cheng-Mayer et al., 1990; 1991) may be modulated by alterations in glycosylation. It is, therefore, important to analyse in detail to what extent glycans of HIV-1 gp160 produced in transgenic plants differ from the natural counterpart and how they affect the function of gp160, in particular, its immunogencity.

Studies on the development of mucosal vaccines against HIV have been shown to induce both mucosal and systemic immune response to HIV (Lehner et al., 1992; Thibodeau et al., 1992; Mestecky et al., 1994; Morrow et al., 1994; Bond et al., 1995; Muster et al., 1995; VanCott et al., 1998; Staats et al., 1996; 1997), which raised the possibility that application of HIV antigens on mucosal surfaces may be a fruitful approach to vaccination against AIDS. AIDS represents an international health crisis that threatens to overwhelm even the best systems of health care delivery. The hardest hit regions are developing countries. Therefore, efficient prevention of AIDS epidemic in these areas is the paradigm for the goal of controlling this disease worldwide. To achieve this goal an efficient vaccine must be developed. However, the key concern of the developing world in the areas of vaccine production and supply can be summed up as access and affordability. For example, a highly effective and safe vaccine against hepatitis B was developed in early 1980s, but its use in developing countries was long
prevented by its high cost. Other vaccines of importance to developing countries, such as *Haemophilus influenzae* type b vaccine and an HIV vaccine (hopeful) will not be widely used for these countries unless they are affordable. Therefore new, effective, safe and cheap candidate vaccines against major infectious diseases will need to be developed and evaluated. This is particular true for a vaccine against HIV as well as malaria and diarrhoeal diseases.

The successful expression of HIV-1 gp160 in transgenic plants provides the possibility for the generation of a safe and cheap mucosal vaccine candidate against HIV infection. However, many issues or technical challenges must still be resolved before plant-based production of HIV-1 gp160 for vaccine candidates gains widespread acceptance. Key areas include (1) optimisation of protein production levels as described above. (2) Test of oral immunogenicity of recombinant antigen gp160 produced in plants. To this end, gp160 produced in plants will be orally administered to animals and induction of protective immune response will be investigated. It is known that in addition to the delivery or presentation of antigen, the type of adjuvant incorporated in a vaccine is also an important determinant of whether a given antigen administered by mucosal route may elicit mucosal immune response. Two bacterial products with the greatest potential to function as mucosal adjuvants are cholera toxin B subunit (CTB) and the heat-labile enterotoxin (LT) (Morris *et al.*, 2000; Xu-Amano *et al.*, 1993). (3) Based on the achievements from experimental transgenic tobacco and potato system described in this thesis, further expression of HIV-1 gp160 protein in edible transgenic plants such as tomatoes and bananas, which can be eaten uncooked to prevent antigen denaturation, may provide the ideal tool for the development of cheap, safe, effective and convenient HIV oral vaccine.

Plant based oral vaccines offer a new approach to vaccination strategies. The demonstration that some antigens of pathogens expressed in transgenic plants are immunogenic when administered orally encourage the study of other antigens expressed in plants with the goal of developing edible vaccine. Viral antigens from hepatitis virus
(HBsAg) (Mason et al., 1992; 1995), norwalk virus (Mason et al., 1996), foot-and-mouth disease virus (Carrillo et al., 1998), hemorrhagic disease virus (RHDV) (Castanon et al., 1999), and porcine transmissible gastroenteritis virus (TGEV) (Tuboly et al., 2000) have been successfully expressed in transgenic plants. Immunisation with recombinant viral proteins expressed in transgenic plants induces a protective immune response in animals. Clearly research on plant edible vaccines has moved from theory to proof-of-principle. The first human clinical trials for a transgenic, plant-derived antigen was approved by US food and drug administration and performed in 1997 (Tacket et al., 1998). Transgenic potatoes constitutively expressing a synthetic bacterial diarrhea vaccinogen (LT-B) were orally delivered to human volunteers. All of IgG, IgA and sIgA anti-LT-B antibodies were significantly increased in tested participants compared to that of those controls. These results demonstrated that transgenic potato plants-derived recombinant LT-B was protected against digestion and proved capable of inducing an immune response in humans. However, challenging questions including whether edible vaccine functions at effector sites in the gut; if oral tolerance could be developed with food-born antigens, remain to be answered. Although many obstacles have yet to be overcome, it is my opinion that they are not roadblocks. The transgenic plant edible vaccines will be a potential new generation of novel, encouraging and prospective vaccine production and delivery systems with its distinguished characteristics including inexpensive production, unnecessary preparation or purification and easy transportation and storage.
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Appendix I: Solution and Media
**SOLUTION AND MEDIA**

**LB (LB/amp; LB/kan)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrpton (Difco)</td>
<td>10 g</td>
</tr>
<tr>
<td>Select yeast extract (Difco)</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl (Fisher Scientific)</td>
<td>5 g</td>
</tr>
<tr>
<td>Deionized H₂O up to 1 litre</td>
<td></td>
</tr>
<tr>
<td>Autoclave</td>
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<tr>
<td>Cool to 55°C,</td>
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<tr>
<td>For LB/amp, add ampicillin at 100 µg/ml.</td>
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</tr>
<tr>
<td>For LB/kan, add kanamycin at 50 µg/ml.</td>
<td></td>
</tr>
</tbody>
</table>

**LB agar (LB/amp; LB/kan) plates**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrpton (Difco)</td>
<td>10 g</td>
</tr>
<tr>
<td>Select yeast extract (Difco)</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl (Difco)</td>
<td>5 g</td>
</tr>
<tr>
<td>Bacto-agar (Difco)</td>
<td>15 g</td>
</tr>
<tr>
<td>Deionized H₂O up to 1 litre</td>
<td></td>
</tr>
<tr>
<td>Autoclave</td>
<td></td>
</tr>
<tr>
<td>Cool to 55°C,</td>
<td></td>
</tr>
<tr>
<td>For LB/amp agar plates, add ampicillin at 100 µg/ml.</td>
<td></td>
</tr>
<tr>
<td>For LB/kan agar plates, add kanamycin at 50 µg/ml.</td>
<td></td>
</tr>
<tr>
<td>Pour into petri dishes (25ml/100mm plate).</td>
<td></td>
</tr>
</tbody>
</table>

**SOB medium (per litre)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrpton (Difco)</td>
<td>20 g</td>
</tr>
<tr>
<td>Yeast extract (Difco)</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl (Difco)</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Autoclave</td>
<td></td>
</tr>
<tr>
<td>Add 10 ml of 1M MgCl₂ and 10 ml of 1M MgSO₄ prior to use, Filter sterilizing.</td>
<td></td>
</tr>
</tbody>
</table>

**SOC medium (per 100 ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOB medium</td>
<td>99 ml</td>
</tr>
<tr>
<td>2 M glucose</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

**YENB medium (per litre)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Select yeast extract (Difco)</td>
<td>7.5 g</td>
</tr>
<tr>
<td>Nutrient broth (Difco)</td>
<td>8 g</td>
</tr>
</tbody>
</table>

**B5 vitamin stock**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myo-inositol</td>
<td>100 mg/ml</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>10 mg/ml</td>
</tr>
</tbody>
</table>
Nicotinic acid 1 mg/ml
Pyridoxine-HCl 1 mg/ml

**MSO medium (per litre)**
- MS salts (Gibco BLR) 4.3 g
- B5 vitamin stock 1 ml
- Sucrose 30 g
- TC agar (JRH Bio Science) 0.8%
- Adjust pH to 5.7.

**MS104 medium**
- MSO
- Benzyladenine (BA) 1.0 μg/ml
- Naphthaleneacetic acid (NNA) 0.1 μg/ml
- Note: Dissolve 50 mg BA in 1 ml 1N KOH, then bring to 100 ml with H₂O for stock solution.

**MS selection medium**
- MS104
- Carbenicillin (AGRI) 500 μg/ml
- Kanamycin (AGRI) 300 μg/ml

**MS rooting medium**
- MSO with 0.6% Agar
- Carbenicillin (AGRI) 500 μg/ml
- Kanamycin (AGRI) 100 μg/ml

**NT-1 media and plate (per litre)**
- MS salts (Gibco BLR) 4.3 g
- Sucrose 30 g
- 20 x MES (pH 5.7) 50 ml
- B₁ - inositol 10 ml
- Miller's I 3 ml
- 2, 4 - D (0.5 mg/ml) 4.4 ml
- Adjust pH to 5.7.

For plates add 8 g/L TC agar (JRH Bio Science), autoclave 25 minutes.

For NTC add carbenicillin (250 mg/ml) to make the final concentration of 500 μg/ml.

For NTKC add carbenicillin to be the final concentration of 100 μg/ml, and kanamycin the final concentration of 300 μg/ml.

**20X MES**
MES 10 g
Adjust pH to 7.5 with 1M KOH
Deionized H₂O up to 1 litre.
Miller’s I
KH₂PO₄ 60 g
Deionized H₂O up to 1 litre.

B₁-inositol
Thiamine (B₁) 0.1 g
Myo inositol 10 g
Bring final volume to 1 litre.

CM medium (per litre)
MS salts (Gibco BLR) 4.3 g
Vitamins (1000 X) 1 ml
Sucrose 20 g
Adjust pH to 5.7
Hazelton agar (JRH Bio Science) 8 g
Autoclave
Pour 60 ml of media per GA-7 Magenta box.

LC1 medium (per litre)
MS salts (Gibco BLR) 4.3 g
Vitamins (1000 X) 1 ml
Sucrose 30 g
NNA stock solution (1 mg/ml) 0.2 ml
BAP stock solution (1 mg/ml) 2.24 ml
GA₃ (1 mg/ml) 10 ml
Adjust pH to 5.7
Hazelton agar (JRH Bio Science) 8.5 g
Autoclave
Pour 30 ml of media per 100 x 20 mm petri plate.

BAP stock solution
To 10 mg benzyl amino purine in a 13 ml glass tube, add 0.5 ml 1N HCl to dissolve.

LC1 c/k medium
Prepare LC1 as above;
Cool to 55°C
Add to each 500 ml portion
Carbenicillin (100 mg/ml) (AGRI) 2.5 ml
Kanamycin (25 mg/ml) (AGRI) 1 ml
Pour into petri plate as above.
LC2 c/k(per litre)
MS salts (Gibco BLR) 4.3 g 
Vitamines (1000 X) 1 ml
Sucrose 30 g
BA stock solution (1 mg/ml) 2.24 ml
GA3 (1 mg/ml) 10 ml
Adjust pH to 5.7
Hazelton agar (JRH Bio Science) 8.5 g
Autoclave
Cool to 60°C
Add to each 500 ml portion:
Carbenicillin (100 mg/ml) (AGRI) 2.5 ml
Kanamycin (25 mg/ml) (AGRI) 1 ml

Tuberisation media
MS salts (Gibco BLR) 4.3 g 
Sucrose 160 g
Kinetin (1 mg/ml) 10 ml
Deionized H2O up to 2 litre. Adjust pH to 5.85
Aliquot 500 ml/1L flask
Add 4.5 g Hazelton agar (JRH Bio Science) per flask
Autoclave
Cool to 55°C
To each 500 ml media, add 9.5 ml filter sterilized ancymidol.
Appendix II: Review I

Immune Responses in Highly Exposed, Persistently Seronegative (HEPS) Individuals

Yanmin Li

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The King's Buildings, West Mains Road, Edinburgh EH9 3JN, UK)
Abbreviations

ADCC antibody-directed cellular cytotoxicity
AIDS acquired immunodeficiency syndrome
bp base pair
CCR CC chemokine receptor
CTL Cytotoxic T lymphocytes
CVF cervicovaginal fluid
CXCR CXC chemokine receptor
env envelope
EUs exposed uninfected individuals
gp glycoprotein
HEPS highly exposed, persistently seronegative
HIV human immunodeficiency virus
HLA human leukocyte antigen
IFN interferon
Ig immunoglobulin
IL interleukin
LTNP long term non-progressor
MHC major histocompatibility complex
MIP macrophage inflammatory protein
M-tropic macrophage tropic
nef negative effector function
NK natural killer cell
PBMC peripheral blood mononuclear cells
PI primary isolate
pol polymerase
RANTES reduced upon activation normal T cell expressed and secreted
rev regulator of expression of virion proteins
RNA ribonucleic acid
SDF stromal cell derived factor
SHIV chimeric SIV/HIV viruses
SIV simian immunodeficiency virus
tat trans-activator of transcription
TCLA T cell line adapted
vif viral infectivity factor
vpr virion protein R
vpu virion protein U
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1.1 Introduction

Primary HIV infection is characterised by a transient viraemia which declines concordant with the onset of the specific cytotoxic T-lymphocyte (CTL) response. Seroconversion, with the generation of HIV-specific antibodies to Gag, Env and other viral proteins also occurs at this time or shortly afterwards (Weiss and Weiss, 2001). The progression time from infection with HIV to the development of AIDS may be affected by the effectiveness of control of infection by the host immune system (Buchbinder et al., 1999; McMichael et al., 2001) as well as host genetic factors such as chemokine receptor polymorphism and human leukocyte antigen (HLA) polymorphism (Michael et al., 1997a), but such factors are ultimately unable to prevent the development of AIDS which features greatly diminished numbers of CD4+ T cells (a massive decline from normal adult range 1200 cells/mm$^3$ to <200 cells/mm$^3$) (Flint et al., 2000; Weiss and Weiss, 2001). However, there is substantial variation in susceptibility to HIV infection and exposure to HIV does not inevitably lead to infection. Thus some individuals who remained HIV seronegative despite being repeatedly exposed to HIV (defined as highly exposed, persistently seronegative (HEPS) individuals or exposed uninfected individuals (EUs)) have been described in different cohorts of at risk individuals including homosexual/heterosexual partners of HIV-seropositive individuals and sex workers (Rowland-Jones et al., 1995a; Simonsen et al., 1998; Plummer et al., 1999). Thus the question raised is how HIV-specific immune response could play a role in protection against HIV-1 infection in HEPS individuals while failing to eradicate HIV in most HIV-1 infected individuals. This paper will focus on the progress in understanding of the factors that determine why HEPS individuals remain uninfected.

1.2 Immune responses against HIV

The immune system in man can be divided into the innate (non-specific) and the acquired (specific) immune systems. The innate immune system such as the skin and phagocytic cells imposes physical barriers against invading pathogens and is particularly important in warding off pathogenic infection presenting at the mucosal cell surface. It has become increasingly clear that it may be involved in primary protection against HIV
infection. For example, sexually transmitted infections, particularly those which disrupt the epithelial barrier and cause genital ulceration, enhance the risk of HIV infection (Simonsen et al., 1988; 1998; Grosskurth et al., 1995). If the innate immune system is overwhelmed, specific immune responses are then relied on. The specific immune responses are divided into humoral and cellular immune responses. They will be considered separately here.

1.2.1 Humoral immune responses

The humoral immune response, which is characterised by the production of large numbers of antibody molecules specific for the invading pathogen, is uniquely adapted to the elimination of extracellular pathogens. This process requires the participation of macrophages and activated helper T cells and B cells.

Early research on the immune response to HIV infection focused on the role of the humoral immune response because of the strong, early detectable antibody response observed in HIV-infected individuals. Antibodies to HIV proteins can appear as early as 2 weeks and as late as 6 months after infection (Allain et al., 1986; Gaines et al., 1988), but generally within 1 to 3 months (Flint et al., 2000). These antibodies are secreted into the blood and on the mucosal surface of the body and can be detected in genital and other body fluid. It was shown that the viral gp120 and gp160 Env proteins were the major targets for human antibody responses (Allan et al., 1985; Barin et al., 1985; Moore et al., 1994). Antibodies specific for other HIV proteins, including p66, p55, gp41, p32, p24, and p17, have also been detected (Carlson 1988; Moore et al., 1994).

Among the various isotypes, IgG₃ antibodies are believed to play a dominant role at all stages of infection, giving rise to ADCC responses and complement-dependent cytotoxicity and neutralising responses (Flint et al., 2000). However, a recent in vitro study showed that IgG₃ from polyclonal anti-HIV antibodies has more potency in neutralising HIV-1 compared to that of IgG₁ and IgG₂ (Scharf et al., 2001). This difference appears to be due to the unique structure of IgG₃ molecule (Cooper et al., 1994). The long hinge region (located between C₇ and C₉2 range of heavy chain) of IgG₃ permits enhanced flexibility of the Fab arms, thus permitting better divalent
binding to multivalent epitopes spaced at a certain ranges of distance from each other. Whether this advantage occurs in vivo remains unknown. If this phenomenon was retained in vivo, strategies which favor IgG₃ may provide a new approach for improvement of passive immunotherapy with antibodies, and HIV vaccines which induce high-titre of IgG₃ antibody response could be advantageous.

In viral infection, the principle role of antibody is neutralisation which prevents cell entry. Following primary infection with HIV-1, neutralising antibodies against HIV-1 are detectable in the serum within 1-2 months after infection (Koup et al., 1994; Moore et al., 1994). A variety of different observations show that early neutralising antibodies tend to be of low titer, are not present in all patients after primary infection, and are only able to neutralise a limited range of isolates (Moore and Ho 1995; Legrand et al., 1997; Moog et al., 1997; Pilgrim et al., 1997; Stamatos et al., 1998). Even following many years of HIV infection, most infected subjects mount only weak neutralising responses against primary HIV-1 isolates (Sattentau et al., 1996; Burton et al., 1997a). A study of 17 recently HIV-1-infected patients followed up for 12 months showed that neutralising antibodies were not detectable before month 3 (Legrand et al., 1997). Similar results have been reported by Moog et al. (1997), who showed that anti-HIV-1 neutralising antibodies against the autologous strains were detected about 1 year after seroconversion. This slow development of neutralising antibodies during primary infection might be one of the reasons the HIV-1 is able to establish persistent infection. Similar delays in neutralising antibody responses have been reported for SIV infections in macaques (Reimann et al., 1994). These observations question whether antibodies that arise in response to HIV infection are critical in controlling HIV infection. However, recent work showed that HIV-specific antibody, capable of inhibiting autologous and heterologous primary strains of HIV-1, in the presence of natural killer (NK) effector cells, is detectable in acutely HIV-1 infected-patients when viremia is declining and as early as 3 days after the onset of symptoms of acute infection. The magnitude of this antibody response was inversely associated with plasma HIV-1 viremia level. Furthermore, antibodies from acutely infected patients likely reduced HIV-1 yield in vitro both by mediating effector cell lysis of target cells expressing HIV-1 glycoproteins.
and by augmenting the release of beta-chemokines MIP-α and RANTES from NK cells (Forthal et al., 2001a). Thus HIV-1-specific antibody could be an important contributor to the early control of HIV viremia. Baba et al. (2000) demonstrated that passive immunotherapy with IgG monoclonal antibodies against the HIV-1 gp120 CD4 binding region and the gp41 neutralising epitope, protected rectally SHIV-infected macaques. Similar results have been reported by Parren et al. (2001), who demonstrated that the neutralising monoclonal antibody (B12) against an epitope overlapping the CD4+ binding site of gp120 protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus (SHIV) when the animals were intravenously given 25 mg of B12 per kg of body weight six hours prior to challenge. Neutralising antibody against the HIV-1 Env glycoprotein is capable of protecting macaque monkeys from chimeric SHIV infection (Shibata et al., 1999). Moreover, HIV-1 serum antibodies against monomeric gp120 from HIV-1 infected individuals can neutralise primary isolates of HIV-1 (Stamatos et al., 1998). These studies indicate neutralising antibodies may play a role in controlling or preventing HIV-1 infection.

There are several possible elements to limit the ability of antibodies to neutralise HIV. The HIV-1 envelope is initially synthesized as a precursor (gp160) that is subsequently cleaved to yield gp120 (an extracellular domain) that is noncovalently associated with gp41 (a transmembrane protein) (Rao et al., 1995). Several features of the HIV-1 envelope limit its ability to be neutralised by antibodies. gp120 is one of the most heavily glycosylated proteins identified to date, and these N-linked glycosylation sites are believed to limit the ability of antibodies to bind to gp120 (Luciw, 1996). In addition, the HIV-1 envelope exists as an oligomer of three gp120-gp41 molecules. Oligomerisation of the HIV-1 envelope blocks the ability of antibodies that bind to epitopes present on monomeric forms of envelope to bind to the oligomeric form. In fact, the majority of antibodies present in HIV-infected people recognise monomeric forms of envelope including monomeric gp120 rather than the mature oligomeric protein (Burton et al., 1997b). These data suggest that the accessibility of epitopes is important in mediating HIV-1 serum neutralisation.

Another factor critical to understanding neutralising antibodies involves the distinction
between T cell line adapted (TCLA) and primary isolates (PIs) of HIV-1. Adaptation to growth in T cell lines generally leads to a selection of HIV-1 virus variants which are not representative of the original input population (Meyerhans et al., 1989) and are generally more easily neutralised than primary virus isolates (Moore et al., 1995). This property appears to result from the great accessibility of several epitopes including the V3 loop and the CD4 binding site in TCLA HIV-1 strains for antibody neutralisation (Moore et al., 1995). As a result of these factors just listed, primary virus isolates are relatively resistant to neutralisation by antibodies (Sattentau et al., 1996; Burton et al., 1997a). However, York et al. (2001) showed that HIV-1 Env-specific monoclonal antibodies against the VP3 loop and the CD4 binding site of gp160, bind equally well to neutralisation-sensitive TCLA Env and neutralisation-resistant PIs Env. This indicates that the different neutralisation sensitivity between TCLA and PIs may not be due to the differences in the ability of antibodies to bind the respective Env complexes. If the antibody binding event is not different for TCLA and PI virions, then the differential outcome of binding (neutralisation or lack thereof) must arise through other factors which are unknown. Further research is needed to explore in detail the process and mechanisms of virus neutralisation, which will shed light on the development of effective HIV vaccines.

1.2.2 Cell mediated immune responses

The cell-mediated immunity (CMI) is the principal controlling/eliminating force against intracellular pathogens (McMichael et al., 1983; McMichael and Rowland-Jones, 2001). CMI is mediated by antigen-specific T lymphocytes and various non-specific cells such as NK cells and monocytes/macrophages of the immune system.

1.2.2.1 CD8+ CTL responses to HIV

Cytotoxic T lymphocytes (CTL) are major contributors to the antiviral T cell immune response. This T cell population carries the CD8+ membrane glycoprotein on the cell surface and recognises and lyses virus-infected cells following binding of the T cell receptor to a viral peptide presented by a class I HLA molecule on the infected cell. The HLA type of an individual thus plays a major role in determining whether an individual
will generate a CTL response to a given epitope. Presentation of viral proteins to CD8+ CTL requires synthesis of antigen within the cell.

HIV-specific CTLs were initially described in 1987 and it was remarkable that human leukocyte antigen (HLA)-restricted CTLs could be readily detected in freshly separated peripheral blood mononuclear cells or in alveolar lymphocytes from HIV-infected individuals, without prior in vitro stimulation with antigen (Walker et al., 1987; Flamand et al., 1998; McBreen et al., 2001). Subsequent studies demonstrated that HIV-1 specific CTLs could be isolated from infected organs, such as spleen (Cheynier et al., 1994), lymph nodes (Hadida et al., 1995; Kuroda et al., 1999), and even from the vaginal mucosa of SIV-infected macaques (Lohman et al., 1995; Wilson et al., 2000). HIV-specific CTLs were also found in individuals exposed but not infected by HIV. The latter may reflect the ability to control virus early in infection (Langlade-Demoyen et al., 1994; Rowland-Jones et al., 1995; 1998a; 2001; Kaul et al., 2001b) and will be discussed in section 1.3.2.2b. In vitro studies showed that HIV-specific CD8+ CTL cells are able to inhibit HIV-1 replication (Yang et al., 1997) and eradicate HIV-1 (Lu et al., 2001).

CD8+ CTLs have been demonstrated against most of the HIV gene products, and are predominantly directed against the structural proteins Gag, Pol and Env (Borrow et al., 1994, Betts et al., 1997; Cao et al., 1997; Wilson et al., 1998; Novitsky et al., 2001), but also target regulatory proteins such as Nef (Novitsky et al., 2001), Tat and Rev (Addo et al., 2001) and the accessory protein Vpr (Altfeld et al., 2001a). The first identified peptide epitope was an HLA-B27-restricted 15-mer in gag (Nixon et al., 1988), now known to be the decamer KRWIILGLNK (Rowland-Jones and McMichael 1993), and subsequently a large number of epitope peptides have been identified and are recorded in the Los Alamo HIV Molecular Immunology Database, New Mexico (Korber et al., 1998). A significant number of CTL epitopes that are located in highly conserved regions of the viral genome and are almost identical among HIV-1 clades might be responsible for the observed cross-clade CTL recognition (Betts et al., 1997; Cao et al., 1997; Durali et al., 1998; Novitsky et al., 2001). Work by Durali et al. (1998) demonstrated that epitopes conserved between the HIV-1 clades A and B appeared...
especially frequent in Gag p24, Gag p17, integrase, and the central region of Nef. However, a series of CTL epitopes including epitopes in Gag 24 that are unique for each HIV-1 subtype might be highly clade specific (Rowland-Jones et al., 1998a; Dorrell et al., 1999, Cao et al., 2000). Thus identification of immunodominant CTL regions and epitopes across the viral genome might help clarify the issue of clade specificity versus cross-clade reactivity for HIV-1 vaccine design.

A striking feature of HIV infection is that HIV-specific CTLs of an infected person can be directed toward multiple epitopes in HIV viral proteins. In HIV-infected individuals, HIV-specific CTLs responses have been described against six different HLA-B*5101 restricted epitopes (Tomiyama et al., 1999); six different HLA-B*3501 restricted peptides (four Pol and two Nef epitopes) (Tomiyama et al., 1997); 11 different HLA-A*2402 restricted Gag epitopes (Ikeda-Moore et al., 1998) and as many as 13 different HLA-A3/11 and HLA-B35/44 restricted Gag, Pol and Nef epitopes (Dalod et al., 1999). Similar results have been reported for SIV-infected Rhesus macaques. Egan et al. (1999) identified CTL responses against as many as five peptides in an SIV-infected macaque. It has also been observed that CD8+ lymphocytes from the same SIV-infected macaque could recognise as many as 14 Mamu-A*01 (a common MHC class I molecule in Rhesus macaque) restricted epitopes in SIV Gag (Allen et al., 2001). Therefore, the CTL repertoire against HIV-1 or SIV may be broad (in the sense of multiple antigens being recognised) and multispecific (in the sense of multiple epitopes being recognised within the same antigen). However, the range of HIV CTL epitopes recognised by infected individuals may vary since it has also been observed that an entire CTL response was directed toward a single epitope in HIV-1 Gag in HIV-infected hemophiliacs over several years (Goulder et al., 1997a).

Studies of CD8+ CTL response at the different stages of disease provide evidence of the importance of HIV-1 specific CD8+ CTL in controlling HIV-1 replication and disease progression. Borrow and co-workers (1994) reported that the level of HIV-1-specific CTL activity in HIV-1 infected patients paralleled the efficiency of control of primary viremia. Patients with strong gp160-specific CTL responses showed rapid reduction of
acute plasma viremia and antigenemia, while in contrast, primary viremia and antigenemia were poorly controlled in patients in whom virus-specific CTL activity was low or undetectable. This observation is consistent with other studies (Ariyoshi et al., 1992; Koup et al., 1994), which demonstrated that the early decline in plasma viral levels shortly after HIV-1 infection coincides with the appearance of HIV-specific CD8+ CTLs. In addition, inverse correlations between frequency of peripheral blood HIV-1-specific CTL and plasma HIV load have been reported (Ogg et al., 1998). A recent study showed that during acute HIV-1 infection, broader HIV-1 specific CTL responses were correlated with lower viral load (Altfeld et al., 2001). The best evidence for the protective immune function of the specific CTL derives from the macaque model of SIV infection. Yasutomi et al. (1993) have detected CTL precursors as early as 4 days after SIV infection, peaking with viremia at around 2 weeks. Subsequent studies confirmed the correlation of SIV-specific CD8+ response with viremia control after SIV primary infection in macaques (Reimann et al., 1994; Chen et al., 1995). Depletion of CD8+ cells results in SIV replication being uncontrolled during primary infection and high virus load in chronically infected rhesus monkeys (Jin et al., 1999). Reappearance of SIV-specific CD8+ T cells resulted in a decrease in viremia again (Schmitz et al., 1999). These data indicate the importance of CD8+ CTL responses that arise in response to HIV-1 infection in controlling HIV replication during primary infection, and a role for cellular immunity in protection to HIV-1 in vivo. In addition to resolving the viremia of acute infection, early generation of HIV-specific CD8+ CTLs may influence the subsequent disease course (Borrow et al., 1994; Koup et al., 1994). It was reported that a more broadly directed CTL response correlates with slower disease progression (Pantaleo et al., 1997). There is an inverse correlation between disease progression and HIV-1-specific CTL activity in HIV-1 infected subjects (Musey et al., 1997). Moreover, HIV-infected long-term nonprogressors (LTNPs) appear to have been associated with strong virus-specific CTL (Klein et al., 1995; Pantaleo et al., 1995). Pontesilli et al. (1998) described that LTNPs maintained the established CTL precursor pool, and that the frequency of gag-specific CTL precursors was inversely correlated with virus load. On the other hand, viral mutation in CTL epitopes can result in viral escape from CTL
recognition, which contributes to disease progression (McMichael 1998; Goulder et al., 1997a). It has been described that CD8+ CTL are capable of exerting significant selective pressure on the viral genome during SIV infection (Allen et al., 2000; Chen et al., 2000) and HIV infection (Borrow et al., 1997), as evidenced by the rapid appearance of epitope escape mutations. Allen et al. (2000) demonstrated that SIV Tat-specific CD8+ T cell responses select for new viral escape variants during the acute phase of infection. Sequencing the entire virus immediately after the acute phase showed that amino-acid replacements accumulated primarily in Tat CTL epitopes, indicating that Tat-specific CTLs may be significantly involved in controlling wild-type virus replication. This implies that responses against viral proteins that are expressed early during the viral life cycle might be attractive targets for HIV vaccine development.

In advanced stages of HIV infection, the numbers of CD8+ T cells drop dramatically and effector CTLs become low and virtually disappear (Landay et al., 1993; Gamberg et al., 1999). Differences between the specificity of HIV-1 specific CD8+ responses has been noted during primary and late HIV infection. The work from Goulder et al. (2001) showed that in subjects with chronic HIV-1 infection, the dominant HLA-A*0201-restricted CTL response is directed against the epitope SLYNTVATL ("SL9") in p17 Gag (residues 77-85), which were not detectable in 11 HLA-A*0201-positive subjects with acute HIV-1. In contrast, multiple responses to other epitopes were evident in 7 of the 11 A*0201-positive subjects with acute HIV-1 infection. Furthermore this study confirmed that the HLA-A*0201 SL9 response emerged later than other CTL response and after viral set point has been reached. These data showed that the HIV-specific CTL responses that are present and even may dominate in HIV-1 chronic infection, may differ substantially from those constitute the initial antiviral CTL response.

In addition to cytolytic responses, noncytolytic CD8+ suppressor activity also appears to play a role in controlling viral replication in vivo. Noncytolytic CD8+ mediated suppression of HIV-1 replication was first described by Walker and coworkers (1986), who showed that HIV could be recovered from PBMCs of asymptomatic individuals only after the CD8+ cells were removed, and that re-introduction of CD8+ cells
suppressed virus production in a dose-dependent manner. Mackewicz et al. (1994) demonstrated that CD8+ suppressive activity was most marked before seroconversion and showed an inverse correlation with plasma viral load in three of seven HIV-1 infected individuals. The CD8+ suppressive activity was further confirm by the work from Blackbourn et al. (1996) who correlated the control of HIV replication in lymph nodes to the non-cytolytic anti-HIV activity of CD8+ T cells. A number of different factors are believed to mediate this inhibition, including the β-chemokines RANTES, MIP-1α, and MIP-1β (Cocchi et al., 1995; Rubbert et al., 1997), macrophage-derived chemokine (Pal et al., 1997) and IL-16 (Baier et al., 1995).

1.2.2.2 CD4+ T cell responses to HIV

CD4+ T helper cells play a critical role in regulating production of antibodies, induction and maintenance of CTL responses, and activation of macrophages and natural killer cells (Walter et al., 1995; Abbas et al., 1996; Ridge et al., 1998; Zajac et al., 1998).

Rosenberg et al. (1997) found that in HIV-1 infected individuals, who maintain normal blood CD4+ T cell counts and appear to control viremia, HIV-1 Gag p24 specific CD4+ T cells proliferative responses were observed, resulting in the elaboration of INF-γ and antiviral β-chemokines. This observation is in agreement with the finding that Gag-specific CD4+ T cells responses were detected in most HIV-1 infected subjects with non-progressive disease (Pitcher et al., 1999). A recent study further demonstrated that CD4+ T cells specific for multiple HIV proteins including Gag, Pol, Env and Nef are readily detectable in 21 of 23 chronically infected patients (Betts et al., 2001). Similarly, SIV-specific CD4+ T cell responses are vigorous in macaques infected with attenuated SIV viruses that are known to be able to induce protective immunity (Gauduin et al., 1999). It has been shown that rapid suppression of viral replication in acutely SIV infected animals by anti-retroviral therapy leads to both a relatively robust CD4+ T cell response and, in some cases, an enhanced ability to control viral replication after subsequent withdrawal of the antiretroviral therapy (Lifson et al., 2000). These studies indicate that HIV-specific CD4+ T cell response may be required to contribute to anti-HIV host defense. Furthermore, T helper cell responses were found to be associated with
HIV-specific CTL responses in HIV-1 infected persons (Kalams et al., 1999a). In a model in which SCID mice were engrafted with PBMC from LTNPs, HIV-1-specific CD8+ T cells were only able to resist subsequent viral challenge when potent gag-specific CD4+ T cell responses were present (de Quiros et al., 2000). Thus HIV-specific CD4+ T cell responses may be required for maintaining a functional and effective CTL responses in HIV infection as has been suggested (McMichael and Rowland-Jones, 2001), which supports the premise that induction of strong HIV-specific proliferative responses may be important for candidate vaccines.

A key feature of HIV-1 is its ability to infect and cause depletion of CD4+ T cells, particularly, activated CD4+ T cells (Veazey et al., 2000). Without potent antiretroviral therapy, most HIV-1 infected persons experience a progressive decline in CD4+ T cells and impairment in T helper function (Clerici et al., 1989; Schrier et al., 1989; Valentine et al., 1998; Musey et al., 1999; Oxenius et al., 2000). There are several hypotheses proposed to explain HIV-mediated depletion of CD4+ cells including accelerated killing of mature T cells, altered movement (CD4+ T cells sequestered into lymphoid organs) leading to the appearance of CD4+ T cell loss, and impaired production of new T cells (reviewed in McCune, 2001). Therefore, it appears that a deficit in these cells is related, in part, to the failure of host immune system to completely control HIV-1 infection.

1.2.2.3 ADCC

Virus-specific antibodies may direct the effector cells which bear Fc receptors, such as natural killer (NK) cells and monocytes/macrophages, to lyse the infected cell through a process known as antibody-directed cellular cytotoxicity (ADCC). Infected cells expressing the HIV envelope or CD4+ cells pulsed with the gp120 molecule are effectively lysed by this process (Lyerly et al., 1987; Ahmad et al., 1994). Antibodies capable of triggering ADCC are predominantly of the IgG1 class and are directed against the envelope proteins (Ljunggren et al., 1988). Antibodies directed against Gag proteins are not as effective at inducing ADCC as antibodies directed against Env proteins (Koup et al., 1989). HIV-1-specific antibodies capable of inducing ADCC decline as disease progresses. This may be related to the level of effector cell function (Tyler et al., 1990).
It has been described that a direct correlation of HIV-1 specific ADCC antibody levels with viral load was only observed in patients with high CD4+ cell counts (Ahmad et al., 2001; Forthal et al., 2001b). These studies indicate that ADCC may be involved in control of viremia, and CD4+ lymphocytes likely play a role in ADCC functions.

1.2.2.4 Natural killer (NK) cells

NK cells represent a discrete lymphocyte subset defined by CD16/56 expression. NK cells are spontaneously cytotoxic to tumour and virally infected targets. As such, they may play a role in natural resistance to HIV-1-associated disorders and other opportunistic infections. NK cells have been shown to be effective in killing HIV-infected cells (Brenner et al., 1993; Ullum et al., 1999; Hultstrom et al., 2000). However, peripheral blood NK cell activity is frequently reduced in HIV-1 infected-patients with HIV-1-induced disease (Brenner et al., 1993), and the impairment of NK cell function is observed throughout the course of HIV-1 infection (Cai et al., 1990; Hu et al., 1995). The causes of NK cell dysfunction in AIDS-related disorders remain unknown. An altered expression of important regulatory receptors has been observed on NK cells from HIV-infected patients. The inhibitory NK receptor CD94 was up-regulated (Andre et al., 1999) and the NK activation molecules CD16 and CD56 down-regulated (Hu et al., 1995). These phenotypical changes as well as the decreased number of NK cells in HIV infection (Hu et al., 1995) may contribute to the decreased NK cell activity. This reduction in NK cell function may be one of causes of immunodeficiency in HIV-1 infection. A recent study of HIV-1 exposed but uninfected (HEPS) individuals in Vietnam showed the increased NK cell cytotoxicity present in HEPS individuals, compared to low-risk donors, indicating that NK cells may, in part, contribute to natural protection against HIV-1 (Scott-Algara et al., 2001).

1.3 HEPS: role for genetic variation and immunity in resistance to HIV-1 infection

There has been increased interest and intense studies in understanding the mechanisms of controlling HIV-1 infection in HEPS individuals. Different immunological features and genetic features have been identified to associate with HEPS status at variable
frequencies (Devito et al., 2000a; 2000b; Kaul et al., 2000; 2001a; Butera et al., 2001; Broliden et al., 2001; Nicastri et al., 2001; Sriwanthana et al., 2001).

1.3.1 Genetic variation

1.3.1.1 Chemokine receptor polymorphism

The chemokine receptors belong to a family of G-protein coupled seven transmembrane receptors. There are five CC (cysteine-cysteine) chemokine receptors (CCR) CCR-1-5 and four CXC chemokine receptors, CXCR-1-4 (Baggiolini et al., 1997). It has been identified that the ligands for CCR5 are the C-C chemokines RANTES (reduced upon activation normal T cell expressed and secreted), MIP (macrophage inflammatory protein)-1α, and MIP-1β (Cocchi et al., 1995). CCR5 is a major coreceptor for R5 HIV-1 entry (Alkhatib et al., 1996; Dragic et al., 1996; Berger et al., 1998). The ligand for CXCR4 is stromal cell derived factor (SDF)-1 (Bleul et al., 1996; Oberlin et al., 1996). CXCR4 is important for X4 HIV-1 entry (Feng et al., 1996; Berger et al., 1998). CCR2 and CCR3 have also been shown to be minor coreceptors utilised by a few strains of virus (Choe et al., 1996; Doranz et al., 1996). CCR3 is involved in vivo in HIV-1 infection of microglial cells of the central nervous system (He et al., 1997). It has been found that CCR2 occurs as two RNA-splicing variants, CCR2a and CCR2b, which differ in their COOH-terminal regions and may affect signal transduction, but not ligand binding (Folks et al., 1988; Steinberg et al., 1991). CCR2b is naturally utilized by SIV isolates in mangabey macaques (Chen et al., 1998).

1.3.1.1a CCR5

It has been demonstrated that a 32-bp deletion (Δ32) in the CCR5 gene leads to a premature stop codon and results in encoding a nonfunctional protein. Liu and coworkers (1996) reported that a homozygous CCR5Δ32 defect in CCR5 gene was found in two HEPS individuals. The encoded protein is severely truncated and can not be detected at the cell surface. Moreover, this defect had no obvious phenotype in the affected individuals. Subsequent studies showed that people who are homozygous for the deleted allele (Δ32) are highly resistant to HIV-1 infection in vivo (Dean et al., 1996;
Huang et al., 1996; Samson et al., 1996; Michael et al., 1997a), and cells from such people are resistant to the infection by HIV-1 M-tropic strains *in vitro* (Connor et al., 1996; Liu et al., 1996; Rana et al., 1997). These results indicate that mutant CCR5Δ32 confers resistance to HIV-1 infection *in vivo* as well as *in vitro*.

Population analyses have shown the CCR5Δ32 mutant allele to occur at a high frequency (8-21.7%) in Caucasians and low frequency (0-6.9%) in all other ethnic groups studied (Liu et al., 1996; Huang et al., 1996; Samson et al., 1996; Zimmerman et al., 1997; Ruchusatsawat et al., 2000). An Δ32 allele frequency of 9.2-10% (Liu et al., 1996; Samson et al., 1996; Martinson et al., 1997) in Europeans and 8-21.7% in Caucasian Americans (Huang et al., 1996; Zimmerman et al., 1997) showed the mutation is highly prevalent in these populations. A recent study showed that CCR5WT/Δ32 (heterozygous genotype) and CCR5Δ32/Δ32 (homozygous genotype) may be associated with protection against HIV infection among high-risk HIV seronegative Caucasian homosexual men (Marmor et al., 2001), indicating the protective role of heterozygous genotype CCR5WT/Δ32 and homozygous genotype CCR5Δ32/Δ32 in HIV-1 infection among Caucasian populations.

Based on the observation that homozygous Δ32 provides resistance to HIV-1 infection in Caucasian populations, the hypothesis that people who carry one deleted and one normal allele (heterozygous) Δ32 genotype can be partially protected against HIV-1 infection, is suggested and demonstrated by a couple of studies (Samson et al., 1996; Hoffman et al., 1997). It was found heterozygous CCR5Δ32 slows the rate of disease progression in infected Caucasian homosexuals (Zimmerman et al., 1997). The presence of two CCR5wt/Δ32 heterozygous genotypes among 139 high-risk HIV-seronegative Africans (1.44%) was reported and PBMCs from these two heterozygous individuals were also found to be less susceptible to HIV-1 M-tropic isolates infection *in vitro* (Kokkotou et al., 1998). Heterozygotes have been shown to express less CCR5 on the surface of cells (Wu et al., 1997), which may result in reduced rate of viral replication and slowed progression to disease.
Although they are extremely rare, a few reports showed homozygotes for CCR5Δ32 are present in HIV infected individuals (Balotta et al., 1997; Biti et al., 1997). Furthermore, further studies demonstrated that the CCR5Δ32 failed to account for most HEPS cases studied (Beyrer et al., 1999; Goh et al., 1999; Sriwanthana et al., 2001). Two studies reported that only 1 of 37 HIV-1 HEPS individuals had the homozygous CCR5 32-bp deletion (Δ32/Δ32) (Akridge et al., 1999; Goh et al., 1999). This observation was confirmed by another study which demonstrated that none of 17 HIV-1 exposed seronegative women had the CCR5 Δ32/Δ32 genotype associated with HIV-1 resistance (Bernard et al., 1999). Studies from other populations including Thai HEPS individuals (Beyrer et al., 1999; Sriwanthana et al., 2001) and Nairobi-based Pumwani HEPS sex workers cohort (Fowke et al., 1998) also showed no CCR5 polymorphism associated with resistance to HIV-1 infection.

1.3.1.1b CCR2b

A mutation in the CCR2b receptor gene has also been reported. The mutation occurs at CCR2b amino acid position 64 which is a valine to isoleucine amino acid substitution (64I) in the first transmembrane domain (Smith et al., 1997). Unlike the CCR5Δ32 allele, which inactivated the major HIV-1 entry coreceptor, CCR2b-64I caused a conservative change and has not shown to effect expression and ligand binding (Kostrikis et al., 1998). Genetic association analysis of five AIDS cohorts (3003 patients) enriched with seroincident subjects (whose the date of seroconversion is known) revealed that individuals expressing the CCR2 mutation 64I showed a delayed progression to AIDS, but that the polymorphism had no influence on the incidence of HIV-1 infection (Smith et al., 1997). This observation was confirmed by subsequent study (Anzala et al., 1998), but questioned by another study which reported that there is no association of CCR2b-64I with delayed AIDS progression (Michael et al., 1997b). It appears that these HIV-1 coreceptor mutant alleles CCR5Δ32 and CCR2b-64I may play a partial role in reducing HIV-1 transmission risk and delaying disease progression.

In addition, a mutation in the 3’ untranslated region of the SDF-1 chemokine gene which causes a glycine to alanine switch at amino acid position 801 (SDF-1 3’A) has been
described. This mutation, in homozygotes, delays the onset of AIDS, furthermore, the SDF-1 3'A effect on disease progression was stronger than either CCR5Δ32 or CCR2b-64I (Winkler et al., 1998). Recently, Sriwanthana et al. (2001) reported a higher frequency of SDF-1 3'A in HEPS women in Thailand, compared to the healthy controls. These data suggested the role of SDF-1 3'A in influence HIV-1 transmission or course of disease. The mechanism of this SDF-1 3'A function is not clear, but might relate to the role of SDF-1 as ligand for CXCR4, which, in turn, might interfere with the interaction between the X4 HIV-1 and the CXCR4 coreceptor.

1.3.1.2 HLA

Human leucocyte antigen (HLA), the term for human major histocompatibility complex (MHC), encompassing class I and class II loci, is highly polymorphic. Class I is encoded by the A, B and C genes and class II by the DP, DQ and DR genes. Class I is expressed on the surface of nearly all nucleated cells and recognised by T cytotoxic cells. Class II is expressed primarily on B cells, dendritic cells and macrophages and is recognised by T helper cells.

Individual differences seen in HIV-1 susceptibility and AIDS, led to investigations of the influence of HLA on HIV infection and disease. An early study showed the HLA types A25, A32, B18, B27, B51 and B57 were associated with slow progression to AIDS; and A23, B37 and B49 were associated with rapid progression to AIDS (Kaslow et al., 1996). Subsequently, a comprehensive analysis of HLA genes in HIV-1 cohort consisting of 200 slow and 75 rapid progressors to AIDS provides further evidence that HLA alleles are associated with fast or slow progression. They identified that HLA class I genes A29, B22, B35 and C16 are associated with rapid progression, whereas B14, B27, B57, C8, C14 are associated with non-progression. Only the HLA class II allele DR11 was influential (Hendel et al., 1999). Carrington et al. (1999) revealed that HLA class I allele B35 and Cw4 were associated with rapid progression to AIDS in Caucasians. Moreover, they found the extended survival of between 28% and 40% of The HIV-1 infected Caucasian individuals who were AIDS-free for over ten years could be attributed to their being fully heterozygous at HLA class I loci, lacking B35 and Cw4.
alleles, or both. A study of an HIV-1 infected long term non-progressor (LTNP) cohort revealed a association between HLA class I subtype B5701 allele and non-progressive infection (Migueles et al., 2000). These data suggest that HLA alleles can affect the course of HIV infection and disease.

Recent studies reported that certain HLA alleles were associated with HIV resistance (MacDonald et al., 2000; 2001; Sriwanthana et al., 2001). Analysis of a longitudinal cohort of HEPS Nairobi and Kenya female sex workers demonstrated that decreased HIV-1 infection risk was strongly associated with possession of a cluster of closely related HLA alleles in the A2/6802 supertype comprising A0202, A0205 A0214 of the HLA-A2 subtype and A6802 of the HLA-A28 subtype. In addition, resistance to HIV-1 infection was independently associated with HLA DRB101 (MacDonald et al., 2000). A later study further showed that the HLA class I alleles A2/6802 were associated with decreased HIV-1 infection risk in perinatally exposed infants (MacDonald et al., 2001). Studies of HEPS Thai female sex workers revealed a high frequency of HLA- A11 (86%) and HLA-B18 (17.6%) in HEPS women compared with low risk healthy controls (56% for A11 and 1.1% for B18) (Beyrer et al., 1999; Sriwanthana et al., 2001). These date indicate that certain HLA alleles may present HIV-derived antigenic peptides which stimulate immune responses, clearing the virus in at least some exposed HIV-1 uninfected individuals.

1.3.2 Immunology of HEPS

There is increasing evidence that a range of immune responses including cellular immune responses (Clerici et al., 1992; Rowland-Jones et al., 1993; 1995a; 1997; 1998a; 1998b; 1999; 2001; Kaul et al., 2001b; 2001d), and IgA responses to HIV-1 (Mazzoli et al., 1997; 1999; Kaul et al., 1999; 2001a; Devito et al., 2000a) are generated in HEPS populations which play an important role in preventing HEPS individuals from HIV-1 infection.

1.3.2.1 Humoral immune response
1.3.2.1a IgA in HEPS individuals

HIV-1 specific IgA mediated mucosal and systemic immunity have been demonstrated in HEPS individuals. A study of sixteen Italian couples discordant for HIV infection, of whom one member was HEPS and another HIV-1 seropositive, demonstrated that HIV-1 specific IgA but not IgG was present in serum, urine and vaginal wash samples from HEPS individuals, whereas both IgA and IgG were observed in those samples from their HIV-1 infected partners (Mazzoli et al., 1997; 1999). 5 of 15 sera from HIV-1 exposed seronegative individuals were shown to be able to neutralise primary HIV-1 isolates and in 2 cases such neutralisation was retained by serum-purified IgA (Mazzoli et al., 1999).

HIV-1 specific IgA was also detected in genital tracts of 16 out of 21 (76%) HIV-1 resistant Kenyan sex workers (Kaul et al., 1999); and in cervicovaginal lavage fluids of 6 of 13 (46.1%) Thai HIV-1 HEPS women (Beyrer et al., 1999). When IgA was purified from plasma, cervicovaginal fluid (CVF) and saliva samples from HIV-1 resistant Kenyan female sex workers, the neutralisation activity of purified IgA was detected in 73% of plasma; 79% of CVF and 73% of saliva samples. The IgA-mediated neutralisation activity present in HEPS is HIV-1 specific because recombinant gp160 could block the activity and IgA depleted samples failed to neutralise HIV-1. In contrast, IgA from plasma, saliva and CVF samples of low risk uninfected HIV-seronegative individuals lacked neutralising activity (Devito et al., 2000a). The cross-clade neutralising activity of IgA from Kenyan HEPS sex workers was demonstrated by a recent study. IgA from both mucosal and systemic sites was able to neutralise primary viral isolates from clade B, clades A and D, which predominate in Kenya. Cross-clade neutralising activity appeared to be broadest in CVF samples and lowest in saliva samples, which indicated that the genital tract has a higher frequency of exposure to HIV-1 than the oral route in these HEPS sex workers (Broliden et al., 2001). Recently, Trabattoni et al. (2001) reported that HIV-1 specific IgA was detected in semen and urethral swabs of all 12 HEPS heterosexual males, but not in healthy controls. 4 of 12 seminal fluids from these HEPS males showed neutralising activity against two HIV-1 primary isolates. These results suggest the HIV-1 specific IgA may play an important
role in protection of HEPS male and female individuals from HIV-1 infection.

The epitopes of HIV-1 recognised by HIV-1 specific neutralisation IgA of serum from HIV-1 exposed seronegative were analysed and compared to those of HIV-infected patients (Pastori et al., 2000). Neutralisation of primary HIV-1 strains of IgA of HEPS individuals was shown to be mediated by the recognition of different epitopes from HIV-1 infected patients. IgA of HEPS exclusively mapped to epitopes within HIV-1 gp41, whereas IgA of HIV-1 infected individuals recognize epitopes within both gp120 and gp41. Furthermore, epitopes on gp41 seen by IgA of HEPS were restricted to aa 581-584 regions of gp41, in contrast, those seen by IgA of HIV-infected patients were identified within aa 589-618 and aa 642-673 regions of gp41. These results have important implications for the development of HIV-1 preventive vaccines.

The ability of HIV-1 specific IgA from HEPS individuals to inhibit HIV-1 epithelial transcytosis has been investigated using a transwell system. The apical-basolateral transcytosis of primary HIV-1 isolates across human mucosal epithelium was modelled using two chambers separated by a nitrocellulose filter on which a tight monolayer of epithelial cells was allowed to grow. The upper chamber represented the apical (luminal) side to which HIV-1 infected PBMCs were added. The lower chamber represented the basolateral (mucosal) side to which antibodies tested were added. The effect on HIV-1 epithelial transcytosis of IgA, isolated from the genital tract, saliva, and plasma of HEPS individuals including a sex worker cohort in Nairobi, Kenya and a discordant couple cohort in Italy, were evaluated. The results showed that purified IgA (three of six CVF samples, five of ten saliva samples, and three of six plasma samples from HEPS individuals) was able to inhibit HIV-1 transcytosis. In contrast, IgA from low risk, healthy control subjects had no inhibitory effect on HIV-1 transcytosis. Moreover, the main part of the inhibition of HIV-1 transcytosis by the IgA antibodies was shown to be at the intracellular level (Devito et al., 2000b). Recently, Broliden et al. (2001) found that IgA purified from saliva, CVF and plasma of Kenya HEPS sex workers could inhibit HIV-1 transcytosis in approximately 50% of samples tested. These results indicated that the mucosal IgA present in HEPS individuals could mediate intracellular
neutralisation in epithelial cells at the mucosal site in a way that has been reported by Bomsele et al. (1998), in which it was demonstrated that the epithelial transcytosis of primary HIV-1 isolates could be blocked by anti-HIV-1 dimeric IgA. Because the most common way of spreading HIV is sexual transmission (Overbaugh et al., 1999), the presence of HIV-1 specific IgA in HEPS individuals in the genital tract could offer a potent first-line defense, and IgA in sera may form a second line of defense, against HIV-1 infection. Thus these observations may, in part, contribute to HIV-1 resistance in HEPS people.

1.3.2.1b Other antibody responses in HEPS individuals

It was reported that anti-CD4 autoantibodies were detected in 33-34% HIV-1 exposed seronegative individuals (Burastero et al., 1996; Lopalco et al., 1999). These antibodies could block HIV-1 driven syncytia formation, and inhibited the ability of CD4 binding to specific anti-CD4 monoclonal antibodies, but did not interfere with the CD4-gp120 interaction (Burastero et al., 1996). A recent study showed that antibodies against HLA class I and CD4 were found in 11 of 17 and 9 of 17 HEPS sera (but none of the low risk control sera) (Lopalco et al., 2000). The correlations between the HEPS status and either anti-HLA antibodies or anti-CD4 antibodies were statistically significant (Lopalco et al., 2000). These results indicated that in some HEPS individuals, the resistance to HIV-1 infection, may be associated with autoantibodies which are possibly specific for cellular antigens involved in the HIV-1 infection process.

1.3.2.2 Cellular immune responses

1.3.2.2a CD4+ T cell responses in HEPS individuals

Specific CD4+ T cell responses to HIV-1 in the absence of anti-HIV IgG antibody detection were first described in 5 of 5 repeatedly exposed, but HIV-1 seronegative, homosexual men. Peripheral blood mononuclear cells (PBMC) from these subjects secreted IL-2 in response to a panel of HIV envelope peptides (Clerici et al., 1992). Similar results were reported from other studies. Mazzoli et al. (1997) found that following HIV-1 Env-stimulation, PBMCs from 9 of 16 Italian HEPS individuals
secreted more IL-2 than those from HIV-1 infected individuals. Kaul et al. (1999) reported that systemic T helper lymphocyte responses to HIV-1 envelope peptides were detected in 11 out of 20 (55%) HIV-1-resistant Kenyan female sex workers. A similar observation was also reported by Fowke et al. (2000) who found that HIV-1 resistant Nairobi female sex workers had significantly increased HIV-1 specific T-helper responses, as determined by in vitro IL-2 production to HIV-1 envelope peptides and soluble gp120 (compared to low-risk seronegative and HIV-1-infected controls). T-helper cell responses to HIV-1 envelope peptides were also detected in 38% newborns of HIV-infected women by measuring IL-2 production. No HIV-1 infections were acquired in those infants in whom T helper cell responses were detected in cord blood at the time of delivery or post-natally through breast-feeding (Kuhn et al., 2001). These data support the suggestion that HIV-1 specific T helper cell responses contribute to natural resistance to HIV-1 infection, possibly by supporting CTL responses in HEPS individuals.

1.3.2.2b CTL responses in HEPS individuals

Rowland-Jones et al. (1993) demonstrated HIV-1 specific CTLs in an uninfected child born to an HIV-1 seropositive mother, which provided a suggestive role of CTL in HIV-1 infection and clearance, although the reactivity was only transient, possibly related to the loss of persistent antigenic stimulation. Further reports soon followed of HIV-1 specific CTLs in exposed HIV-1 seronegative (HEPS) individuals. Detels et al. (1994) described that higher levels of neutrophils and CD8+ cells were present in repeatedly exposed HIV-1 seronegative homosexual men than in HIV-1 seropositive males. Rowland-Jones et al. (1995a) demonstrated the presence of HLA class I restricted HIV-2-specific CTL in 5 of 6 HEPS Gambian female sex workers. HIV-specific CTL were subsequently found in 6 out of 15 highly exposed and apparently HIV-resistant Kenyan sex workers, predominantly towards epitopes highly conserved between B clade and the Kenyan A & D clades of HIV-1 (Rowland-Jones et al., 1998a, 1998b). The strong implication from these studies is that the presence of HIV-1-specific CTL may, in part, contribute to HIV-1 resistance in HEPS. The hypothesis was further supported by a number of studies within HIV-1 discordant partners (of whom, one repeated high-risk
sexual activity with an HIV-1-infected partner, but retained HIV-1 seronegative (HEPS) status), which demonstrated HIV-specific CTLs present in 35% (Akridge et al., 1999); 36% (Goh et al., 1999) and 41.1% (Bernard et al., 1999) of HEPS individuals with the absence of inherited CCR5Δ32 defects. These results indicate that cellular immunity rather than inheritance of the Δ32 CCR5 mutation accounts more often for persistently HIV-1 resistant cases. Up to date, HIV-specific CTLs have now been described in a number of studies in HEPS cohorts of the sex workers (Rowland-Jones et al., 1995a; 1998a; Kaul et al., 2000; 2001b, 2001d; Sriwanthana et al., 2001); HIV-1 discordant couples (Zerhouni 1997; Akridge 1999; Bienzle et al., 2000; Goh et al., 1999; Nicastri et al., 2001; Trabattoni et al., 2001); homosexual men (Paxton et al., 1996; Schmechel et al., 2001); infants borne to HIV-1 infected mothers (Rowland-Jones et al., 1993; de Maria et al., 1994); health care workers with occupational exposures (Pino et al., 1995) and recently intravenous drug users who remain HIV seronegative despite exposure to HIV through needle sharing with HIV infected partners (Makedonas et al., 2001).

The presence of local HIV-specific IgA mediated humoral immune responses (see section 1.3.2.1a), raised the interest and possibility that local HIV-specific cellular immune responses, may be present in HEPS individuals, which has recently been given credence by Kaul and his colleagues (2000; 2001d). They have demonstrated that HIV-specific CD8+ CTL responses were found in 11 of 16 (69%) cervix samples from HIV-1-resistant HEPS and none from low risk individuals using an IFN-γ ELISPOT assay. Furthermore, HIV-1-specific CTL responses in the cervix directed against the same epitopes as the systemic CTL responses in 9 of 11 (82%) resistant sex workers. The frequencies of HIV-specific CD8+ cells were slightly higher in cervix than in blood in HEPS sex workers, whereas in HIV infected individual cervical CTL response frequencies were lower than in blood, which indicated that there was relative enrichment of CD8+ CTLs in the genital mucosa in HIV HEPS compared with HIV infected individuals. These data suggest that mucosal cellular immunity may play an important part in resistance to HIV-1 heterosexual transmission in HEPS people. The mechanism by which these mucosal CTL could function remains unclear. It has been demonstrated HIV-1 can cross into the submucosa by transcytosis across a tight epithelial barrier, a
process that can be inhibited by HIV-specific IgA (Bomsel et al., 1998; Devito et al., 2000b), and that such virus can infect CD4+ and CCR5+ T cells and macrophages present in this region (Patterson et al., 1998; Yeaman, et al., 1998). Furthermore, the cervical submucosa is well supplied with CD8+ T lymphocytes (Olaitan et al., 1996), and CD3+ cytolytic activity is present in the genital tract (Olaitan et al., 1996; Patterson et al., 1998; Yeaman et al., 1998). These data lead to the hypothesis that HIV-1 specific cervical CTLs in the submucosa may target susceptible host cells, which have undergone productive infection by HIV-1 after viral transcytosis across the epithelial membrane, and thus may play a role in HIV infection and clearance.

Shearer and Clerici (1996) proposed that the resistance against HIV infection in HEPS individuals who exhibit HIV specific cellular immune responses may be in part, acquired immunity through “natural immunisation”. Accidentally-acquired immunity may therefore protect from HIV infection. This possibility has been supported by work from Zhu and colleagues (1999), who have detected HIV-1 viral sequences from bulk CD4+ lymphocytes in a minority of HEPS subjects. However, the phenomenon of HIV-1 resistance is not absolute. Maintenance of HIV-1 natural immune resistance will require ongoing antigenic priming, which has been demonstrated by Kaul and his coworkers (2001c; d). They have found that 12 (approximately 10%) of Kenyan HIV-1 resistant sex workers, who used to be HIV-1 resistant despite high exposure to HIV-1 (11 of 12 had had an estimated 527 unprotected exposure for an average of 6.9 years), eventually became HIV-1 seropositive despite preceding HIV-1 specific CD8+ responses. Comparison of immunological and behavioral variables between late seroconverters and persistent HEPS sex workers controls revealed that seroconversion was correlated with a reduction in sex work over the preceding year which resulted in waning of HIV-specific CD8+ responses. A break from sex work was associated with a loss of HIV-specific CD8+ responses, although restarting sex work led to the return of HIV-1 specific CTL responses. Subsequently, it was found there is an association between the duration of uninfected HIV exposure and both the frequency and magnitude of HIV epitope-specific CTL responses (Kaul et al., 2001b). These findings suggested that ongoing HIV exposure may be necessary to maintain an efficient HIV specific CTL response in HEPS
individuals, as is seen in antiretroviral therapy treated chronic HIV-1 infection patients whose levels of HIV-1 specific CTL responses declined after suppression of viremia with potent antiretroviral therapy (Dalod et al., 1998; Kalams et al., 1999b; Ogg et al., 1999a; Nixon et al., 1999). Similar results were also found in health care workers and infants born to HIV-1 infected mother, after a single exposure to HIV by needle-stick injury or at birth, HIV-specific cytotoxic and helper T lymphocyte responses can be detected but fall below the limits of detection within 2–8 months (Rowland-Jones et al., 1993; Pinto et al., 1995).

It has been showed that HIV-1 specific CTLs detected in HEPS individuals were specific for HLA class I restricted epitopes within HIV-1 Env, Gag, Pol, Nef and Rev (Rowland-Jones 1995a; 1999; Kaul et al., 2001b; d; Schmechel et al., 2001). HIV-specific CTL in two groups of African HEPS female sex workers shows extensive cross-clade reactivity between different viral sequences. In a small group of Gambian HIV resistant women exposed to both HIV-1 and HIV-2, studied over 4 years, HLA-B35-restricted CTL were repeatedly detected and exhibit cross-reactivity between the HIV-1 and HIV-2 sequences of the CTL epitopes (Rowland-Jones et al., 1995a). In Kenyan HIV-1 resistant female sex workers, HIV-1 specific CTLs were detected directed towards epitopes conserved between HIV-1 clades B, A and D (Rowland-Jones et al., 1999). To elucidate the mechanism of protective HIV-1 specific CTL responses in HEPS individuals, Kaul et al. (2001b; c) have recently compared the specificity of CTL epitopes between 91 HEPS and 87 HIV-1 infected Kenya sex workers. The results revealed that at certain HLA class I restricted HIV-specific CTL responses in HIV-infected women tended to be against epitopes rarely or never recognised in HEPS individuals, and at these same alleles, responses in HEPS women tended to recognise epitopes that were subdominant or unrecognised in infected women. Furthermore, these differences in epitope specificity were only seen for responses restricted by HLA class I A2, A24, A6802, B14 and B18 which were shown to be associated with HIV-1 resistance (MacDonald et al., 2001). It was also found that later seroconverters in HEPS sex workers was associated with an epitope specificity switch to those preferentially recognised by HIV-1 infected sex workers. These data suggested that there may be functional differences between
specificity of CTLs for different epitopes.

The HIV-1 specific CTL responses in HEPS population are generally low in magnitude and breadth (Herr et al., 1998; Rowland-Jones et al., 1998b; Kaul et al., 2000; 2001b; Schmechel et al., 2001; Sriwanthana et al., 2001). It has been shown that the CTL responses in HIV infected individuals were approximately tenfold stronger than those in HEPS individuals (Kaul et al., 2000; 2001b; d). HIV-1 specific CTLs are believed to be important in controlling viremia and preventing disease progression in HIV-infected individuals (Borrow et al., 1994; Ogg et al., 1998; 1999b), but are ultimately unable to prevent the development of AIDS and death (Wodarz and Nowak 1999). Thus the question raised is how HIV-specific CTL could play a role in protection against HIV-1 infection in HEPS individuals while failing to eradicate HIV in HIV-1 seropositive individuals. To answer this, based on the described observation in HEPS cohorts as above, Rowland-Jones (2001) recently proposed a hypothesis that the quality of T cell response, rather than its magnitude, in HEPS individuals may be different from HIV-1 infected individuals and thus may be an important factor to provide the HEPS status. Both the quality of CTL responses and the presence of effector T cells in the genital tract (encountering virus following sexual exposure) may be of importance. Animal models suggested that murine mucosal rather than systemic (splenic) HIV-specific CTL were necessary to confer resistance to mucosal viral transmission (Belyakov et al., 1998). A study in SIV-infected rhesus macaques has shown that class I HLA-restricted SIV Env specific CTL responses in the jejunal lamina propria was correlated with protection from SIV challenge (Murphey-Corb et al., 1999). These studies indicate that HIV-specific CTL present in the genital tract are likely to be important in mediating protective immunity against sexual transmission of HIV-1. The specificity of CTL responses were directed towards conserved regions of HIV-1 which were likely to be functional importance to the virus. As discussed above, resistance to HIV-1 is, in part, related with specific class I HLA molecules (MacDonald et al., 2001), these alleles might be represented in particularly efficient HIV-specific CTL or present specific protective epitopes (Kaul et al., 2001d). It has been described that low levels of perforin and low production of INF-γ were detected in CD8+ T cells of chronically HIV infected subjects.
(Appay et al., 2000), which indicated the functional impairment of HIV specific CD8+ cells in HIV-1 infected individuals. However, such parameters (function of CD8+ T cells) have not been studied in HEPS population, and it is worth to investigate the intact function of CD8+ T cells and its association with resistance to HIV infection in HEPS individuals.

In addition, noncytolytic activity of T cells also appears to play a role in resistance to HIV infection in HEPS individuals. The factors responsible for such protection have not been clearly identified. Recent work from Butera and colleagues (2001) showed an association between production of a novel viral suppressive activity with resistance to infection among Thai HIV-1 HEPS female sex workers. Further study showed that this activity was the unique product of CD4+ T cell and CD16+ monocyte cocultures. The exact mechanism by which of this novel viral suppressive factor could function remains unclear.

1.4 Conclusion

Identification of the components of protective immunity is crucial for the development of effective prophylactic and therapeutic vaccine strategies. Analysis of HIV-specific responses in highly exposed but persistently HIV seroneagtive (HEPS) individuals may thus provide a unique resource to elucidate the components and correlates of protective immunity to HIV. So far considerable progress has been made toward understanding of the factors that determine the HEPS status. These results suggest that a combination of factors, including IgA mediated humoral immune responses, both mucosal and systemic cellular immunity and also host genetic factors, may be involved in the resistance to HIV infection in HEPS people. These findings are of great importance to help define factors related to protective immunity against HIV which is desirable for an effective HIV vaccine. More research is necessary to shed further light on the mechanism of the genetic and immunological correlates of this condition.

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Appendix III: Review II

Overview of Human Immunodeficiency Virus (HIV) Type 1 Vaccines

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Abbreviation

AIDS acquired immunodeficiency syndrome
ALVAC Albany vaccine
BCG *Bacille Calmette-Guérin*
bp base pair
CTB Cholera toxin B subunit
CTL Cytotoxic T lymphocytes
3D-MPL 3-deacylated monophosphoryl lipid
dNA deoxyribonucleic acid
env envelope
GM-CSF granulocyte macrophage colony stimulating factor
gp glycoprotein
HAART highly active antiretroviral therapy
HEPS highly exposed, persistently seronegative
HIV human immunodeficiency virus
HSV herpes simple virus
IFA incomplete Freund's adjuvant
IFN interferon
Ig immunoglobulin
IL interleukin
LTR long terminal repeats
mAb monoclonal antibody
MF microfluid
MIP macrophage inflammatory protein
MTP-PE muramyl tripeptide-dipalmitoyl phosphatidylethanolamine
MVA modified vaccinia virus Ankara
nef negative effector function
NYVAC New York vaccine
PND principal neutralising domain
pol polymerase
QS *Quilaja saponaria*
RANTES reduced upon activation normal T cell expressed and secreted
rev regulator of expression of virion proteins
RT reverse transcriptase
SCID severe combined immunodeficiency
SFV Semliki forest virus
SHIV chimeric SIV/HIV viruses
SIV simian immunodeficiency virus
tat trans-activator of transcription
TNF tumour necrosis factor
VEE Venezuelan equine encephalitis
vif viral infectivity factor
VLP virus-like particle
vpr virion protein R
vpu virion protein U
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1.1 Introduction

HIV was first identified in 1983 and subsequently recognised as an etiologic agent for acquired immunodeficiency syndrome (AIDS). In spite of extensive prevention programs, the HIV/AIDS epidemic is still spreading worldwide, particularly in developing countries. WHO estimates that there are thirty-six million people living with HIV/AIDS; 22 million people have died and 15,000 new infections occur each day (Piot et al., 2001). While highly active antiretroviral therapy (HAART) has provided therapeutic benefit to those who can obtain and tolerate it, at least 95% of all people infected with HIV live in regions with limited resources such as sub-Saharan Africa, where recently developed antiretroviral treatments are neither affordable nor widely available (Schwartlander et al., 2001). Therefore, the development of an effective HIV-1 vaccine remains the best strategy for controlling the HIV epidemics. This overview will focus on different vaccine approaches that have been tested to date in animal models and human clinical trials.

1.2 The challenge for the development of an effective HIV vaccine

The extraordinary genetic diversity of HIV-1 is a major problem to overcome in the development of an effective vaccine that is universally effective against the various clades and viral strains (Coffin, 1995; Wei et al., 1995). HIV-1 rapidly mutates during infection, resulting in generation of viruses, which are able to escape immune recognition (Borrow et al., 1997; Price et al., 1997). Because a significant proportion of HIV-specific neutralising antibodies and cytotoxic T lymphocytes (CTL) responses are type specific and capable of neutralising (for neutralising antibodies) or killing (for CTL) the strain of virus from which the immunogen derived, but not other divergent strains (Goudsmit et al., 1991; Carmichael et al., 1996), this genetic diversity has fostered efforts to define and structure immunisation strategies that induce broadly immune response or utilise multivalent HIV vaccines.

Definitive immunological correlates of protection against HIV-1 infection remain uncertain, which poses considerable difficulty for the development of an HIV-1 vaccine.
(Nabel, 2001). Like other lentiviruses, HIV can integrate into the host genome where it can remain in a latent form that does not express HIV structural proteins and is thus less likely to be recognizable by the immune system (Chun et al., 1997). This capacity of HIV-1 to achieve true latency poses another difficulty to HIV-1 vaccine development.

Finally, transmission of HIV-1 predominately occurs across a mucosal surface (Kresina et al., 1999), therefore mucosal immunity may be required to prevent the sexual transmission of the virus. However our knowledge of mucosal infections and the immune responses responsible for defending against it is quite limited.

1.3 Protective immunity against HIV-1

The development of an effective vaccine must be based on an understanding of the humoral and cellular immune responses which impact HIV-1 infection and are likely to have a critical role in the clinical outcome. Neutralising antibody and CTL are major effectors of anti-viral immunity. Definitive immunological correlates of protection against HIV remain undefined, but much is known about HIV-specific protective immune responses associated with long term survival and maintenance of low viral loads (Deacon et al., 1995; Kirchhoff et al., 1995; Pontesilli et al., 1998), and natural resistance to HIV (Rowland-Jones et al., 1995; Kaul et al., 2001). It is clear that multiple aspects of immune response have potential to contribute to protective immunity (Dittmer et al., 1999).

1.3.1 Cell-mediated immunity

CTLs are also known as killer T cells, which recognise, bind and kill cells that display foreign antigens. Evidence for the protective role of cell-mediated immunity against HIV-1 come from a variety of observations. During acute infection the appearance of the CD8+ CTL response is temporally related with the control of HIV-1 replication (Borrow et al., 1994; Koup et al., 1994) and a strong HIV-specific CD4+ T cell mediated response is correlated with viral load (Rosenberg et al., 1999). Studies on individuals remaining uninfected despite multiple exposures (termed as HEPS, highly exposed persistently seronegative) have demonstrated HIV-1 specific cellular immune response,
including HIV specific CD8+ activity and T-helper responses (Rowland-Jones et al., 1993; 1995; 1998; Kaul et al., 2000; 2001), suggesting that transient infection may occur inducing protective immune response mediated by CTLs. In addition, HIV-specific CTL responses are also associated with long-term non-progressors, who have HIV-1 persistent infection but do not progress to AIDS for greater than 12 years. Some of these individuals are infected with HIV isolates which replicate poorly (Deacon et al., 1995; Kirchhoff et al., 1995). However, others are infected by HIV isolates with the capability of normal replication but have maintained a strong and broad HIV-1 specific immune response including HIV-specific CD4+ T cell proliferation (Rosenberg et al., 1997) and strong CD8+ CTL activity (Dyer et al., 1999; Pontesilli et al., 1998), which are believed to be responsible for their delayed disease progression.

Probably the best evidence for the importance of CTLs in the control of HIV has come from studies in non-human primate models. The CTL response is the best correlate of viremia control after simian immunodeficiency virus (SIV) primary infection in macaques (Reimann et al., 1994). This concept is further supported by recent studies showing that depletion of CD8+ cells results in SIV replication being uncontrolled during primary infection and high virus load in chronically infected rhesus monkeys (Jin et al., 1999; Schmitz et al., 1999). There are now several observations using different vaccine strategies suggesting that HIV-specific CTL play a major role in inhibiting HIV replication. Early studies using SIV vaccines demonstrated that induction of CD8+ CTL responses with weak or absent antibody responses correlates with reduction of viral load and delayed disease progression (Hirsch et al., 1996). Subsequent studies have shown similar patterns (Buge et al., 1997; Allen et al., 2000; Davis et al., 2000; Leno et al., 2000; Ourmanov et al., 2000; Barouch et al., 2001b). Protection of animals immunised with attenuated SIV was associated with the development of CD8+ CTL (Miller et al., 1997; Johnson and Desrosiers, 1998; Wyand et al., 1999). Taken together, the apparent importance of CTLs in controlling HIV replication as discussed above has had a large impact on vaccine development, where many recent efforts have centered on eliciting cellular immune response (Robinson et al., 1999; Allen 2000; Leung et al., 2000; Barouch et al., 2001b; Gorse et al., 2001; Nilsson et al., 2001).
1.3.2 Humoral immunity

Antibodies which are capable of neutralising HIV infectivity are often undetectable or at low titre during acute HIV-1 infection (Legrand *et al.*, 1997; Moog *et al.*, 1997). These observations suggest that neutralising antibodies that arise in response to HIV infection may not play a major role in limiting viral replication. However, this does not mean that neutralising antibodies can not prevent HIV infection or such an immune response can not be induced by a vaccine. A variety of different observations support the concept that it is important for a successful HIV vaccine to induce neutralising antibodies against the virus.

Passive antibody studies have suggested that HIV/SIV infection may be preventable by anti-HIV/SIV antibodies (Mascola *et al.*, 1999; Baba *et al.*, 2000; Parren *et al.*, 2001). Studies evaluating polyclonal anti-HIV-1 antiserum (Prince *et al.*, 1991); monoclonal anti-V3 antibody in HIV-1 infected chimpanzees (Emini *et al.*, 1992); monoclonal antibody against an epitope overlapping the CD4+ binding site of gp120 in SHIV-infected macaques (Parren *et al.*, 2001) and polyclonal serum in SIV-infected macaques (Putkonen *et al.*, 1991) have shown that when sufficient levels of antibodies are present prior to challenge that viral infection can be prevented. Passive prophylaxis using a human monoclonal antibody (mAb) has protected SCID (severe combined immunodeficiency) mice from infection with two primary HIV isolates (Gauduin *et al.*, 1997). Passive transfer of IgG from an SIV-infected long term nonprogressor can delay disease progression when given to macaques at the time of SIV infection (Haigwood *et al.*, 1996). Importantly, neutralising antibody against the HIV-1 envelope glycoprotein is capable of protecting macaque monkeys from chimeric SIV/HIV-1 (SHIV-1) infection (Shibata *et al.*, 1999). Passive prophylaxis with neutralising antibodies has protected rhesus macaques against vaginally transmitted infection (Mascola *et al.*, 1999; Parren *et al.*, 2001), indicating the ability of antibody to confer prevention at a mucosal surface, which may also be of great importance for a successful HIV vaccine. *In vitro* studies showed that IgG3 polyclonal anti-HIV has more potency in neutralising HIV-1 compared to that of IgG1 and IgG2 (Scharf *et al.*, 2001). If this phenomenon exist *in vivo*, strategies
which favour IgG3 may provide a new approach for improving HIV vaccines. More recently, it has been demonstrated that HIV-specific antibody from a patient with acute HIV infection can inhibit HIV replication in the presence of natural killer effector cells (Forthal et al., 2001). These observations support the concept that induction of neutralising antibody responses may be important in vaccine induced protection.

1.4 Animal models for HIV-1 vaccine evaluation

Animal models are of great importance for evaluation of different strategies for inducing protective immunity against HIV infections. Although transgenic mice and rabbits have been engineered which are susceptible to HIV infection, non-human-primates represent relevant model for head to head comparison of vaccine candidates. For studies evaluating HIV vaccine, these non-human-primate challenge models can be divided into three categories: HIV-1 chimpanzee model, SIV-macaque model and SHIV-1 macaque model (Nath et al., 2000). Chimpanzees are susceptible to HIV-1 infection, which can establish a long-term persistent infection. However, infected chimpanzees rarely develop disease and are limited in quantity, which is a problem for vaccine testing (Kumar et al., 2001a). The SIV-macaque model uses an SIV virus, which can persistently replicate to very high level in macaque. Similar to HIV-infected human beings, infected animals can develop an AIDS-like disease. This model could be potentially used for the establishment of correlates of protection. Although the SIV and HIV-1 in general have substantial nucleotide sequence homology, the envelopes of these viruses are quite divergent (Nath et al., 2000) Therefore, the meaningful interpretation of the immune response must be assessed carefully. Differences in important envelope epitopes between HIV-1 and SIV have limited the utility of this SIV-macaque model for evaluating HIV-1 Env-based vaccine candidates. In efforts to resolve this problem in the SIV-macaque model, the chimeric SIV/HIV-1 (SHIV-1) virus was constructed by replacing SIV env with HIV-1 env (Nathanson et al., 1999). Therefore SHIV-1 has the gag and pol structural genes of SIV and the env gene of HIV-1. This SHIV-1 macaque model makes it possible to test HIV-1 envelope immunogens directly in an animal model.
1.5 HIV vaccines strategies

1.5.1 Subunit vaccine

Initial efforts to elicit vaccine-induced immune responses to HIV-1 have focused on the viral envelope glycoprotein (Fast et al., 1993). The viral envelope glycoprotein contains the CD4 binding site, the region that attaches to human cells and thus has a pivotal role in the early events of virus attachment and entry into the target cell. Importantly, neutralising antibodies against HIV-1 found in the sera of infected individuals are predominantly directed against this glycoprotein (Poignard et al., 1996; Kwong et al., 1998; Wyatt et al., 1998; Saphire et al., 2001), and antibodies to HIV gp160 have been detected in genital secretions, urine samples, tears, milk, nasal washing, intestinal fluid, cervical fluid and saliva (O’Shea et al., 1990; Sun et al., 1990; Thongcharoen et al., 1992; Wolff et al., 1992; Constantine et al., 1994; Janoff et al., 1994; Mestecky et al., 1994). Furthermore, experimental stimulation of neutralising antibodies to HIV was first achieved by immunisation of animals with either recombinant or purified natural gp120 (Lasky et al., 1986; Robey et al., 1986). Moreover, this glycoprotein also contains epitopes that elicit cellular immune responses (Kahn et al., 1995; Mooij et al., 1998). For these reasons, vaccines based on genetically engineered HIV envelope proteins either gp120 or gp160 have been the best studied to date.

The efficiency of gp120 vaccines have been extensively studied in non-human primate animals. Early studies in the HIV-1 infected chimpanzee model indicated that rgp 120 vaccine could protect against challenge with either HIV-1 LAI or HIV-1SF2 (Berman et al., 1990; 1996; Girard et al., 1991; Bruck et al., 1994). More encouraging results have been obtained using HIV-1 gp160s from HIV-1LA1 and HIV-1MN and V3-MN peptides in two chimpanzees in which immunised animals were protected against intravenous infection with a heterologous HIV-1 strain HIV-1SF2. The neutralising antibody to HIV-1LA1 or HIV-1MN detected in immunised chimpanzees suggested that humoral immune responses had mediated this protection against heterologous HIV-1 infection (Girard et al., 1995).

In the rhesus macaque model, immunisation with recombinant SIV surface glycoprotein
has had only limited success in inducing protection against challenge with pathogenic SIVmac strains. In one study, immunisation with recombinant envelope protein from SIVmac could induce virus-binding but no neutralising antibodies were produced and no protection against intravenous challenge of cell-free virus was observed (Giavedoni et al., 1993). Subsequent studies further showed that rgp120 from HIV-1 failed to protect macaques against infection with SHIV-1 virus (Stott et al., 1998). The failure of this vaccine to prevent replication of the virus may be associated with the lack of CTL response as the monomeric rgp120 is known to be a poor inducer of CTL responses (Corey et al., 1998; Kumar et al., 2000). This inability of inducing CTL responses of monomeric gp120 was improved by using oligomeric gp120 as vaccine immunogen. Luke et al. (1996) reported that protection against the autologous pathogenic SIVmac has been achieved in all three rhesus macaques immunised with SIV gp130 (surface protein of SIV) oligomers. A similar result was obtained using oligomeric HIV-1 Env protein gp140 which is an oligomeric structure composed of uncleaved gp120 linked to the ectodomain of g41. Strong neutralising antibodies against a homologous virus was elicited in rhesus macaques immunised with oligomeric HIV-1 gp140. After intravenous challenge with SHIVHXB2, three of the four vaccinated macaques exhibited no evidence of virus replication (Earl et al., 2001), suggesting the possibility that immunisation with oligomeric, rather than monomeric, envelope proteins may improve protective efficacy. However, these studies uniformly demonstrated that immunisation with recombinant viral envelope protein failed to elicit broadly neutralising antibody response against both homologous and heterologous HIV-1 strains, and there was a lack of CTL responses in immunised animals. This may be attributed to intrinsic properties of the HIV envelope glycoprotein including multimeric complex present on the virion surface, extensive glycosylation and marked genetic variability. It has been reported that antigens generated in mammalian cells induced higher level of neutralising antibodies against HIV-1 than those produced in insect cells (Dolin et al., 1991), baculovirus (Keefer et al., 1994) or yeast systems (Keefer et al., 1996), probably due to more natural glycosylation achieved in mammalian cells than in non-mammalian cells (Belshe et al., 1994; Graham et al., 1996). This demonstrated that the degree of glycosylation of the recombinant envelope
protein of HIV-1 has a critical effect on its ability to induce neutralising antibodies. The use of monomeric gp160/120 may contribute to the reduction of immunogenicity of subunit vaccines. In addition to the poor induction of CTL responses by monomeric gp120 as described above, it has also been reported that antibodies induced against monomeric forms of the HIV-1 envelope proteins failed to efficiently neutralise primary HIV-1 isolates because of the failure to generally recognise oligomeric envelope proteins present on HIV virions (Sattentau, 1996; Burton et al., 1997).

In view of the extreme diversity of HIV-1, it is important to develop vaccination strategies that generate and sustain significant level of cross-reactive neutralising antibodies and cellular immune responses. Consideration of the unique properties of the HIV-1 envelope glycoprotein including multimeric complex present on the virion surface, extensive glycosylation and marked genetic variability must be made. It might be possible to induce strong and/or broadly neutralising antibody response and cellular immune responses by following strategies. (i) A vaccine containing a cocktail of envelope protein representing the majority of prevailing HIV-1 isolates. A recent study showed that macaques immunised with polyvalent vaccine consisting of gp120 proteins from multiple HIV-1 viral isolates (LAI, RF, 89.6, AD8, Bal and DH12) generated broader neutralising antibodies against three or more HIV-1 isolates, (albeit this breadth of neutralisation was limited almost entirely to the virus strains used for vaccination), than that in macaques immunised with monovalent vaccine containing gp120 of HIV-1DH12 only. These results suggested that mixtures of HIV-1 envelope glycoproteins could induce increased breadth of immune responses against multiple HIV-1 strains (Cho et al., 2001). (ii) Modification of HIV-1 envelope proteins (such as loop deletion) to increase their immunogenicities by increasing the exposure of conserved neutralisation epitopes (Cao et al., 1997; Reitter et al., 1998). In a recent study, the modified HIV-1SF162 envelop protein with partial deletion of the second hypervariable region (V2) was more effective than unmodified protein in eliciting potent neutralising antibodies not only against the homologous HIV-1, but also against several heterologous primary HIV-1 isolates in immunised rabbits. But in rhesus macaques, only those immunised with the modified immunogen elicited neutralising antibodies against several heterologous
primary HIV-1 isolates. These results suggested that specific modifications introduced in HIV-1 envelope proteins could elicit potent cross-reactive neutralising antibodies against heterologous primary HIV-1 isolates (Barnett et al., 2001).

Recombinant envelope protein-based subunit vaccines have been extensively tested in phase I/II human clinical trials (Klein 2001, Mascola and Nabel 2001), and the bivalent subunit vaccines comprising recombinant gp120 proteins from HIV-1MN and a primary HIV-1 isolate are currently being tested in phase III trial since 1998 (in North America) and 1999 (in Thailand). Efficacy results of these phase III trials are expected to become available 2-3 years later (Mulligan et al., 1999; Migasena et al., 2000). Generally, the typical vaccination schedule in phase I/II clinical trial has consisted of 0, 1, and 6 month injections with different doses of 15-600 μg immunogen for each injection, followed by a booster injection at 12 to 18 months (Graham et al., 1998). The principal findings related to immune responses generated by HIV-1 recombinant subunit vaccine in human clinical trials include: (I) HIV-1 envelope protein based vaccine have been generally well tolerated and immunogenic in HIV-1 seronegative individuals (Dolin et al., 1991, Graham et al., 1996; Keefer et al., 1997; Migasena et al., 2000; Nitayaphan et al., 2000), and particularly were also safe and well tolerated in neonates born to HIV-1 infected women (Cunningham et al., 2001). (II) Use of adjuvants affect the magnitude of antibody responses, although moderate or severe local reaction including pain, erythema and swelling, and/or systemic reaction including fever, headache, malaise, myalgia, nausea and rash occurred (Keefer et al., 1996; 1997; McCormack et al., 2000). A novel adjuvant QS-21, an amphipathic molecule from the soapbark tree *Quilaja saponaria*, was demonstrated to be able to promote a reduction in antigen (recombinant soluble HIV-1MN gp120 protein) dose by up to 100 fold without affecting magnitude of antibody generation, suggesting that QS-21 may provide an effective adjuvant to reduce the dose of a soluble protein immunogen (Evans et al., 2001). A combination of adjuvants was also investigated. HIV-1 seronegative volunteers received recombinant 200 μg monomeric HIV-1 rgp120w61D derived from a dual co-receptor tropism isolate HIV-1ACH320 in 50 μg 3D-MPL (3-deacylated monophosphoryl lipid A) and 50 μg QS-21 together with a novel oil and water emulsion, induced antibody titres as high as those
seen in HIV infection, albeit the quality of the antibodies remained different in that there was no evidence of primary isolate neutralisation. Cellular immunity was also enhanced in immunised subjects in terms of lymphoproliferative responses, but HIV-1 specific CD8+ cytotoxicity was not demonstrated (McCormack et al., 2000). Studies reported that HIV-1 rgp120 formulated with an adjuvant, consisting of an emulsion of polysorbate 80 and sorbitan trioleate and the oil-in-water emulsion is microfluidised (MF59), can induce type-specific and cross-reactive neutralising antibodies against homologous and heterologous HIV-1 strains in immunised HIV-1 seronegative volunteers (Nitayaphan et al., 2000). HIV-1 specific lymphoproliferative responses were induced in all 49 rgp120/MF59 vaccinated participants, but CD8+ cytotoxic T lymphocyte activity was shown in only one participant (Graham et al., 1996). The use of aluminum hydroxide (alum) and incomplete Freund's adjuvant (IFA) as HIV-1 rgp160/120 vaccine adjuvants in human clinical trials were also reported (Pialoux et al., 1995; Migasena et al., 2000). The effect of addition of an immunomodulator, muramyl tripeptide linked covalently with dipalmitoyl phosphatidyl-ethanolamine (MTP-PE) which is a synthetic lipophilic derivative of the naturally occuring compound muramyl dipeptide in mycobacterial cell wall, to a candidate HIV-1 vaccine Env 2-3 (Chiron Biocine Co.) formulated with MF59, was investigated. Results showed that 15 of 30 Env 2-3 in MTP-PE/MF59 vaccinees developed severe, although self-limited systemic and/or local reactions, compared to adverse effects in 2 of 18 subjects who received Env 2-3 in MF59. No significant difference was observed between those two immunised groups in terms of induction of neutralising antibodies to HIV-1Sp2 and lymphoproliferative responses to the immunogen Env 2-3. These results suggested that the addition of MTP-PE significantly increased reactogenicity, but had little, if any, effect on immunogenicity (Keefer et al., 1996). (III) Recombinant HIV-1 envelope proteins, formulated in any available adjuvant, rarely induce a CD8+ CTL response (Graham et al., 1996; Connor et al., 1998; McCormack et al., 2000; Nitayaphan et al., 2000), but lymphoproliferative responses to intact HIV-1 glycoproteins have been readily detected in immunised HIV-1 seroneagtive individuals (Belshe et al., 1993; Cooney et al., 1993; McCormack et al., 2000; McElrath et al., 2000; Schooley et al., 2000), including neonates and infants born to HIV-1 infected
women (Borkowsky et al., 2000). (IV) Neutralising antibodies induced by recombinant HIV-1 envelope proteins are generally type-specific which can only neutralise homologous HIV-1 strains, but not primary HIV-1 isolates (Graham et al., 1996; Mascola 1996, McCormack et al., 2000; McElrath et al., 2000; Nitayaphan et al., 2000), with a relatively short half-life (declined about 10-fold in 6 months after the last boost (Migasena et al., 2000)). (V) Even though some participants in Phase I/II trials who were immunised with HIV envelope protein vaccine had significant neutralising antibodies against vaccine isolates have in their sera, they still have become infected during or following immunisation (Berman PW et al., 1997; Connor et al., 1998; Graham et al., 1998; Locher et al., 1999; Migasena et al., 2000), suggesting that immunisation with such HIV-1 vaccines is less than 100% effective in preventing or rapidly clearing infection. This feature remains a major obstacle of HIV vaccine development.

In addition to recombinant envelope protein-based vaccines, HIV-1 regulatory proteins such as Tat and Rev, RT have also been tested as potential vaccine candidates (Cafaro et al., 1999; Osterhaus et al., 1999; Pacheco et al., 2000). The data reported by Cafaro and coworkers (1999) showed that monkeys, immunised with a biologically active Tat protein, were protected against challenge by SHIV.

1.5.2 Live recombinant vaccines

Live recombinant vaccines are recombinant viral vectors expressing SIV/HIV immunogens. The viral vectors are either replication defective or live-attenuated viruses. Live recombinant vaccines can mimic antigen presentation that occurs during natural viral infection, and thereby offer advantages including the potential to present antigens in a natural form (i.e., with correct conformation, glycosylation, and oligomerization), and the ability to induce CD8+ CTL responses. Potential disadvantages of live recombinant vectors include their potential to cause disease in vaccinees, especially in immunocompromised hosts, and the possibility that pre-existing immunity against the vector could render the vaccine ineffective, as has been demonstrated in a vaccinia based vaccine (Cooney et al., 1991).
A number of different live viral recombinant vectors have been developed and used for the development of HIV vaccines, including poxviruses (Ourmanov et al., 2000; Seth et al., 2000a), adenoviruses (Zhong et al., 2000), poliovirus (Crotty et al., 2001), herpes simplex virus (HSV) (Murphy et al., 2000), alphaviruses such as venezuelan equine encephalitis (VEE) viruses (Davis et al., 2000) and semliki forest virus (Mossman et al., 1996; Berglund et al., 1997), rhinoviruses (Smith et al., 1998), rabies virus (McGettigan et al., 2001), Sendai virus (Kano et al., 2000) and influenza virus (Palese et al., 1997).

Among these recombinant viral vectors, the poxvirus family, notably vaccinia and canarypox, have been widely used as HIV-1 vaccine candidates. Primate studies showed that recombinant vaccinia vectors expressing SIV gag proteins can induce SIV Gag specific CTL responses in vaccinated rhesus monkeys (Shen et al., 1991). And the SIV Nef specific CTL responses, induced by a recombinant vaccinia virus expressing SIV Nef, has been shown to significantly reduce viral loads in immunised macaques following intravenous challenge with pathogenic SIV_{MAC} (Gallimore et al., 1995). However, a recombinant vaccinia virus expressing the surface glycoprotein of SIV_{MAC} was unable to provide effective immunity against intravenous challenge with pathogenic virus in immunised rhesus macaques with undetectable neutralising antibodies (Giavedoni et al., 1993).

Due to safety concerns regarding vaccinia virus as a vaccine vector and the fact that immunosuppression was a contraindication for vaccination with vaccinia virus, recent efforts have focused on the highly attenuated poxviruses NYVAC (New York vaccine), ALVAC (Albany vaccine) and MVA (modified vaccinia Ankara). NYVAC is a Copenhagen strain of the vaccinia virus with mutations that attenuate its virulence. ALVAC is a canarypox recombinant which is unable to complete its replicative cycle but is able to synthesize foreign proteins in non-avian species. MVA is a vaccinia virus strain that was attenuated by undergoing 570 passages in primary chicken embryo fibroblasts, and contains six large genomic deletions that reduce its pathogenicity (Blanchard et al., 1998). The limited replicative ability of these virus strains enhances safety, albeit at the cost of reduced immunogenicity.
Studies with macaques have demonstrated the ability of recombinant HIV-1-NYVAC/ALVAC vectors to induce HIV-specific neutralising antibodies and T-cell proliferative responses, and the efficiency of HIV-1-NYVAC/ALVAC vaccines in cross protecting macaques from intravenous challenge with HIV-2 (Abimiku et al., 1995). Similar protection was also demonstrated in rhesus macaques immunised with recombinant HIV-2-ALVAC/NYVAC (Franchini et al., 1995). However, Girard et al. (1995) reported that two chimpanzees immunised with the live recombinant canarypox virus expressing gp160 proteins of HIV-1MN failed to induce protection from intravenous inoculation of HIV-1SF2. Similar results were seen in macaques immunised with recombinant HIV-2-ALVAC/NYVAC vectors and subsequently boosted with either recombinant vectors or HIV-1 gp120. Immunised animals became infected following challenge with HIV-2, although either T cell proliferative activity (for HIV-2-ALVAC) or neutralising antibodies (for HIV-2-NYVAC) was elicited (Myagkikh et al., 1996), suggesting this prime/boost immunity is not sufficient to confer protection in immunised animals. Interestingly, Benson et al. (1998) demonstrated that vaccination with NYVAC vector expressing SIV gag, pol and env genes could induce protection of macaques against intrarectal exposure of pathogenic SIVmac251, but did not protect macaques following intravenous challenge, although either neutralising antibodies (immunised with NYVAC-SIV vector alone) or CTL responses (immunised with both NYVAC-SIV vector and NYVAC-IL-12/IL-2 vector) were induced in immunised animals. These data indicated that the route of exposure may have an important implication in the evaluation of vaccine efficacy in SIV/macaque model.

Studies with MVA vectors expressing SIV Gag-Pol (Seth et al., 2000a) or SIV Gag-Pol and HIV-1 Env (Barouch et al., 2001a) defined the role of CTL in control of viremia. In these two studies, immunisation with recombinant MVA vectors induced potent Gag-specific CTL responses. Of note, the magnitude of the vaccine elicited CTL responses prior to SIV or SHIV challenge was associated with the magnitude of reduction in viremia following challenge.

Both NYVAC- and ALVAC-based HIV-1 vaccine candidates evaluated in human
clinical phase I/II trials have demonstrated good safety profiles (Pialoux et al., 1995; Clements-Mann et al., 1998; Belshe et al., 2001). An ALVAC based vector (vCP205) encoding multiple HIV-1 gp120, p55 and protease has been initially assessed in a phase II trial. 435 volunteers including people at both higher and lower risk for HIV infection received vCP205 with/without gp120 boosting. More (94%) subjects given vCP205 plus gp120 compared with 56% of those who received vCP205 alone, developed neutralising antibody to HIV-1MN. CD8+ CTL cells were detected in 33% of volunteers given vCP205, with or without gp120 (Belshe et al., 2001). These data suggested that weak neutralising antibody responses induced by live recombinant vectors, as has been demonstrated (Graham et al., 1992), can be heightened by boosting with recombinant envelope proteins. Elicitation of both potent humoral and cellular immunity are desirable characteristics of an HIV vaccine regimen. Therefore, various vaccinia-based vaccines are being assessed in prime-boost approaches to extend the potency and breadth of the immune response and to induce the protective humoral and cellular immunities. In a more recent study, a combination vaccine regimen consisting of a canarypox vector containing Env, Gag, and Pol, in combination with a recombinant gp120 subunit protein resulted in neutralising antibodies in 91%, and CD8+ CTL responses in 62% of vaccinees. Boosting with HIV-1 rgp120 did not increase the CTL responses to HIV-1 envelope proteins, but did enhance the magnitude and frequency of neutralising antibodies to HIV-1MN (AIDS Vaccine Evaluation Group, 2001). Similar results were also demonstrated in other clinical trials including priming with HIV-1 gp160-vaccinia followed by boosting with rgp160 in vaccinia-naïve adults (Graham et al., 1993); priming with HIV-1 gp160-vaccinia and boosting with HIV-1 rgp120 (Corey et al., 1998); priming with ALVAC based vector expressing HIV-1 gp120, gp41, gag and protease followed by boosting with rgp120 in HIV-1 seronegative volunteers (Evans et al., 1999), and priming with ALVAC-HIV-1 gp160 and boosting with rgp120 in HIV-1 uninfected adults (Clements-Mann et al., 1998). More frequent and long-lived (6 months after the last immunisation) HIV-1 CD8+ CTLs and/or neutralising antibodies were induced by priming with a live recombinant viral vector followed by a booster with recombinant HIV-1 envelope proteins, than by immunising with live recombinant vector or
recombinant HIV-1 envelope protein alone. It is encouraging that CTL responses induced by the ALVAC-HIV-1MN Env/Gag/protease vaccine can recognise viruses belonging to genetically diverse HIV-1 clades in HIV-1 uninfected volunteers (Ferrari et al., 1997), and that sera from recipients of a vaccine consisting of ALVAC based recombinant vector vCP205 and rgp120 from HIV-1SF2 displayed neutralising activity against several HIV-1 primary isolates from clades B and C (Verrier et al., 2000). One can expect that protection against the wide range of HIV isolates affecting the populations of worldwide geographic regions might be achieved by formulating live recombinant HIV vaccines consisting of multiple HIV-1 genes from a number of HIV-1 isolates, together with a rgp120 booster regimen. However, subsequent phase III trials with those HIV-1 live recombinant vaccines are necessary to evaluate the efficacy in preventing HIV infection in uninfected vaccinees, or in slowing disease progression among vaccinees who become infected. Furthermore, the level and types of immune responses that were induced by the vaccines compared with the protective efficacy must be investigated.

Several other live viral vectors for HIV vaccine research are also actively being developed and applied in the SIV/macaque model. Recombinant adenovirus vaccines have the feature of targeting antigen-presenting dendritic cells (Zhong et al., 2000), and have been shown to be highly immunogenic in non-human primates (Sullivan et al., 2000). A recombinant adenoviral vector expressing SIV Env elicited SIV-specific humoral, cellular and mucosal responses, resulting in decreased viral loads following vaginal challenge with SIVMAC251 (Buge et al., 1997). Replication-deficient adenoviral vectors expressing HIV Env could induce HIV-specific humoral and CTL responses in immunised mice (Bruce et al., 1999).

Crotty et al. (1999) showed that recombinant poliovirus vectors expressing SIV p17 and gp41 proteins could induce mucosal, cellular and humoral immune responses in immunised cynomolgus macaques. Subsequent studies demonstrated that vaccination with a Sabin poliovirus vector cocktail expressing SIV gag, pol, env, nef and tat proteins successfully induced complete protection in 2 of 7 immunised macaques against vaginal
challenge with pathogenic SIV\textsubscript{mac251} (Crotty \textit{et al.}, 2001). This successful primate protection using a viral library cocktail vaccination approach suggests that immunisation with an array of defined antigenic sequences could be an effective strategy to induce protection against diverse HIV stains.

Other studies have shown that recombinant rabies virus-based vectors expressing HIV-1 envelope glycoproteins from a laboratory-adapted HIV-1 strain (NL4-3) and a primary HIV-1 isolate (89.6) could induce humoral immune responses against HIV-1 gp160 (Schnell \textit{et al.}, 2000), and cross-reactive CTLs against a variety of different HIV-1 envelope proteins from different HIV-1 isolates in immunised mice (McGettigan \textit{et al.}, 2001). An attenuated propagation-defective HSV vector expressing SIV Env and Nef antigens was demonstrated to be able to induce anti-SIV envelope antibody responses, and protection against intrarectal challenge with pathogenic SIV\textsubscript{MAC239} in 2 of 7 immunised rhesus monkeys (Murphy \textit{et al.}, 2000). The sequential immunisation of cynomolgus monkeys with recombinant semliki forest virus and MVA both expressing SIV\textsubscript{MAC32H} Rev and Tat, at four weeks intervals, could also induce protection against disease caused by intravenous challenge with SIV\textsubscript{MAC32H} (Osterhaus \textit{et al.}, 1999).

A few bacterial vectors are being developed and used for the development of HIV vaccines, including attenuated strains of \textit{Salmonella typhimurium}, \textit{Bacille Calmette-Guérin} (BCG) (an avirulent strain of \textit{Mycobacterium bovis}) and \textit{Listeria monocytogenes}. Recombinant \textit{Salmonella}-based vectors expressing HIV-1 gp120 were able to induce antibody responses, with the absence of CTL responses detected, in immunised mice (Fouts \textit{et al.}, 1995). Another non-viral vector expressing HIV antigens that is in development is \textit{Listeria} (Frankel \textit{et al.}, 1995). Primary study showed that a hyperattenuated \textit{Listeria monocytogenes} vector expressing HIV antigens could induce HIV specific CD8+ T cell responses in immunised mice (Friedman \textit{et al.}, 2000). Several SIV genes including \textit{nef}, \textit{gag}, and \textit{env} have been expressed by recombinant BCG strains. Immunisation of mice via mucosal or parenteral routes with those recombinant BCG vectors expressing SIV proteins induced specific IgA antibodies as well as specific IgG and CTL responses (Lim \textit{et al.}, 1997, Lagranderie \textit{et al.}, 1998). Subsequently,
immunisation of rhesus macaques of recombinant BCG constructs expressing SIV Gag, Pol, Env and Nef proteins elicited SIV specific IgA and IgG antibodies, and CTL responses as well as T cell proliferation, which demonstrated that recombinant BCG vectors can induce concomitant humoral and cellular immune responses to SIV (Leung et al., 2000).

1.5.3 Whole inactivated HIV-1

One of the first vaccine approaches tested in the SIV/macaque model was formalin-inactivated whole SIV particles. Early studies showed that macaques immunized with inactivated SIV could be protected against intravenous challenge with up to 1000 animal infectious doses of pathogenic SIV strains grown in human cells, which generated considerable enthusiasm for the feasibility of generating an AIDS vaccine (Desrosiers et al., 1989; Murphey-Corb et al., 1989). Enthusiasm waned, however, when it was reported that it was the result of immune response to direct towards xenoantigens in the vaccine preparations rather than toward epitopes of SIV (Stott 1991; Cranage et al., 1993). Antibodies to human cell surface molecules and HLA molecules, which are incorporated into the viral envelope during the process of budding from an infected cell, might be responsible for mediating such protection (Arthur et al., 1992). This was further supported by the observations that immunised animals were not protected against challenge with SIV_{MAC} grown in monkey peripheral blood mononuclear cells (Cranage et al., 1993), and the fact that macaques immunised with HLA class II molecules purified from human cells can induce protection against intravenous challenge with infectious SIV grown in human cells (Arthur et al., 1995).

Because there are potential risks associated with incomplete inactivation of the virus stock and the failures of early SIV vaccine preparations, research devoted to developing whole inactivated HIV-1 as a primary vaccination strategy has been limited. In addition, inactivating HIV-1 without disrupting or losing potential neutralising epitopes (such as shedding of gp120 from virions) poses another difficulty to develop an inactivated whole HIV-1 vaccine. Chemical inactivation of HIV and SIV particles by 2,2'-dithiodipyridine, and production of replication defective SIV_{MNE} mutants by genetic modification, was
demonstrated to be able to preserve antigenic structure on the surfaces of SIV\textsubscript{MNE} and HIV\textsubscript{MN} (Arthur \textit{et al.}, 1998; Rossio \textit{et al.}, 1998; Gorelick \textit{et al.}, 1999). A recent study showed that the combined use of heat and formaldehyde could inactive HIV-1. Moreover, these virus preparations were able to enhance the binding capacity to monoclonal antibodies directed towards conformation-dependent neutralisation epitopes, suggesting greater exposure of the epitopes following treatment (Grovit-Ferbas \textit{et al.}, 2000). These data indicate that it may be possible for development of inactivated HIV-1 vaccines without disrupting or losing potential epitopes which can induce both humoral and cellular immune responses.

1.5.4 Live attenuated HIV-1 vaccines

The live attenuated HIV-1 vaccines refer to a live HIV-1 virus from which one or more disease-promoting genes have been deleted. Live attenuated SIV vaccines have achieved the most impressive vaccination results to date in inducing protection against infection with pathogenic virus. In 1992, Daniel \textit{et al.} provided a landmark study which extensively prompted the attenuated vaccine approach. In his study, 6 rhesus monkeys infected with a \textit{nef} deleted SIV (SIV\textsubscript{MAC239}\textit{Δnef}) remained healthy in the context of both virus burdens and CD4+ lymphocyte concentration for more than 3 years after inoculation with the mutated virus, indicating that deletion of auxiliary genes of the virus can be the means for creating a safe live attenuated vaccine to protect against AIDS. Most importantly, 4 Rhesus monkeys vaccinated with SIV\textsubscript{MAC239}\textit{Δnef} were completely protected against intravenous challenge with wild-type, pathogenic SIV\textsubscript{MAC} by all criteria including no decline of CD4+ lymphocytes, low virus burdens as with the attenuated virus observed before challenge and no evidence of wild-type SIV\textsubscript{MAC} virus in the immunised monkeys. Later, a live attenuated SIV\textsubscript{MAC239}\textit{Δ3} produced by deletion of \textit{nef}, \textit{vpr} and upstream sequences (US) in the U3 region of Long terminal repeats (LTR) of SIV\textsubscript{MAC239} was also demonstrated to be able to induce protection against intravenous challenge with pathogenic SIV\textsubscript{MAC251} (Wyand \textit{et al.}, 1996), SHIV\textsubscript{DH12} (Shibata \textit{et al.}, 1997), or SHIV\textsubscript{89.6} and SHIV\textsubscript{SM660} (Wyand \textit{et al.}, 1999) in immunised rhesus monkeys. In addition to inducing protection against intravenous challenge, a live
attenuated SIV\textsubscript{MACC8} with deletion of a 12bp in the nef/3'-LTR could protect immunised macaques against infection with intrarectal challenge with either pathogenic SIV\textsubscript{MACJ5} and SHIV composed of SIV\textsubscript{MAC239} expressing the HXBc2 env, tat and rev genes (Cranage \textit{et al}., 1997) or pathogenic SIV\textsubscript{SM} (Nilsson \textit{et al}., 1998), suggesting that a live attenuated SIV could induce protection against mucosal challenge with pathogenic virus. In a recent study, sequential immunisation of macaques with two different attenuated vaccines SHIV-4ΔnefΔvpu (vaccine-I) and SHIV\textsubscript{PPC}Δvpu (vaccine-II) induced long-term (during 85 weeks observation period) virus-specific immune responses and conferred protection against AIDS caused by intravenous challenge with heterologous simian human immunodeficiency virus SHIV\textsubscript{89.6P} in the context of controlling virus replication. All four immunised rhesus macaques developed binding antibodies against both the vaccine-I and -II envelope glycoproteins but neutralising antibodies only against vaccine-I. Vaccine virus-specific CTLs recognised homologous as well as heterologous pathogenic SHIVs. These results showed that the graded immunisation schedule can induce an effective immune response that was associated with protection against AIDS caused by a pathogenic heterologous SHIV (Kumar \textit{et al}., 2001b). It has been demonstrated that the protection induced by this class of vaccine in immunised macaques was associated with development of either CD8+ CTL (Cranage \textit{et al}., 1997; Joag \textit{et al}., 1998; Kumar \textit{et al}., 2001b), or the production of specific neutralising antibodies (Wyand \textit{et al}., 1996), suggesting both cell-mediated immunity and humoral immunity were involved in protective immunity (Johnson \textit{et al}., 1998).

However, the major concern regarding the development of a live attenuated HIV vaccine relates to safety concerns, including reversion of the vaccine strain to virulence and recombination with endogenous retroviral sequences to produce new infectious and potentially pathogenic virus. For instance, an \textit{in vitro} study showed that deleted HIV-1 vaccine strains can evolve into fast-replicating variants by multiplication of remaining sequence motifs and therefore their safety are not guaranteed (Berkhout \textit{et al}., 1999). Baba \textit{et al}. (1995) was the first to report that an attenuated SIV with multiple deletions in nef, vpr and the negative regulatory element (NRE) was demonstrated to be pathogenic.
in orally exposed neonatal macaques. Subsequently, it has been showed that this attenuated SIV was also pathogenic in orally or intravenously vaccinated infant macaques as well as in intravenously immunised adult macaques (Baba et al., 1999). Therefore, attention has been drawn to the development of more highly attenuated SIV vaccines by deleting several areas of accessory genes including nef, vpr, vpx, vif and upstream sequences (US) in the U3 region (Desrosiers et al., 1998), and nef, vpr, vpx, vif, tat and rev (Guan et al., 2001). However, greater attenuation is also likely to decrease the probability of inducing protective immunity, as demonstrated by the fact that antiviral immunity was inversely associated with degree of attenuation in immunised rhesus macaques (Lohman et al., 1994). Thus these highly attenuated constructs need to be evaluated for their long-term safety as well as ability to induce protective immune responses as SIV vaccine candidates. Given the lack of a suitable animal model to evaluate attenuation of HIV strains, it will be challenging to develop attenuated HIV strains that combine both efficacy and safety.

1.5.5 Virus-like particle -based vaccines

Virus-like particles (VLP) are non-infectious HIV-1 look-alike particles that contain the viral proteins required for virion assembly, but do not contain the viral RNA genome. These non-replicating, conformationally correct particles can have multiple antigenic components, and therefore have the potential to elicit broadly directed immune responses. The gag precursor protein pr55 of HIV and SIV can self assemble in budding noninfectious virus-like particles in the absence of other viral components (Peters et al., 1997; Notka et al., 1999; Paliard et al., 2000). Immunisation of rhesus macaques with recombinant HIV-1ІІІІ derived chimeric pr55(gag)/gp120 VLP induced Gag- and Env-specific humoral responses and CTL activities, but was unable to provide any protection against challenge with SHIVІІІІ (Wagner et al., 1998). Similar results occurred in macaques vaccinated with SIV pr55 VLP modified by either inserting HIV-1 gp160 derived peptides into the pr55 precursor, or integrating the complete HIV-1 gp120 into the particle membrane. Immunised animals became infected following challenge with SHIV, although SIV specific humoral and cellular immune responses were induced.
However, macaques immunised with pr55 VLP presenting complete gp120 managed to clear virus faster than nonimmunised controls, which is correlated with the accelerated appearance of neutralising antibodies postchallenge (Notka et al., 1999). Paliard et al. (2000) demonstrated that HIV-1 derived pr55 VLP could induce CTL responses against multiple HIV-1 pr55 epitopes in immunised rhesus macaques. Importantly, these CTLs could recognise naturally processed peptides and were long lived (>8.5 months). These results suggested that HIV derived pr55 VLPs may be a good HIV vaccine candidate in combination with approaches priming humoral immune responses, which is desirable for an effective HIV vaccine.

The yeast-derived virus-like particle immunogen, Typ17/p24 VLP (p24-VLP) expressing HIV-1 p24 and p17, is the only VLP-based vaccine which has entered human clinical trials in seronegative volunteers (Weber et al., 1995) as well as in HIV-1 infected individuals as a potential therapeutic vaccine (Kelleher 1998; Klein et al., 1997; Peters et al., 1997). The Ty transposon found in yeast (Saccharomyces cerevisiae) encodes the protein p1, which has the capacity to self-assemble into particles. When p24 gag proteins are fused to the carboxy terminus of the p1 protein, the resulting fusion proteins assemble into 50 nm virus-like particles that can be purified and used as an antigen. Weber et al. (1995) conducted a phase I trial. Sixteen healthy male subjects were subcutaneously immunised with either 100 μg or 500 μg p24-VLP three times at weeks 0, 4 and 12 and 500 μg intramuscular injection for the fourth immunisation. HIV-1 p24 antibodies were detected in 4 (25%) subjects after the third immunisation and in 11 (70%) subjects after the fourth injection. However, no p24 specific CTL responses were elicited. This study indicated that p24 VLP vaccine is immunogenic and well tolerated in healthy people.

1.5.6 Peptide-based vaccines

Peptide-based vaccines refer to chemically synthesised pieces of HIV-1 proteins (peptides) known to stimulate HIV-1-specific immunity, which are highly safe as an immunogen. In some studies, this class of vaccine has been presented as lipopeptides, a synthetic peptide bound to fatty acids. These fatty acids make it easier for the peptides to
enter the cells (Pialoux et al., 2001). As discussed in section 1.2, the major problem to overcome in the development of an effective vaccine is extraordinary genetic diversity of HIV-1. Therefore peptide-based vaccines may be able to induce broadly immune response against infection with different HIV-1 strains by providing multiple epitopes in a single vaccine (Pialoux et al., 2001; Toledo, 2001).

Studies in animal models showed that peptide vaccines were safe and immunogenic (Mortara et al., 1998). Immunisation of guinea-pigs with a HIV-1MN principal neutralising domain (PND) peptide based vaccine covalently conjugated to Pseudomonas aeruginosa toxin subunit A (TA), generated neutralising antibodies against the homologous strain HIV-1MN and two heterologous strains HIV-1IIIb and HIV-1RF (Cryz et al., 1995). This result suggested that a peptide-based HIV vaccine could generate broad antibody responses against homologous and heterologous HIV-1 strains.

Peptide-based vaccines have been extensively evaluated in human phase I clinical trials. Most peptide-based vaccines have been based on the peptide sequences of the HIV-1 V3 loop (Cryz et al., 1995; Rubinstein et al., 1995; Gorse et al., 1996; Kelleher et al., 1997; Toledo 2001). It was demonstrated that the immunisation route could affect the immunogenicity of a prototype synthetic HIV-1MN V3 region peptide vaccine. In one phase I trial, HIV-1 uninfected healthy adult volunteers were immunised intramuscularly with 20, 100 or 500 µg of vaccine formulated with alum adjuvant on days 0, 28, and 168. The results showed that the vaccine was safe and well-tolerated. Induction of binding antibody to V3 peptide was vaccine dose-related and was detected in 9 of 10 subjects in 500 µg dose group. V3 peptide stimulated lymphocyte proliferation was detected in 15 of 20 subjects tested after vaccination (Gorse et al., 1996). However, in an other phase I/II trial, 24 HIV-1 seronegative men were subcutaneously immunised with the 100 or 500 µg HIV-1MN V3 peptide vaccine at 0, 1, and 6 months. Antibodies against the immunogen were only detected in 4 immunised subjects. Lymphoproliferative responses to immunogen were demonstrated in only 7 of 19 vaccine recipients tested (3 from 500 µg group and 4 from 100 µg group) (Kelleher et al., 1997). Recently, it was demonstrated that this HIV-1 MN V3 peptide vaccine, when encapsulated in
biodegradable micro-spheres composed of co-polymers of lactic and glycolic acids and administered by the oral route, failed to induce humoral, cellular, or mucosal immune responses in a phase I clinical trial (Lambert et al., 2001).

CTL responses have received great attention in the context of controlling the viral load and progression from HIV-1 infection to disease (Pontesilli et al., 1998), but it seems that a synthetic vaccine consisting of a single CTL epitope is unlikely to be immunogenic in vivo (Brander et al., 1996). Therefore, peptide vaccines consisting of multi-epitopes including HIV-1 CTL epitopes to elicit a defined spectrum of CTL-specificities as well as humoral immune responses have been investigated (Woodberry et al., 1999; Toledo et al., 2001). Two studies showed that peptide epitope vaccines consisting of HIV-1 gp120 epitopes for Th cell, CTLs and B cells (Bartlett et al., 1998) or Gag CTL epitopes known to be restricted by HLA-A33, -B8, -B27, -B35, and -Bw62 (Seth et al., 2000b), used as a therapeutic vaccine, may not be a sufficiently potent immunogen to significantly augment HIV-1-specific CTLs and to decrease virus load in HIV-1-seropositive individuals. This may be due to non-generation of optimal immune responses. Studies in seropositive persons which demonstrated cross-clade recognition of defined HIV-1 CTL epitopes in HIV-1 Gag (Bertoleti et al., 1998; Lynch et al., 1998), Env (Wilson et al., 1998) and Gag precursor pr55 (McAdam et al., 1998) may raise the possibility that a Gag-Env based vaccine may be able to induce broad immune responses against different HIV-1 strains. In a phase I trial, the efficacy of a six lipopeptide mixture including two Gag peptides, one Env peptides and three Nef peptides, as a vaccine immunogen was investigated. Twenty-eight healthy HIV-1 seronegative volunteers received the lipopeptides, with or without the adjuvant QS-21. The results showed that the vaccine was safe and well tolerated. Specific IgG antibodies against the Nef and Gag proteins were detected in 25 out of 28 volunteers (89%). The T cell proliferation response to at least one peptide was detected in 19 (79%) of the 24 subjects tested, with the majority had induced a multispecific proliferative response, that is, cells from volunteers proliferated to two (5 of 19), three (5 of 19), four (3 of 19) or five peptides (1 of 19). Anti-HIV CD8+ cytotoxic lymphocytes could be tested in 24 volunteers, with the polyepitopic responses were detected in 7 of 13 responders. Cytotoxic responses against
the whole Nef proteins from HIV-1\textsubscript{LAI} (clade B) were found in 3 of 4 tested volunteers, with cross-reactions to the proteins from HIV-1\textsubscript{MN} (clade B) and HIV-1\textsubscript{Bangui} (clade A) strains detected in one of two tested volunteers (Pialoux \textit{et al.}, 2001). These results demonstrated that these lipopeptides could induce both HIV-1 specific cellular and humoral immune responses in vaccinated healthy volunteers and might be a promising candidate for an AIDS vaccine.

1.5.7 DNA vaccines

DNA vaccines are naked DNA (purified DNA alone, with no other molecules) that encodes for one or several HIV-1 proteins. The administration of DNA vaccine would offer advantages in terms of the vaccine's simplicity and stability, and theoretically should result in the production of antigenic proteins in a natural form and thus have raised hopes for generating long-lived, broad humoral and cell-mediated immunity. Several experimental HIV-1 DNA prototype vaccines have been developed including DNA constructs encoding HIV-1 structural proteins Env (Wang \textit{et al.}, 1993; 1995; Lekutis \textit{et al.}, 1997; Shiver \textit{et al.}, 1997; Cher pelis \textit{et al.}, 2001; Ross \textit{et al.}, 2001), Gag (Gorelick \textit{et al.}, 2000a; 2000b; zur Megede \textit{et al.}, 2000; Yoshizawa \textit{et al.}, 2001), Env and Gag (Yasutomi \textit{et al.}, 1996), and reverse transcriptase (RT) (Isagulians \textit{et al.}, 2000). DNA constructs have been made encoding HIV regulatory and/or accessory proteins Nef (Moureau \textit{C et al.}, 1999), Tat (Caselli \textit{et al.}, 1999; Cafaro \textit{et al.}, 2001), Nef, Rev and Tat (Hinkula \textit{et al.}, 1997; Calarota \textit{et al.}, 1998; Tahtinen \textit{et al.}, 2001) and Vif, Vpu and Nef (Ayyavoo \textit{et al.}, 2000); and structural and regulatory proteins Env and Rev (MacGregor \textit{et al.}, 1998; Kim \textit{et al.}, 2001a).

The feasibility of HIV-1 DNA vaccines have been evaluated in animal models including mice and non-human primates (Wang \textit{et al.}, 1995; Lekutis \textit{et al.}, 1997; Calarota \textit{et al.}, 1998; Gorelick \textit{et al.}, 2000a, 2000b; Isagulians \textit{et al.}, 2000; Wang \textit{et al.}, 2000; Tahtinen \textit{et al.}, 2001). In rodent models, HIV-1 DNA vaccine immunisation could elicit potent HIV-1 specific humoral (Moureau \textit{et al.}, 1999; Lu \textit{et al.}, 1995) or cellular immune responses (Wang \textit{et al.}, 1995; 1993; Yasutomi \textit{et al.}, 1996; Ayyavoo \textit{et al.}, 2000; Tahtinen \textit{et al.}, 2001). Importantly, successful elicitation of both humoral and cellular
immune responses (Wang et al., 1993; Hinkula et al., 1997; Shiver et al., 1997; Caselli et al., 1999; Isaguliants et al., 2000; zur Megede et al., 2000) as well as mucosal immune responses (Wang et al., 1997; Yoshizawa et al., 2001) was observed in immunised mice. It was found that the immunisation route could affect the strength of the immune response induced by DNA vaccine. A 3 to 75 fold stronger T cell response was seen in mice receiving DNA constructs encoding HIV-1 regulatory genes nef, rev and tat epidermally, compared to that in animals receiving intramuscular injections (Hinkula et al., 1997). The immunogenecity of HIV-1 regulatory proteins Nef, Rev and Tat was compared in a recent study. It has been shown that the HIV-1 nef constructs were the most immunogenic raising both humoral and cell-mediated immune responses in immunised mice, compared to HIV-1 rev construct which only elicited CTL-response against the corresponding protein, whereas, HIV-1 tat construct was shown to be a poor immunogen in all respects (Tahtinen et al., 2001).

Several viral challenge studies have been performed in non-human primates to evaluate the protective capacity of immune responses induced by DNA-based vaccines. Vaccination of chimpanzees with DNA constructs encoding HIV-1 proteins (Env, Rev and Gag/Pol) induced specific cellular and humoral immune responses. Animals immunised with DNA constructs were completely protected from infection following challenge with HIV-1SF2 (Boyer et al., 1997). However the meaningful interpretation of this result remains uncertain because HIV-1SF2 is nonpathogenic and does not replicate at high levels in chimpanzees (Nath et al., 2000; Kim et al., 2001a). Early studies in macaques elucidated that DNA vaccines encoding SIV Env proteins or all of the structural and regulatory proteins have been unsuccessful in protecting against infection with SIV following challenge, although either both neutralising antibody and cytotoxic T-lymphocyte responses (Lu et al., 1996) or Th1 and Th2 cytokine responses as well as CD8+ CTLs (Haigwood et al., 1999) were observed in vaccinated animals. However, the loss of CD4+ T cells could be prevented in macaques immunised with SIV mutant (a four amino acid deletion) nucleocapsid DNA constructs, with humoral immune responses but no cellular immune responses observed prior to challenge (Gorelick et al., 2000a; 2000b). It was found that a DNA vaccine encoding HIV-1SFI62AV2 envelope
induced lymphoproliferative responses and potent neutralising antibodies in immunised rhesus macaques. To evaluate the protective role of the anti-HIV Env antibodies elicited by the DNA vaccine, the CD8+ T lymphocytes of vaccinated macaques were depleted by intravenous administrations of the anti-CD8 monoclonal antibodies prior to intravenously challenge with SHIV_{162P4}. The vaccinated animals showed lower peak viremia levels, rapidly cleared plasma virus and delayed seroconversion, compared to unvaccinated animals. These results demonstrated the protective role of anti-HIV-1-Env antibody responses induced by HIV-1 DNA vaccine (Cherpelis et al., 2001). Another study showed that protection from pathogenic virus challenge could be achieved in 3 of 8 rhesus macaques immunised with a multicomponent DNA vaccines encoding HIV Env/Rev and SIV Gag/Pol. Following challenge with pathogenic SIV_{MAC239} and subsequently SHIV_{89.6P}, three vaccinated animals exhibited no detectable virus, whereas control animals showed CD4+ cell loss, high viral burden and subsequently failed to thrive. Cellular immune responses were observed in the protected macaques, suggesting their important role in protection (Kim et al., 2001a). Therefore, it appeared that a DNA vaccine can induce both humoral and cellular immune responses in primates model and they all play an important protective role in immunised animals, suggesting that an effective HIV-1 vaccine should be able to provide both protective humoral and cellular immune responses.

Different strategies have been investigated to boost the immune responses induced by DNA vaccine. A great deal of attention has focused on two strategies: prime/boost strategy and cytokine adjuvants. The prime/boost strategy includes boosting the immune response induced by DNA vaccine with recombinant proteins or live recombinant viral vectors. DNA immunisation alone can induce potent specific cellular immune responses but with only transient or low level neutralising antibody responses (Haigwood et al., 1999; Verschoor et al., 1999). It has been demonstrated that relatively high titer neutralising antibody responses induced by DNA immunisation could be generated by employing a subsequent boost with a recombinant HIV-1 protein (Letvin et al., 1997; Richmond et al., 1998; Putkonen et al., 1998; Yoshizawa et al., 2001). Moreover, it was found that immunisation with HIV-1 env DNA followed by a final immunisation with
HIV-1HXb2 \textit{env} DNA plus recombinant HIV-1\textsubscript{IIB} \textit{Env} protein could completely protect immunised monkeys from infection after intravenous challenge with a chimeric virus SHIV\textsubscript{HXb2} \textsuperscript{C} (Letvin \textit{et al.}, 1997). The potent immunity and protection observed in this study suggest that a DNA prime/DNA plus protein boost regimen might be a potential vaccine strategy to prevent HIV-1 infection.

The immune responses induced by HIV-1 DNA vaccine can be enhanced by boosting with recombinant live vectors including modified vaccinia Ankara (MVA) (Hanke \textit{et al.}, 1999; Allen \textit{et al.}, 2000); recombinant fowlpox virus (Kent \textit{et al.}, 1998; Robinson \textit{et al.}, 1999) and recombinant vaccinia vector (Fuller \textit{et al.}, 1997). A recent study showed that DNA vaccine (encoding multiple SIV proteins including Gag, Pol, Vif, Vpx, and Vpr and HIV-1\textsubscript{89.6} \textit{Env}, Tat and Rev) priming followed by boosting with a recombinant MVA encoding the SIV Gag and Pol and HIV-1 \textit{Env}, led to robust CD8\textsuperscript{+} T cell response and prevent vaccinated macaques from developing disease following intrarectal challenge with a pathogenic SHIV\textsubscript{89.6p} (Amara \textit{et al.}, 2001), indicating that recombinant live vectors appear to augment the cellular response primed by DNA vaccine. However, the degree to which boosting with either recombinant proteins or recombinant live vectors will increase the ability of vaccinated animals to control pathogenic virus challenges remains to be determined.

Another strategy to increase humoral and cellular immune responses induced by DNA vaccine involves the co-administration of plasmid encoding cytokines or co-stimulatory molecules B7 molecules. Experiments in murine model showed the augmentation of DNA vaccine-induced HIV-1 specific cellular immune response by co-administration of plasmids encoding cytokines including IL-2 (Xin \textit{et al.}, 1998); IL-12 (Kim \textit{et al.}, 1997, 1999; Boyer \textit{et al.}, 2000b); IL-15 (Xin \textit{et al.}, 1999a); IL-18 (Billanot-Mulot \textit{et al.}, 2001); the combination of IL-12 and GM-CSF (Okada \textit{et al.}, 1997; Kim \textit{et al.}, 1999; Liu \textit{et al.}, 2001); IL-2/IL-15 and TNF-\alpha (Kim \textit{et al.}, 1998a); and co-stimulatory molecules B7 (Kim \textit{et al.}, 1998b; Santra \textit{et al.}, 2000). The humoral immune response induced by DNA vaccine could be increased by co-administration with plasmids encoding cytokines including IL-2, IL-4; IL-5 and IL-10 (Kim \textit{et al.}, 1998a; 1999). Co-administration of
DNA with a plasmid encoding the chemokine RANTES or MIP-1α increased HIV-1 specific antibody responses and CTL activities as well as mucosal immune responses (Lu et al., 1999; Xin et al., 1999b). However, there are limited data reported from studies in primates. Kim et al. (2001b) studied the effects of cytokine gene adjuvants to enhance the level of cell-mediated immune responses in rhesus macaques. DNA vaccine constructs encoding for HIV env/rev and SIV gag/pol proteins were co-delivered to animals with plasmids encoding for IL-2, IFN-gamma or IL-4 cytokines. The results showed that co-administration of IL-2 and IFN-gamma DNA constructs resulted in enhancement of antigen-specific T cell-mediated immune responses. It was also demonstrated that plasmids encoding IL-2/Ig, a fusion protein consisting of IL-2 and the Fc portion of IgG, which has IL-2 functional activity and a prolonged half-life in vivo (Nickerson et al., 1996), can substantially augment immune response when co-administrated with DNA vaccine. The study showed that Gag-specific CTLs amounted to 18-40% of total circulating CD8+ T cells in rhesus monkeys that received the DNA vaccines encoding SIV<sub>MAC239</sub> Gag and HIV-1<sub>89.6p</sub> Env plus IL-2/Ig protein or plasmid, compared to 6-20% of total circulating CD8+ T cells in animals received DNA vaccines only, suggesting that the co-administration of IL-2/Ig can substantially augment immune responses elicited by DNA vaccine in primates (Barouch et al., 2001b).

Phase I trials to evaluate the safety and immunogenicity of DNA constructs as an HIV vaccine candidate have been conducted in HIV-1 uninfected healthy volunteers. An HIV-1 env/rev DNA vaccine was administered intramuscularly to HIV-1-seronegative persons at a dose of 100 or 300 μg at 0, 4, 8, and 24 weeks. Subjects who received the 300 μg dose exhibited antigen-specific T cell immune responses. This study supports the premise that HIV-1 DNA vaccine antigens can stimulate immune responses in immunised volunteers, and further studies to enhance DNA vaccine immunogenicity are required (Boyer et al., 2000a).

Notably, a phase I trial of the first DNA vaccine specifically designed to combat the clade A HIV-1 virus, which is the most prevalent strain in many parts of Africa, was officially launched in Oxford in 2000 and later in Kenya in 2001. The trial was
conducted in low risk healthy volunteers and is to evaluate the safety and immunogenicity of the DNA vaccine. Results are expected to be ready in about two years (Fazal et al., 2000).

1.5.8 Prime/boost strategy

A prime-boost strategy refers to a vaccination regimen involving a primary vaccination with one vaccine, typically a DNA vaccine or a recombinant vector vaccine to induce cellular immune responses, followed by booster shots of a subunit vaccine to stimulate antibody production (Letvin et al., 1997; Tartaglia et al., 1998; Cho et al., 2001). This strategy is under active investigation not only for the potential additional effects of the vaccine on eliciting more effective immunity against HIV-1 infection, but also for the potential that vaccine combinations may augment the breadth of both humoral and cellular immune responses against HIV-1 infection. It has been demonstrated that immunisation with live recombinant viral vectors (e.g., vaccinia vector) or DNA vaccines, which allow de novo synthesis of antigens, elicit CD8+ CTL responses (Steinman et al., 1998; Cho 2000; Barouch et al., 2001b), and with recombinant proteins can induce neutralising antibodies (Graham et al., 1996; Connor et al., 1998; McCormack et al., 2000; Nitayaphan et al., 2000). HIV-1 vaccination prime-boost strategies consist of priming with live virus vectors or HIV-1 DNA vaccines expressing HIV-1 proteins and boosting with subunit proteins or recombinant viral vector vaccines expressing HIV-1 proteins, may induce more potent HIV-1 specific cellular immune responses as well as humoral immune responses, than that induced by immunisation with a DNA vaccine or a live recombinant vaccine alone (Amara et al., 2001; Cho et al., 2001; Nilsson et al., 2001; Yoshizawa et al., 2001). Macaques vaccinated with a recombinant Semliki forest virus (SFV) expressing multiple SIV structural and regulatory proteins and boosted with a recombinant MVA expressing the same SIV proteins as the recombinant SFV vector efficiently induced both humoral and cellular immune responses (Nilsson et al., 2001). Pigtailed macaques primed with recombinant vaccinia virus expressing HIV-1 gp160 and boosted with HIV-1 gp120 proteins developed neutralising antibodies against multiple HIV-1 isolates (Cho et al., 2001).
To generate both cellular and humoral immune responses, a phase I trial for evaluating the regimen of priming with recombinant vaccinia vector expressing HIV-1 gp160_{LAI} and boosting with HIV-1 rgp120_{SF2}, rgp120_{LAI}, rgp120_{MN} or rgp160_{MN} were conducted in 56 vaccinia naïve subjects. Neutralising antibodies against homologous and heterologous HIV-1 strains occurred in all vaccinees receiving live recombinant vaccinia vectors and rgp120 proteins. 26 out of 51 subjects tested demonstrated Env-specific CTLs. These results demonstrated that vaccination regimens in which one component elicits primarily CTLs and the other neutralising antibodies may offer the promise for the development of an effective HIV-1 vaccine strategy (Corey et al., 1998).

To date, different prime-boost strategies have been tested including sequentially immunising with viral vectors and subunit proteins (Corey et al., 1998; Belshe 2001), naked DNA and subunit proteins (Letvin et al., 1997P; Putkonen P et al., 1998; Cherpelis et al., 2001), naked DNA and recombinant viral vector vaccines (Amara et al., 2001); live attenuated HIV and live viral vector vaccines (Walther-Jallow et al., 2001), by combining two different recombinant viral vectors (Nilsson et al., 2001), and also triple combinations (naked DNA, recombinant viral vector and then purified subunit proteins) (Lockey et al., 2000).

1.6 Conclusion

Development of an effective vaccine against HIV-1 is an important goal in the fight against AIDS. The observed role of CTL in the control of HIV-1 replication suggests that it is essential for an effective HIV-1 vaccine to induce strong HIV-1 specific CTL responses. The vaccine should also be able to induce antibodies neutralising HIV primary isolates, and importantly be capable of generating immunity at the mucosal surfaces, the primary route of transmission. At present, in the absence of definitive information about the mechanisms of protective immunity, induction of these responses remains an initial benchmark by which to judge candidate HIV vaccines.

Live attenuated vaccine has been shown to induce efficient protection against pathogenic virus challenge in primate model, but its safety has been questioned. Recombinant viral
protein and whole inactivated viruses are limited by their inability to induce CTL-
response. In contrast, naked DNA and live recombinant vector approaches can induce
cellular immune responses, but these immune response are unlikely to be of sufficient
magnitude to achieve protective immunity against challenges with a pathogenic SIV/HIV
strain. A great deal of attention has been focused on prime/boost strategies, which have
shown that DNA or live recombinant viral vector priming followed by recombinant
protein or live recombinant viral vector boosting, could significantly augment HIV-
1/SIV specific immune responses. However, the safety and efficacy of regimens which
induce immune responses correlating protection from HIV-1 infection of candidate HIV-
1 vaccines need to be validated in human clinical trials. Despite the improvements and
encouraging developments which have taken place over the past several years,
substantial progress is still needed to achieve the goal of developing a safe and effective
HIV-1 vaccine.

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