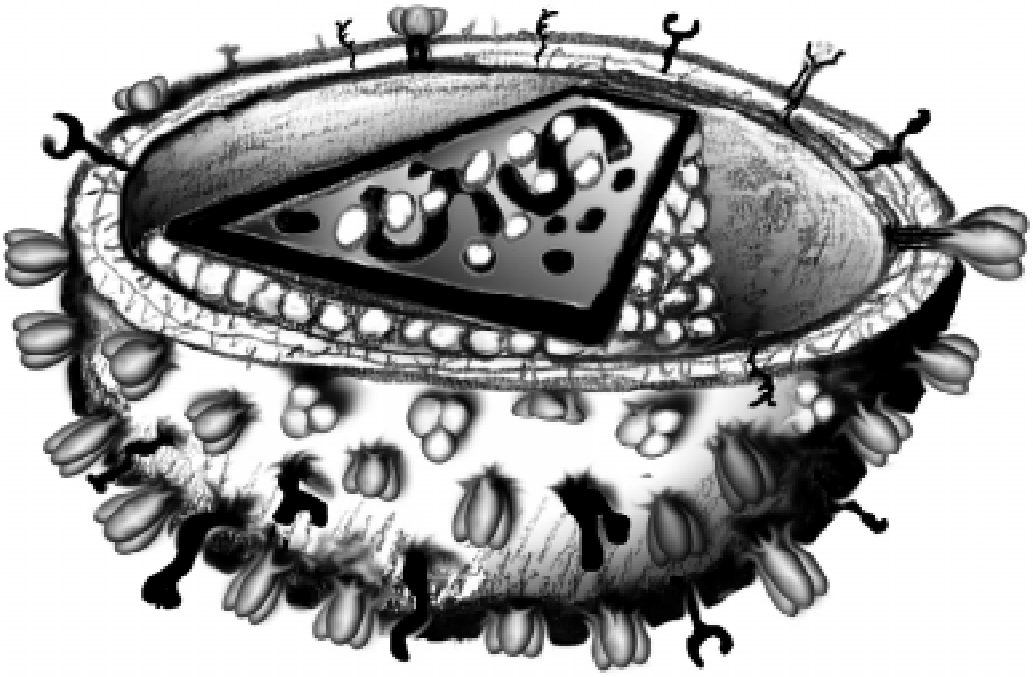
Immune and autoimmune responses to HIV-1 in mucosa and other tissues

Peter Lundholm



SMITTSKYDDSINSTITUTET

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From
The Swedish Institute for Infectious Disease Control
Microbiology and Tumorbiology Center
Karolinska Institute
Stockholm, Sweden

IMMUNE AND AUTOIMMUNE RESPONSES TO HIV-1 IN MUCOSA AND OTHER TISSUES

PETER LUNDHOLM

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The scientist does not study nature because it is useful to do so. He studies it because he takes pleasure in it, and he takes pleasure in it because it is beautiful.

Jules Henri Poincaré 1854-1912

To the memory of Gerda Eliason, my Grandmother

ELECTRONIC VERSION

The Swedish Institute for Infectious Disease Control
Microbiology and Tumorbiology Center
Karolinska Institute
Stockholm, Sweden

IMMUNE AND AUTOIMMUNE RESPONSES TO HIV-1 IN MUCOSA AND OTHER TISSUES

AKADEMISK AVHANDLING

som för avläggande av doktorsexamen i medicinsk vetenskap vid Karolinska Institutet, offentligen försvaras vid Mikrobiologiskt och Tumörbiologiskt Centrum föreläsningssalen, Doktorsringen 13E, Karolinska Institutet, fredagen den 29 oktober 1999, kl 9.00

av

PETER LUNDHOLM

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STOCKHOLM 1999

SUMMARY

The aim of these studies was to evaluate routes and devices for administration of HIV-1 DNA vaccines. In particular we were interested in mucosal routes for the induction of immunity at mucosal surfaces.

We studied the effects of a novel mucosal route, jet-injection, for induction of systemic and local responses. This technique was found to be a rapid and easy way of adminisering plasmid DNA. A combination of the genes encoding the HIV-1 regulatory proteins rev, nef, tat and the structural genes *gp160* and *p24* was administered by intramuscular, intradermal and mucosal routes. B cell responses measured by *in vitro* B cell Ig production and serology showed high titers and high frequencies of IgG and IgA induction. The jet-injection technique elicited predominantly IgG2a and was able to induce local mucosal IgA and IgG antibodies. Intranasal administration of the plasmids induced mucosal IgA, found in lung washings, faeces and vagina, as well as high titers of humoral systemic responses. Continued immunization also induced cytotoxic T cells after intramuscular injections.

Mucosal jet-injections and intramuscular injections of HIV-1 DNA constructs encoding the *nef, rev, and tat* regulatory genes were evaluated in humans. Mucosal and systemic IgA and IgG specific for the gene products were present during the vaccination but were not increased. This was at variance with the non-infected animals. Oral mucosal transudate collected from the site of immunization was analyzed for mucosal cytokines. IFNγ and IL-2 were synthesized at the local site. High concentrations of IL-2 were induced and persisted for several months.

Analysis of mucosal biopsies from the site of immunization revealed a massive infiltration of mononucleated cells as well as granulocytes at various depths of the biopsy on the vaccinated side compared to the control side. Inflammatory T-cell subsets on the immunized side consist of both $CD4^+$ and $CD8^+$ T-cells. The majority of these T-cells expressed the memory phenotype CD45 RO. Additional signs of activation were the increased expression of HLA-DR associated invariant chain (CD74) and increased presence of dendritic cells (S-100) both in the epithelium and in the underlying T-cell infiltrate. Thus, Th1 responses were preferentially induced with the mucosal jet-injection technique in preclinical animal studies, as well as in the clinical trial with HIV infected humans.

The possibility that disease progression would be accentuated by autoimmune induction was evaluated in the light of several reports on gp160/HLA molecular mimicry and anti-leucocyte immunity in HIV infected individuals. Could autoantibodies be induced by immunization? Sera from individuals vaccinated with HIV-1 envelope antigen rgp160 revealed low levels of humoral responses to synthetic peptides representing defined regions reported to exhibit molecular mimicry to self-antigens. Although autoimmunity in HIV-1 infection may play a role in the pathogenesis of HIV-1 progression to AIDS, rpg160 immunization did not contribute to elevated levels of autoimmune antibodies.

Malignancies in the lymphoid system may alter the B cell responses to HIV-1. This hypothesis was tested in patients with HIV-1 related lymphoma. The lymphoma patients with low levels of $CD4^+$ cells had responses similar to asymptomatic patients. Symptomatic patients, on the other hand, exhibited high levels of IgG subclasses to HIV-1 antigens. The patterns of IgG subclass reactivities were also broader than those for either the asymptomatic or the lymphoma patients. This indicates an extensive effect on the humoral immune response in progression of HIV infection.

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PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Hinkula, J., Lundholm, P. and Wahren, B. (1997) Nucleic acid vaccination with HIV regulatory genes: a combination of HIV-1 genes in separate plasmids induces strong immune responses. Vaccine 15, 874-878.

II. Lundholm, P., Asakura, Y., Hinkula, J., Lucht, E. and Wahren, B. (1999) Induction of mucosal IgA by a novel jet delivery technique for HIV-1 DNA. Vaccine 17, 2036-2042.

III. Asakura, Y., Lundholm, P., Kjerrström, A., Benthin, R., Lucht, E., Fukushima, J., Schwartz, S., Okuda, K., Wahren, B. and Hinkula, J. (1999) DNA-Plasmids of HIV-1 induce systemic and mucosal immune responses. Biol Chem 380, 375-379.

IV. Lundholm, P., Wahren, M., Sandström, E., Volvovitz, F. and Wahren, B. (1994) Autoreactivity in HIV-infected individuals does not increase during vaccination with envelope rgp160. Immunol Lett 41(2-3), 147-53.

V. Lundholm, P., Lucht, E., Svedmyr, E., Rudén, U., Linde, A. and Wahren, B. (1998) Immunoglobulin G abnormalities in HIV-1 infected individuals with lymphoma. Immunotechnology 4, 29-36.

VI. Lundholm, P., Leandersson, A-C., Calarota, S., Engström, G., Lucht, E., Hinkula, J., Sandström, E., Christensson, B., Bratt, G. and Wahren, B. Human mucosal T cell activation following intraorally jet-injected HIV-1 DNA. 1999. In manuscript.

Contents

ABBREVIATIONS

aa	amino acid
AIDS	acquried immunodeficiency syndrome
ADCC	antibody dependent cellular cytotoxicity
APC	antigen presenting cell
ASC	antibody secreting cell
B cell	B lymphocyte
bp	base pair
CCR5	CC chemochine receptor
CD	Cluster of differentation
cDNA	complimentary DNA
CXCR4	CXC chemokine receptor 4
CTL	cytotoxic T lymphocyte
DNA	deoxyribonucleic acid
DLN	draining lymph nodes
DTH	delayed type hypersensitivity
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
gag	group antigen
GALT	gut-associated lymphoreticular tissue
gp41	transmembrane envelope glycoprotein (41kD)
gp120	surface envelope glycoprotein (120kD)
gp160	precursor envelope glycoprotein (160kD)
HAART	highly active antiretroviral therapy
HIV-1/2	human immunodeficiency virus type 1/2
HLA	human leucocyte antigen
IgG, IgA, IgM	immunoglobulin isotypes G, A, M
IL	interleukin
LT	lymphoid tissue
LTNP	long term non progressor
LTR	long terminal repeate
Mab	monoclonal antibody
MHC	major histocompatibility complex
MIP-1α/β	macrophage inflammatory protein-1α/β
MØ	macrophage
NALT	nasopharyngeal-associated lymphoreticular tissue
Nef	negative regulatory factor
NSI	non-synsytium inducing
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
pol	polymerase
RANTES	regulated upon activation, normal T cell expressed and secreted
Rev	regulator of virion protein
RNA	ribonucleic acid
RT	reverse transcriptase
SI	syncytium inducing
SS	Sjögren´s syndrome
sIgA	sectretory IgA
SIV	simian immunodeficiency virus
tat	transactivator
T cell	T lymphocyte
TCR	T cell receptor
ICAM-1	intercellular adhesion molecule-1
LFA-1	lymphocyte function-associated antigen 1
Th1/Th2	T helper cells type 1/2
Vif	virion infectivity factor
Vpr	viral protein R

INTRODUCTION

The virus

Early reports of an increased incidence of *Pneumocystis Carinii* pneumonia and Kaposi´s sarcoma in previously healthy homosexual men were concluded to be due to immunosuppression, with decreasing CD4+ T-lymphocytes as a common finding (CDC, 1981a; CDC, 1981b; Gottlieb *et al*., 1981). The causative agent was identified in 1983 (Barre-Sinoussi *et al*., 1983) and 1984 (Gallo *et al*., 1983) and the disease was named acquired immunodeficiency syndrome (AIDS) (CDC, 1982). The virus, named human immunodeficiency virus (HIV) in 1986 (Coffin *et al*., 1986), was identified as a primate lentivirus belonging to the family of retroviruses. Isolation of an additional human retrovirus in West Africa in 1986 (HIV-2), immunologically distinct from HIV-1 but closely related to Simian immunodeficiency virus (SIV), led to conclusions about the African origin of HIV-1 and HIV-2. The most probable origin of HIV in man is by cross species transmission from monkeys to humans in Africa.

The vast majority of HIV transmissions occurs through contact of mucus membranes during vaginal or anal sexual intercourse. It is also well established that needle exchange during intravenous drug abuse can transmit HIV. Vertical transmission from mother to child is now a problem mostly in developing countries where antiviral therapy does not exist. The use of contaminated blood-products was a problem before these could be tested for HIV contamination.

Since the first cases were identified in 1981, HIV infection has spread as a devastating global pandemic; the World Health Organization (WHO) estimates that by the year 2000 there will be approximately 40 million adults and children living with HIV/AIDS, two thirds of them in sub-Saharan Africa. Every day, 7000 young people get infected with HIV, five young persons every second (UNAIDS, 1998).

Since HIV infection in Sweden was first reported to the Swedish Institute for Infectious Disease Control in 1985, a total of 5017 cases have been reported to date (June 1999); 1985 persons have died and 279 have left the country (Department of Epidemiology, SMI).

Worldwide, the predominant virus is HIV-1. Both HIV-1 and HIV-2 are transmitted by sexual contact through blood and they appear to cause clinically indistinguishable AIDS. However, HIV-2 is transmitted less easily, and the period between initial infection and illness is longer. We currently know of at least 10 genetically distinct subtypes (A to J) of HIV-1 within the major group (group M). In addition, group O (Outliers) comprises a distinct array of very heterogeneous viruses. The subtypes are unevenly distributed throughout the world. For instance, subtype B is mostly found in the Americas, Japan, Australia, the Caribbean and Europe; subtypes A and D predominate in sub-Saharan Africa; subtype C in South Africa and India; and subtype E in the Central African Republic, Thailand and other countries of southeast Asia. Subtypes F (Brazil and Romania), G and H (Russia and Central Africa), I (Cyprus), and group O (Cameroon) have a low prevalence. Most subtypes occur in Africa, although subtype B is less prevalent there (UNAIDS, 1998).

The distribution of HIV-1 genetic subtypes in Sweden has changed from the early dominant prevalence of subtype B to the present situation where all subtypes have been identified in Sweden (Alaeus *et al*., 1997).

As with all retroviruses, two copies of single stranded RNA carry the genomic information (Figures 1 and 2). Several essential enzymes are unique for the virus: reverse transcriptase (RT), ribonuclease H (RNase H), protease (PR) and integrase (IN), and are thus attractive for therapeutic interventions. Antiviral drugs target the functions of these enzymes without impairing the cellular functions and several RT and PR inhibitors have been approved for use in HIV infection. Compared to the prototypic retrovirus, lentiviruses are far more complex with, in the case of HIV, six additional genes. Essential for replication, the Tat and Rev regulatory genes are also of interest for drug development. However, only anti-Tat agents have gone into clinical trials and the results were not encouraging, with the anti-tat treated (Ro 24-7429, a Tat antagonist) patients responding significantly worse than nucleoside analogue treated patients regarding CD4 counts, p24 antigenemia and viral load (Haubrich *et al*., 1995). Anti-Rev drugs are not available for clinical use. Several functions for viral replication and HIV-1 pathogenesis have been described for the remaining four accessory genes of HIV: nef, vif, vpr and vpu. The encoded gene products are relatively small (80 to 210 amino acids) regulatory proteins with often multiple functions and therapeutic agents against them are not available.

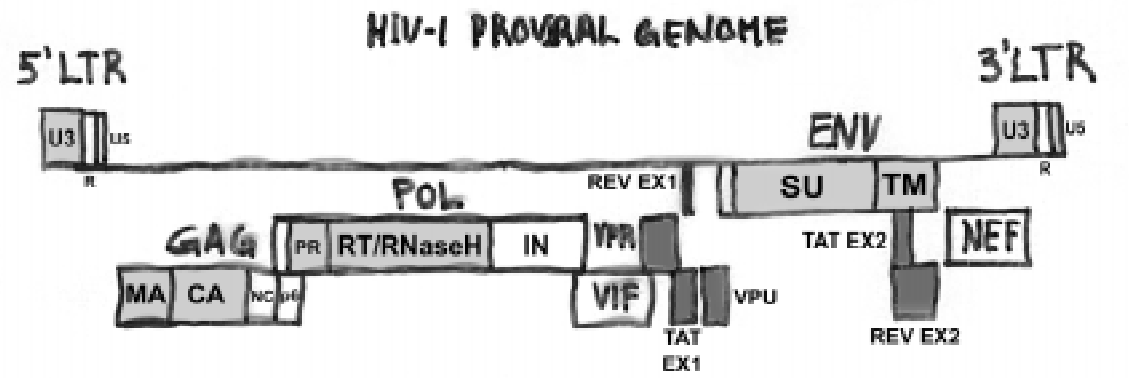


Figure 1. **HIV-1 genomic organization.** Identical flanking long terminal repeat (LTR) promotors with the three major genes that are shared with all retroviral genomes: Group antigen (GAG) precursor generates capsid (CA) p24, matrix (MA) p17, nucleocapsid (NC) p7, and the p6 protein with unknown function. Polymerase (POL) of the GAG-POL precursor is processed to form protease (PR) p10, reverse transcriptase (RT) p66/p51, ribonuclease H (RNase), and integrase (IN) p32. Envelope (ENV) gp160 is cleaved to form gp120 (SU) surface and gp41 (TM) transmembrane envelope. Two exons each code for the proteins Rev and Tat. Clustered on the 3´ side we find the accessory genes nef, vif, vpu, and vpr (adopted from (Miller and Sarver, 1997)).

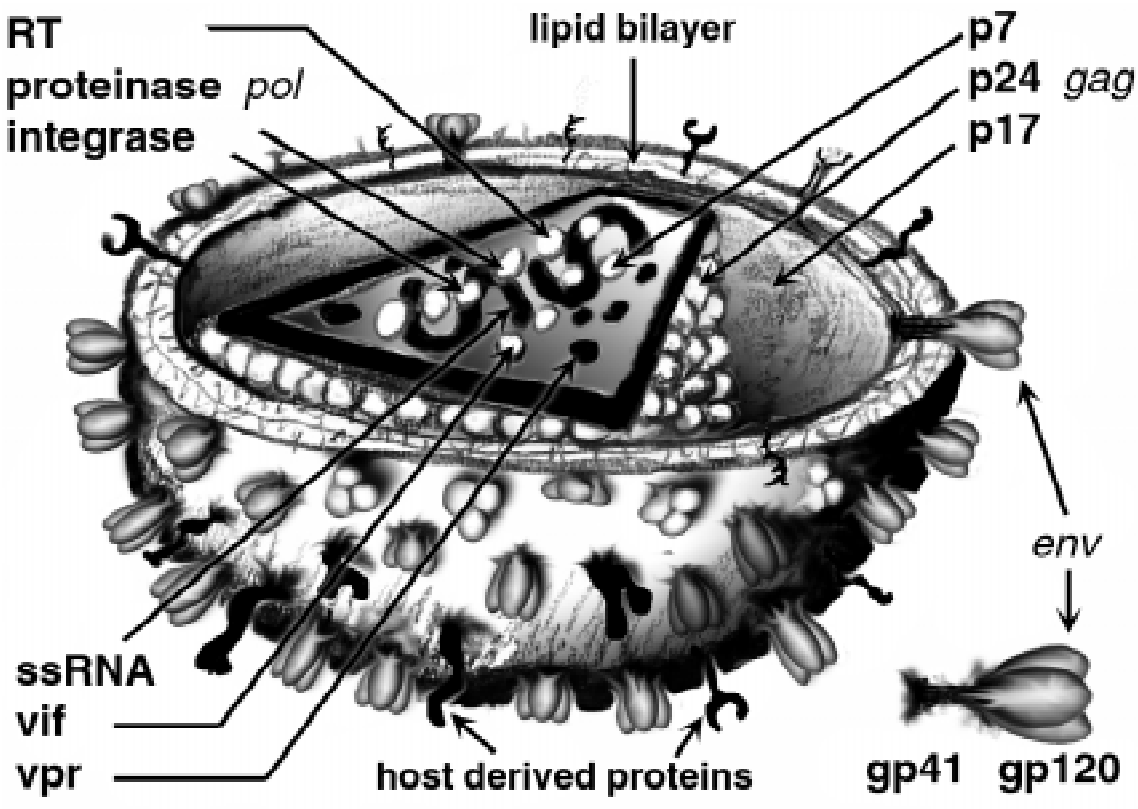


Figure 2. **Structure of HIV-1**. The two single stranded RNA (+ss, 9.2kB) are situated in the core of the virion associated with the nucleocapsid (NC) p7 proteins. The encoded viral enzymes reverse transcriptase (RT) p66/p51, proteinase (PR), ribonuclease H (RNase), and integrase (IN) p32 are packed into the core together with the genomic RNA and associated NC proteins. The core consists of the capsid (CA) p24 protein. The matrix (MA) p17 protein is associated with the lipid bilayer surrounding the nucleocapsid. The envelope gp120 and transmembrane protein gp41 are non-covalently linked to the membrane. More host-derived cellular proteins than envelope proteins are contained in the lipid bilayer of the viral membrane (Table 2).

Infection of cells

The life cycle of HIV is initiated by the receptor-binding of the virus to susceptible host cells. This is accomplished by the high affinity binding of the viral envelope gp120 to the cell-surface CD4 molecule. The CD4 antigen is an essential and specific component of the receptor (Dalgleish *et al*., 1984; Klatzmann *et al*., 1984) and thereafter coreceptors of the chemokine receptor family interact with the gp120 molecule (Figure 3) (Alkhatib *et al*., 1996; Feng *et al*., 1996; Wang *et al*., 1999).

The chemokines macrophage inflammatory protein 1a (MIP-1α), MIP-1β and RANTES were shown to block macrophage-tropic (M-tropic) viral infectivity in vitro (Cocchi *et al*., 1995). M-tropic HIV-1 strains are responsible for viral transmission and are the prevalent viral types isolated from asymptomatic individuals. During disease progression, T-cell-tropic (T-tropic) viral strains emerge that are more able to infect certain CD4+ cells (Figure 3).

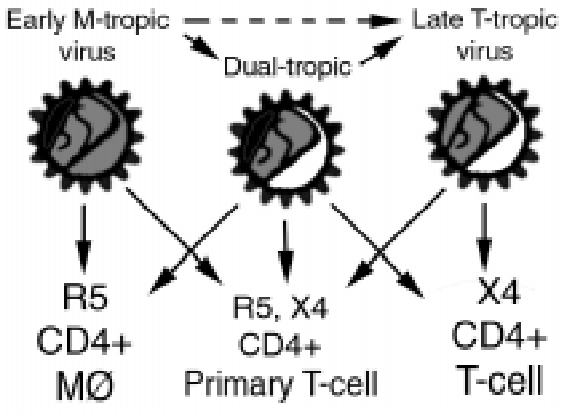


Figure 3. **Model for coreceptor usage and HIV-1 tropism.** M-tropic strains are specific for CCR5 and infect primary macrophages and primary CD4+ T cells. Dual-tropic strains use both CXCR4 and CCR5, and can infect continuous CD4+ T cell lines, macrophages, and primary T cells. T-tropic strains emerge late in the infection and are specific for CXCR4. T-tropic strains infect continuous CD4+ T cell lines and primary CD4+ T cells.

CXCR4 was shown to act as a coreceptor required for cellular entry by T-tropic but not M-tropic strains of HIV-1 (Feng *et al*., 1996). After the subsequent discovery of a chemokine receptor, CCR5, specific for the chemokines MIP-1a, MIP-1b and RANTES (Samson *et al*., 1996), several researchers demonstrated that CCR5 was an entry cofactor for M-tropic isolates of HIV-1 (Moore *et al*., 1997). CXCR4 usage determines the biological phenotype for all subtypes of HIV-1. Thus, it is suggested that the genetic subtypes may differ in virulence, tissue tropism, and transmissibility (Tscherning *et al*., 1998).

The viral envelope gp120 is derived from a precursor protein, gp160, which is glycosylated in the endoplasmatic reticulum and forms oligomers, probably trimers (Wyatt and Sodroski, 1998). The protein is cleaved in the Golgi which produces the mature proteins gp120 and gp41 (Figures 2 and 4). The gp120 and gp41 are maintained in relatively stable trimers. During the gp120 interaction in viral binding, the CD4 – gp120 binding induces conformative changes in gp120, exposing the binding site for the chemokine receptors (Figure 4) (Lapham *et al*., 1996).

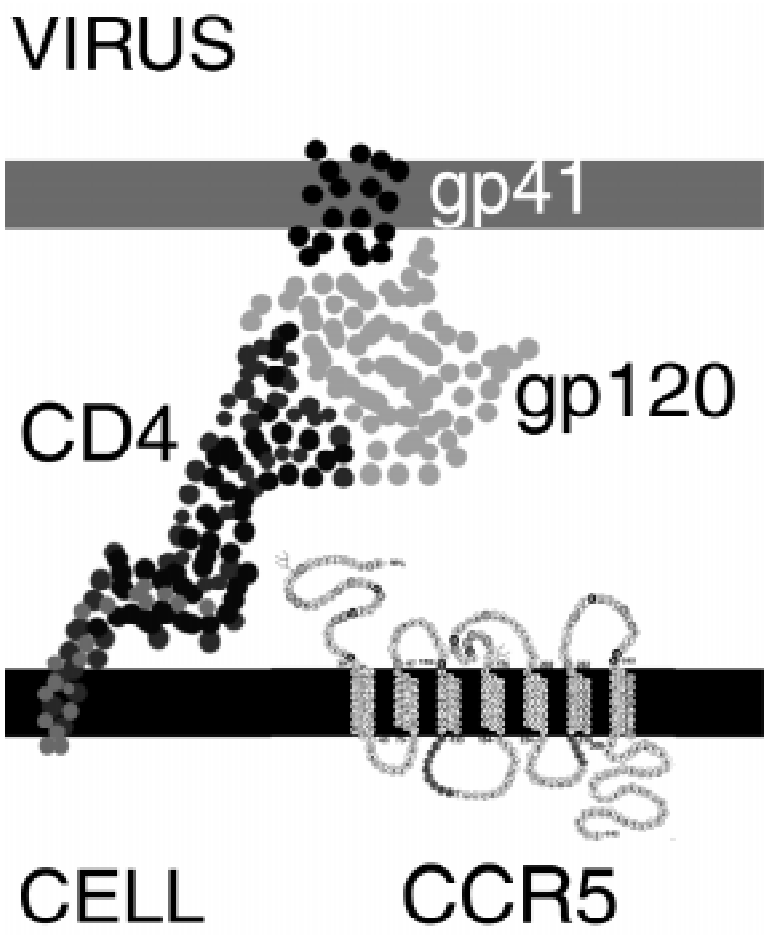


Figure 4. Model for HIV-1 binding and entry using the CCR5 coreceptor. Upon initial binding of gp120 (SU) to the CD4 receptor, gp120 (SU) undergoes a conformational change that exposes the coreceptor-binding determinants. The interaction of gp120 (SU) with the coreceptor triggers gp120 (SU)/gp41 (TM) (Env) to undergo additional conformational changes, leading to extension of gp41 and insertion of the fusion sequence into the target cell membrane.

The binding results in entry of the virus by a fusogenic portion of gp41, leading to fusion of the two membranes. The virion is partly uncoated and the viral RNA is transcribed by reversed transcriptase into double stranded DNA. This is an error-prone process leading to mutations, deletions and recombinations. The newly synthesized DNA is transported to the nucleus and incorporated by integrase into the host cell genome. This proviral DNA is dependent on host transcriptional factors and will stay latently integrated in the genome for a variable length of time or until the host cell is activated.

HIV INFECTION

Viral infections are often transmitted through the mucosa. HIV-1 enters through intact or damaged mucosal epithelium and infects the underlying Langerhans cells and macrophages in the submucosa of the rectum or vagina. However, recent studies indicate that CD4+ cells are also preferentially infected initially (Stahl-Hennig *et al.*, 1999; Veazey *et al.*, 1997). The infected cells disseminate to regional lymph nodes where viral replication occurs (Spira *et al.*, 1996).

Primary HIV infection

A general viremia follows, accompanied by lymphadenopathy, fever and flu-like symptoms in 50 to 70 percent of the cases. The infection is widely disseminated during the primary infection, as seen by the dramatic loss of CD4+ T cells, leading to an initial immunodeficiency. The seeding of virus during primary infection, especially in the lymphoid organs, may influence the subsequent course of HIV infection. Moreover, as CTL responses develop, viremia falls dramatically (Koup *et al.*, 1994). Strong HIV-1 specific antibody responses develop during the first weeks after initial infection (seroconversion) but antibody present immediately after infection is not neutralizing (Moore *et al.*, 1994) (Figure 5). However, this immunity is apparently inadequate to suppress viral replication completely, since HIV expression persists in lymph nodes even with undetectable viremia.

Latency

The term clinical latency is misleading. After primary infection, a mounting of HIV specific humoral and cell mediated immune response is initiated successfully and virus is not readily detectable in plasma. However, this period is characterized by a vigorous battle between the virus and the immune responses. An enormous amount of virus and CD4 cells are killed each day and outbreaks of transient viremia are commonly observed during this time. Although CD4 levels are maintained for a variable period of time, continuous depletion of CD4 cells is seen in most patients.

Progression of HIV infection

Advances have been made in elucidating the genetic, immunologic, and virologic factors in HIV-infected individuals who either progress rapidly or do not progress to acquired immunodeficiency syndrome (AIDS). In addition, natural immune responses to HIV may be protective in rare individuals, as evidenced by the detection of HIV-specific immune responses in HIV-negative individuals who have been exposed to the virus many times (Heeney *et al.*, 1999; Mazzoli *et al.*, 1997; Williams *et al.*, 1999). The clinical progression in HIV-1 infection follows a number of diverse courses. Rapid progressors constitute 10% of HIV infected individuals and develop AIDS within 2 to 3 years of HIV infection. Approximately 5 to 10% of HIV-infected subjects, nonprogressors, are clinically asymptomatic after 7 to 10 years. The remaining subjects with HIV-infection, typical progressors, develop AIDS within a median time of approximately 10 years from initial infection. Approximately 10 to 20% of HIV-infected individuals will be AIDS-free 20 years after infection (Haynes *et al.*, 1996).

Development of clinical disease

The progressive deterioration of the immune system that occurs in most patients with HIV infection inevitably leads to an AIDS-defining illness. Opportunistic infections or neoplasms, as well as severe and persistent constitutional signs and symptoms, are inclusions for the diagnosis of AIDS (CDC, 1985; CDC 1992). HIV-related oral manifestations are increasing prevalent during late progression to AIDS (Lucht et al 1991).

PATHOGENESIS OF HIV INFECTION

How does HIV-1 induce the acquired immunodeficiency syndrome (AIDS)? Several theories have been put forward since HIV was discovered and the matter remains controversial (Pantaleo *et al*., 1993). Most individuals living with HIV-1 infection maintain relatively stable numbers of CD4+ T cells, strong cytotoxic T cell (CTL) responses and low numbers of HIV-1 infected cells in the blood, all indicators that the virus is under substantial immune control. However, at some point after the primary infection, the immune system fails, and most infected individuals develop their first symptoms of AIDS.

For some time it was believed that HIV-1 weakens the immune system by either increasing viral diversity (Nowak *et al*., 1991) or causing immune dysregulation (Miedema *et al*., 1990), whereas others have suggested that the immune response to the virus is actually harmful (Zinkernagel, 1995). Several studies have also implied that differences in viral pathogenicity may contribute to the development of AIDS (Connor *et al*., 1993; Koot *et al*., 1993). The abnormal and defective T cell reactivity to HIV-1 was considered to contribute to progression and could be detected early in the infection (Shearer and Clerici, 1991; Wahren *et al*., 1986).

How can we explain the immunological steady state during asymptomatic HIV infection and what triggers the events leading to the decline in CD4+ T cells and AIDS? Some suggest the enormous turnover of CD4+ T cells exhausts the immune system. At some point the lymphopoietic system fails, leading to the onset of AIDS.

The level of HIV-1 RNA in plasma is a strong predictor of disease progression. Still, it is large numbers of productively infected cells, not free virus in blood, that may be most harmful for the immune system. The lymphoid tissue (LT) is the main site of production and storage of the virus (Pantaleo *et al*., 1991). LT biopsies have therefore been used to measure residual virus load in patients with undetectable viremia. Measurement of cell-associated HIV-1 RNA in blood was recently reported to reflect the virus load in LT better than HIV RNA in plasma (Yerly *et al*., 1999). Efficient control of the number of HIV infected cells is of utmost importance to contain the infection. Combining immunotherapy with antiviral drugs may best inhibit HIV-1 replication. The ultimate goal of such approaches would be to clear the virus completely from the body.

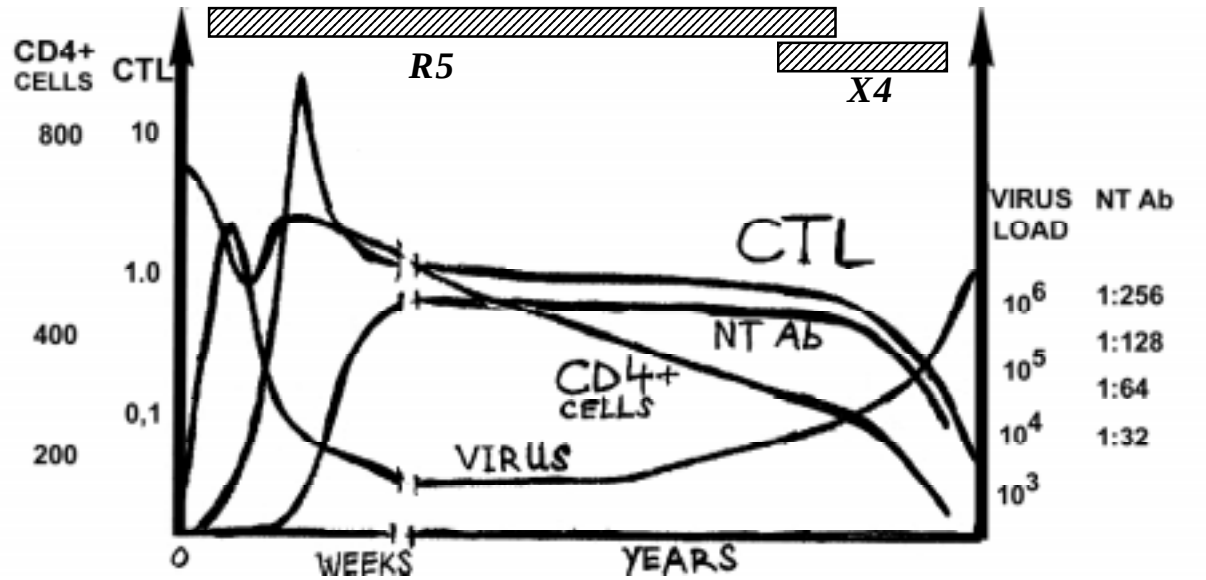


Figure 4. **Immune responses and viremia during HIV infection.** Neutralizing antibody titer (NT ab) (Moore *et al*., 1994) and percent effector CTL in PBMC (Moss *et al*., 1995; Pantaleo *et al*., 1994; Calarota *et al*., 1998). CD4+ T cell count (x10^6/ml) and virus load as RNA copies/ml blood (Ho *et al*., 1995; Wei *et al*., 1995). Predominating viral tropism shown as lines on top of graph: R5; CCR5 (M-tropic) and late emergence of X4; CXCR4 (T-tropic) virus isolates.

HOST IMMUNE RESPONSES

Hypergammaglobulinemia

Immune dysfunction in AIDS involves B cells as well as T cells, with both quantitative and qualitative changes in B cell activation. HIV-1 infection leads to abnormal B cell activation with *in vivo* polyclonal activation and B cell hyperreactivity, such as hypergammaglobulinemia (Lane *et al*., 1983; Shirai *et al*., 1992; Yarchoan *et al*., 1986). Chronically infected individuals often exhibit elevated plasma IgG up to twice the normal levels, although the major portion of these antibodies are not HIV-specific. *In vitro* cultures of B cells from HIV-1 infected also spontaneously secrete antibodies to a higher degree but these respond poorly to B cell mitogens (Lane *et al*., 1983; Yarchoan *et al*., 1986).

Increased serum antibodies are probably a direct consequence of HIV infection or the antigens derived from the virus (Morris *et al*., 1998). Elevated serum IgA was correlated to poor prognosis, possibly due to a profoundly disregulated intestinal B cell activation in HIV infected individuals (Eriksson *et al*., 1995; Munoz *et al*., 1988). Polyclonal activation is rapidly responsive to decreases in viral replication caused by combination antiviral therapy (Morris *et al*., 1998). The decrease of antibody secreting cells (ASC) was seen soon after HAART and the hypergammaglobulinemia declined gradually. These observations are confirmed in measurements of IgG concentrations (Bratt *et al*., 1989) performed in HIV infected patients attending the Söder hospital clinic (Figure 5; Dr Göran Bratt, personal communication). There is a slow decline in serum IgG after HAART.

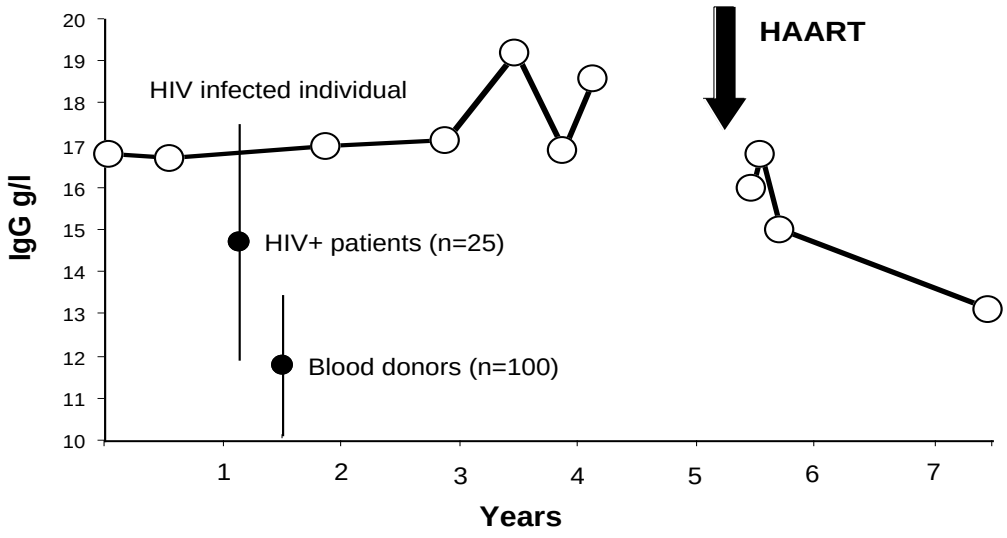


Figure 5. **Development of serum IgG levels in a representative patient starting HAART**. The level of IgG in blood donors (n=100) and HIV+ non-treated patients (n=25) plotted as solid circles (courtesy of Dr Göran Bratt, Venhälsan, Söder Hospital, Stockholm).

Specific humoral immune responses

The immune system mounts an enormous effort to control the replication and spread of HIV-1. The infection leads to a generalized immune activation with stimulation of different T cell subsets and high levels of polyclonal antibody production (Fauci *et al*., 1984) with specificities against a large range of epitopes of different HIV proteins.

Antibody response in the course of HIV-1 infection has been extensively studied to elucidate the role of the humoral arm in a strategy to eliminate free virus. The HIV-1 specific humoral responses to several HIV-1 determinants evolve during the first year of infection. The most dominant B cell determinants are the Env (gp120 and gp41) and Gag (p24 and p17) proteins. Anti-env titers remain high throughout the infection, in contrast to anti-gag antibody responses, which decline during severe immunosupression and are thus a surrogate marker for the progression of HIV infection to AIDS (Sheppard *et al*., 1991).

IgG subclasses

Antiviral IgG subclass reactivity consists mainly of IgG1 and IgG3 (Linde, 1985; Sundqvist *et al*., 1984). IgG is the dominant anti HIV-1 immunoglobulin isotype and its restriction in subclass patterns has been described (Broliden *et al*., 1989; Jansson *et al*., 1992; Khalife *et al*., 1988; Ljunggren *et al*., 1988). In the case of reactivity to whole HIV-1, there is an IgG1 and IgG3 restriction followed by a restriction to synthesis of IgG1 during late progression of the disease (Sundqvist *et al*., 1986).

Neutralizing antibodies

Neutralizing antibodies may be a component of the initial control of HIV replication (Albert *et al*., 1990; Moore *et al*., 1994). However, as HIV variants emerge over time, new variants frequently are not neutralized by autologous sera, and in some cases, antibodies against newly emerging HIV variants may enhance HIV replication *in vitro*, although the significance *in vivo* of enhancing antibodies is controversial. A randomized trial of passive immunotherapy of HIV-infected patients suggested that the administration of heat-inactivated plasma from HIV-infected individuals every 2 weeks for 1 year could slow the progression to AIDS in the recipients (Vittecoq *et al*., 1995). Thus, antibodies appear to be involved in protective immunity against the progression of HIV infection, although the specificities of anti-HIV neutralizing antibodies that might be protective in patients remain unresolved. Despite the potential to exert potent antiviral effects, antibodies are not able to suppress virus replication completely in infected hosts. The efficacy of the humoral immune response in limiting the spread of virus in vivo is compromised by at least two factors: the relative resistance of primary virus isolates to neutralization, and the temporal pattern with which neutralizing antibodies are generated. During natural HIV infection, disassembled envelope glycoproteins apparently elicit most of the antibodies directed to these viral components. Thus, antibodies do not recognize the functional envelope glycoprotein complex (Figure 4). Specific antiviral antibodies are detected early in the infection but most of these antibodies lack the ability to inhibit virus infection. HIV infection is already established when neutralizing antibodies emerge (Figure 5) (Moore *et al*., 1994; Wyatt and Sodroski, 1998).

Mucosal immune responses

Viral infections are often transmitted through the mucosa. Immunity to HIV-1 at the mucosal surface could prevent primary infection and dissemination to regional lymph nodes. As an easily accessible surface for administration of vaccines, the oral mucosa has several experimental advantages over other mucosal sites. Sampling and monitoring the site of immunization are easily accomplished without invasive techniques.

The immune system is divided into two functionally independent compartments: the systemic immune system, comprising the bone marrow, spleen and lymphoid system, and the mucosal immune system, represented by lymphoid tissues within the mucosa and secretory glands. Differences in effector functions separate these two compartments. Mucosal humoral immunity is mediated by antibodies predominantly of the IgA isotype that are selectively transported to external secretions. The overwhelming majority of the secretory IgA (sIgA) is produced locally in the mucosa (Ogra *et al*., 1999).

Nonspecific innate defense mechanisms and specific local antibodies protect the vulnerable mucosal surface. Local antibodies, particularly secretory immunoglobulin A (sIgA), are the primary effectors classically responsible for the elimination of mucosal pathogens. sIgA antibodies are significantly associated with protection against mucosal pathogens (Kraehenbuhl and Neutra, 1992; Michetti *et al*., 1992; Ruggeri *et al*., 1998). The exact mechanisms by which sIgA antibodies protect against invading pathogens are not well understood. Several suggestions have been put forward: preventing contact of the pathogens with epithelial surfaces, formation of immune complexes, clearance by peristalsis, transcytosis of immune complexes, antigen capture during transport through epithelial cells, and intracellular neutralization with or without neutralizing antibodies (Brandtzaeg *et al*., 1999; Childers *et al*., 1989; Mazanec *et al*., 1992; Mestecky, 1988).

Intraepithelial and submucosal lymphocytes are present in the oral mucosa and participate in the immune response against foreign antigens. Mucosal T cells capable of both cytolytic activity and cytokine production may actively participate in the early defense against HIV-1 infection (Lohman *et al*., 1995). Local mucosal CTL may be critical for the success of a vaccine against viruses, as recent work demonstrated in a murine rectal challenge model (Belyakov *et al*., 1998). The cytolytic T cell response contributes to the control of HIV viral replication. It suppresses the viremia both during early infection and for a variable period during which the patient remains healthy even though the viremia is maintained at a high level by continuous rounds of viral replication and reinfection of blood cells (Ho *et al*., 1995; Wei *et al*., 1995). Plasma viremia is thought to reflect the viral burden in tonsillar tissues. However, measurement of cell-associated HIV-1 RNA in blood was recently reported to reflect the virus load in LT better than HIV RNA in plasma (Yerly *et al*., 1999). The percentage of provirus-containing cells in tonsils is around 50-fold higher than in blood (Rosok *et al*., 1997). HIV infection elicits a Th1 cytokine expression in lymphoid tissue; this response is reduced after the initiation of highly active HIV therapy (HAART) and proviral reservoirs remain in lymphatic tissue (Andersson *et al*., 1998). Impaired expression of perforin has been suggested to contribute to the inability of CTL to eliminate HIV infected cells locally in the lymphatic tissue (Andersson *et al*., 1999). It would therefore be of interest to further stimulate the specific immune reactivity to HIV and boost the Th1 anti-HIV responses.

AUTOIMMUNITY

Immunological deprivation is considered to be an explanation for the development of monoclonal B cell lymphomatous proliferation in HIV infection analogous to the situation following bone marrow transplantation. Both cell-mediated and humoral immune responses become severely impaired as the disease progresses. This is due to loss of the regulatory function of CD4+ T lymphocytes and defective or increased production of immunoregulatory or proinflammatory cytokines (Clerici *et al*., 1993). Immune activation becomes inappropriate and detrimental effects will predominate as a consequence of the impaired regulation of both T and B cell functions.

Clinical and pathological evidence of autoimmunity has been associated with HIV disease progression (Morrow *et al*., 1991). The high affinity binding of gp120 to the cell surface CD4 molecule initiates the replication cycle of the virus. Since the HLA class II is the natural ligand for the CD4 molecule in antigen presentation, Ziegler and Stites have hypothesized that autoimmune reactions could result from crossreactivity between the CD4-binding receptor site on the viral gp120 envelope and HLA class II molecules (Ziegler and Stites, 1986). This hypothesis has been extended to an idiotypic network model described by Hoffmann and colleagues which postulates that HIV and allogeneic stimuli act synergistically to cause AIDS, suggesting an opposite approach to vaccine development, that is, to induce tolerance to HIV rather than to stimulate the immune response (Hoffmann *et al*., 1991).

Habeshaw and Dalgliesh hypothesized an induction of a graft-versus-host-like disease by HIV gp120 acting as an alloantigen structurally resembling HLA class I and class II with subsequent chronic autoreactivity (Habeshaw *et al*., 1992). These models are based on similarities between HIV and HLA class II molecules and on the finding that the binding sites for gp120 and HLA class II overlap in the first immunoglobulin-like domain of CD4 (Moebius *et al*., 1992). Reports on the production of anti-gp120 antibodies in mice and macaques never exposed to HIV suggested cross-reactivity between HIV and HLA, further supporting the concept that autoimmunity could participate in HIV disease progression (Fossey, 1991; Hoffmann *et al*., 1991; Kion and Hoffmann, 1991; Morrow *et al*., 1991; Ziegler and Stites, 1986).

Links to autoimmune disease

Autoantibodies typical of non-organ-specific autoimmune disease have been reported in HIV-1 infected patients (Argov *et al*., 1991; Muller *et al*., 1992), also suggesting an autoimmune component in the pathogenesis of HIV. In addition, sera from primary Sjögren's syndrome patients (SS), characterized by lymphoid infiltration of salivary and lacrimal glands and the presence of serum anti-Ro (SS-A) and anti-La (SS-B) autoantibodies, were found to react with HIV p24 in 30% of the patients (Deas *et al*., 1998; Fraziano *et al*., 1996; Talal *et al*., 1990). Thus, retroviruses were considered in the etiology of autoimmune diseases (Flescher and Talal, 1991; Ugen *et al*., 1994). The production of autoantibodies in SS patients is now suggested to be induced by elevated levels of cytokine producing cells leading to B cell activation (Halse *et al*., 1999). The evolution of autoimmune mechanisms during HIV infection cannot exclude possible progression to immunoproliferative malignancy. The evolution of autoimmunity in HIV infection is also related to clonal restriction of antibody response, as repeatedly shown in autoimmune diseases potentially evolving to malignancies (Silvestris *et al*., 1995). It was recently proposed that lymphocyte reactive autoantibodies represent an immunoregulatory cytotoxic mechanism that is activated after chronic immune stimulation and is engaged by HIV to deplete host lymphocytes (Dalgleish, 1995; Wang *et al*., 1998b).

Table 1. **HLA sequences with similarities to sequences in HIV-1 gp160**

Peptide	Origin	Location	Sequence	Reference
p19	HLA cl II DR	142-156	**V V S T** G **L** I Q **N G** D W T F Q	Young, 1988
ml 43	HIV-1 gp120	261-275	**V V S T** Q **L** L L **N G** S L A E E	
p13	HIV-1 gp41	152-166	V A **E Q** L **R A** Y L **D** G T **C** V E	Habeshaw *et al*., 1992
p14	HLA cl II DR	67-81	L L **E Q** R **R A** A **V D** T Y **C R** H	
p15	HIV-1 gp120	498-512	P T K A K **R** R V **V** Q R E K **R** A	
p18	HLA cl II DR	102-116	Y P **S** K T Q P L **Q** H H N **L L** V	Lewis *et al*., 1990
ml 225	HIV-1 gp41	532-546	A A **S** V T L T V **Q** A R L **L L** S	
p16	HLA cl II DR	19-33	N G T E R **V** R F **L** E **R** H F H N	Lewis *et al*., 1990
p17	HLA cl II DR	45-59	G E Y **R** A **V E** E **L G R** P D A **E**	
ml 274	HIV-1 gp41	777-791	I A A **R** I **V E** L **L G R** R G W **E**	
p12	HLA cl II DR	17-31	F F N **G T** E **R V** R L L E R **R** V	Golding *et al*., 1988
ml 283	HIV-1 gp41	835-849	V A E **G T** D **R V** I E V L Q **R** A	Koshino *et al*., 1995
—	HIV-1 gp120	261-275	V V S **T Q L** L L **N G** S L A E E	Zaitseva *et al*., 1992
—	FAS antigen	275-280	V **Q L I** R **N**	Susal *et al*., 1993
—	HLA DR II beta		V V S **T** G **L I** H **N G**	Young, 1988
—	HLA DR alpha	28-40	**E E** H **V I** I Q **A** E **F** Y L **N**	Zaitseva *et al*., 1992
—	HIV-1 gp120	270-282	**E E** V **V I** R S **A** N **F** T D **N**	

Molecular mimicry

Many viral proteins share epitopes or regions homologous to host-cell proteins, as evidenced by the identification of sequence homologies and cross-reactive antibodies to such regions (Table 1) (Fujinami and Oldstone, 1985; Oldstone, 1987; Oldstone, 1998). Molecular mimicry was linked to autoimmune diseases in the early 1980s with the development of technologies to make monoclonal antibodies. The first report demonstrated crossreactivity between measels virus and herpes simplex virus with a human protein (Fujinami *et al*., 1983). Since then, several researchers have reported amino acid sequence homologies between HIV derived proteins and host-cell molecules (Bjork, 1991; Koshino *et al*., 1995; Lewis *et al*., 1990; ReiherIII *et al*., 1986; Susal *et al*., 1993; Vega *et al*., 1990; Yamada *et al*., 1991). Golding and coworkers described homology and crossreactive antibodies between HIV envelope gp41 (amino acids 837-844) and a highly conserved region on the HLA DR ß1-chain (amino acids 19-25) (Golding *et al*., 1988) (Table 1). Affinity-purified IgG antibodies from seropositive patients exhibited immunosuppressive activity of lymphocyte proliferation caused by antigen presentation (Golding *et al*., 1989).

Young reported a sequence similarity between a conserved region of HIV envelope gp120 (amino acids 261-270) and the HLA DR ß-chain (amino acids 142-151) (Young, 1988). Pugliese et al more recently reported that a synthetic peptide corresponding to a region on HIV-1 gp120 stimulated PPD-specific and autoreactive T cell proliferation (Pugliese *et al*., 1992). Interestingly, earlier work concerning this region revealed the benefit of the immunological response as antibodies directed to this region of gp120 block virus infectivity without affecting the virus binding to the CD4 receptor (Ho *et al*., 1988) and a peptide derived from the HLA DR sequence (amino acids 254-268) inhibits HIV-induced syncytia formation (Lewis *et al*., 1990).

Table 2. Suggested functions of cellular constituents in HIV infection

Virion-associated host molecule or induced antibody	**Effect on life cycle of HIV-1**	**Referenses**
CD11a/CD18 (LFA-1)	Synergy in virus neutralization between plasma and anti-LFA-1 antibody	Gomez and Hildreth, 1995
CD54 (ICAM-1)	Increased ability to bind to and infect CD4+ cells, enhancement of virus infectivity, diminished sensitivity to antibody-mediated neutralization, greater susceptibility to virus infection for cells bearing activated LFA-1	Castilletti et al., 1995; Fortin et al., 1997; Fortin et al., 1998; Rizzuto and Sodroski, 1997
CD55 and CD59	Protect virions from complement-mediated destruction	Saifuddin et al., 1995
HLA-DR	Neutralization of virus infection with anti-HLA-DR antibody, increased ability to bind and infect CD4+ cells, enhancement of virus infectivity, immunization with HLA-DR conferred protection against SIV	Arthur et al., 1992; Arthur et al., 1995; Cantin et al., 1997b; Castilletti et al., 1995
MHC class I	Immunization with MHC class I conferred protection to SIV infection	Chan et al., 1995
MHC class II	Rendered virions able to present superantigen, enhancement of virus infectivity	Cantin et al., 1997a; Rossio et al., 1995
Anti-cell antibodies	Protection against SIV infection	Stott, 1991

Abbreviations: HIV-1, human immunodeficiency virus type 1; ICAM-1, intercellular adhesion molecule-1; LFA-1, lymphocyte function-associated antigen 1; MHC, major histocompatibility complex; SIV, simian immunodeficiency virus. Adopted from Tremblay et al., 1998.

Host derived proteins

HIV-1 virions acquire and display more host cellular proteins than envelope proteins (Arthur *et al*., 1992; Saarloos *et al*., 1997). A role of host proteins (Figure 1; Table 2) in the pathogenesis of HIV-infection was suggested as virus infectivity was enhanced by the incorporation of host derived proteins into the lipid bilayer of budding viruses (Cantin *et al*., 1997a; Cantin *et al*., 1997b). Immunity to HIV could also be conferred by host derived determinants in the virus, as evidenced by virus neutralization of antisera against HLA-DR (Arthur *et al*., 1992). Protection against simian immunodeficiency virus (SIV) infection in macaques was also demonstrated by vaccination with MHC class I (Chan *et al*., 1995) and class II (Arthur *et al*., 1995). This is similar to the surprising early finding, reported by Stott, where anti-cell antibodies in macaques conferred protection against SIV infection. Equivalent protection was achieved in animals immunized with the inactivated SIV grown in human cells or the control animals immunized with the human cell line (Stott, 1991). These findings raise the possibility of developing effective vaccines by allo-immunization (Shearer *et al*., 1999).

Cellular immune responses

Most HIV-infected patients make substantial early anti-HIV CTL responses that are responsible for reducing the early viremia and a small proportion of these clones survive as memory cells. Clones that do not rapidly expand may contribute to the memory cell pool from which the subsequent CTL responses are made (Ahmed and Gray, 1996). Some of the highly expanded CTL clones may be eliminated entirely, to be replaced by clones that do not respond to the same degree. The strength of the CTL response decreases and stabilizes during the course of infection and is maintained at a level that relates to the virus load. The critical factor in the relationship between CTL levels and virus load may be the rate of CTL clonal expansion (Nowak and Bangham, 1996). HIV is capable of escaping, or even turning off, cytotoxic T cell responses by mutating T cell epitopes. Virus escape and development of new CTL responses specific for subdominant epitopes could contribute to the heterogeneous CTL response seen in many HIV-infected patients. Escape of virus by mutations could originate from failure of the mutated peptide to bind to the presenting HLA molecule or altered interactions with T cell receptors (TCR) (McMichael and Phillips, 1997).

During the latent phase of HIV infection, CD8+ T cells are thought to be important in the elimination of productively infected cells and for control of the viral load (Walker and Plata, 1990). CTLs may also be involved in the immunopathogenesis of HIV infection. Antigen-presenting cells (APC) may be targets by direct killing of the virus-expressing cells. Tissue damage after the release from CTLs of certain cytokines, such as tumor necrosis factor alpha/beta and interferon gamma during the process of cytolysis, may indirectly promote elimination of lymphocytes (Levy, 1993; Zinkernagel, 1995). Patients whose immune systems recognize fewer immunodominant HIV epitopes have a more stable and effective immune response to HIV than those whose CTL responses are against multiple, less dominant epitopes in a hypothetical mathematical model (Nowak *et al.*, 1995). For vaccine design, the model suggests that the major response should be directed against conserved epitopes even if they are subdominant.

A generalized activation of the immune system precedes the progression to AIDS, manifested by elevated serum concentrations of neopterin, soluble interleukin-2 receptor, soluble CD8, and beta sub 2-microglobulin, and with activation of a large proportion of CD8+ T cells (Levy, 1993; Pantaleo *et al.*, 1994). The chronic stimulation of the immune system is maintained by circulating virions and infected cells and viral particles trapped in the lymph nodes and spleen.

Further evidence for the cytopathic effect of the virus and the involvement of CTLs in the control of HIV infection was established with the use of tetrameric complexes. A significant inverse correlation was observed between HIV-specific CTL frequency and plasma RNA viral load. In contrast, no significant association was detected between the clearance rate of productively infected cells and the frequency of HIV-specific CTLs (Ogg *et al.*, 1998).

INTERVENTION

The efforts to develop preventive human immunodeficiency virus type-1 (HIV-1) vaccines, as well as to determine the feasibility of treating HIV infection by boosting immunity, require a better understanding of the correlates of protective immunity to HIV infection.

Boosting specific immune responses may increase the decay of the latent reservoir, thereby improving the chance of inducing HIV-1 remission. An added concern is the recent observation that specific immunity to HIV-1, both cellular and humoral, declines after effective drug therapy (Morris *et al.*, 1998; Ogg *et al.*, 1999; Spiegel *et al.*, 1999; Wu *et al.*, 1999). Thus, exploring ways to increase the decay of the latent reservoir by boosting specific immunity using candidate HIV-1 vaccines seems particularly worthwhile (Ho, 1998). However, special care needs to be taken not to induce additional autoimmunity, as discussed previously in this thesis.

Antiviral chemotherapy

The use of potent antiviral drugs has revolutionized the treatment of HIV infection and led to a better understanding of the viral dynamics of the infection (Figure 6). Upon initiation of highly active antiretroviral therapy (HAART), viral replication is reduced to a minimum, as reflected by measurements of plasma viremia. The reduction of viral plasma RNA is bi-phasic, with a 99 percent reduction in the first two weeks owing to the elimination of free virus or productively infected cells and a slow decline in the second phase when elimination of long-lived infected cells occurs. These data are the basis for a mathematical model of HIV viral elimination. To eliminate all virus from the body, an estimated 2-3 years of potent antiviral therapy would be needed (Perelson *et al.*, 1997; Perelson *et al.*, 1996). The reservoir of latently infected cells in lymphoid tissues (LT) is, however, not readily eliminated (Chun *et al.*, 1997; Finzi *et al.*, 1997; Wong *et al.*, 1997). The effects of HAART are profound even in these tissues, with a >99.9 percent clearance of virus from LT 6 months after potent therapy and a reversal of the pathological changes in the LT architecture (Cavert *et al.*, 1997; Haase, 1999; Zhang *et al.*, 1999).

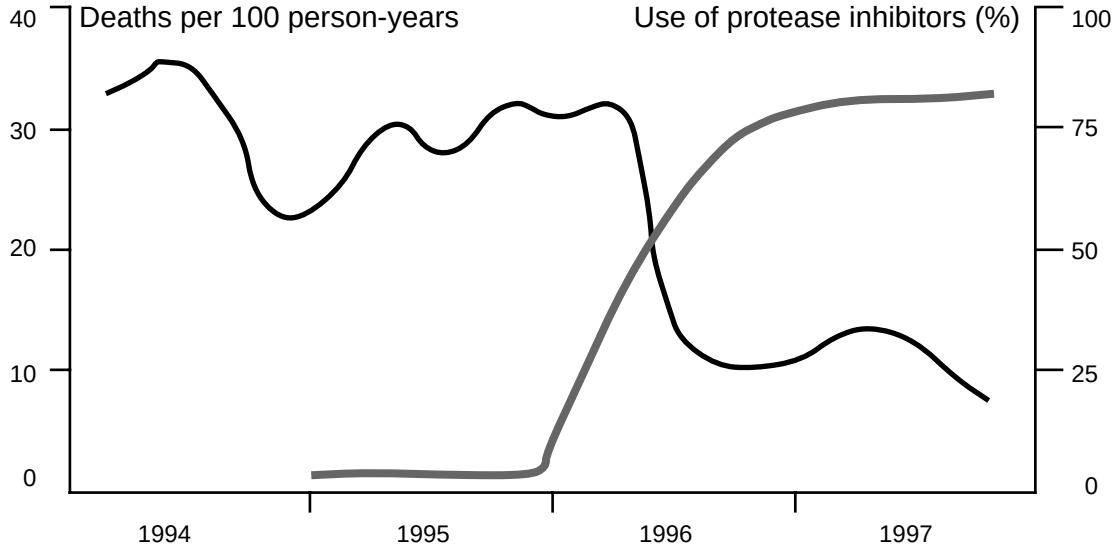


Figure 6. **The use of combination antiretroviral therapy** including a protease inhibitor significantly reduced the death rates in HIV infected patients with CD4 counts below 100 x10^6/l (adopted from Palella *et al.*, 1998).

However, the reduction of antigenic material available to the immune system also reduces the HIV specific T cell repertoire (Ogg *et al*., 1999; Spiegel *et al*., 1999; Wu *et al*., 1999). HIV specific B cell responses, as well as B cell hyperreactivity as seen by hypergammaglobulinemia, also decline after HAART (Morris *et al*., 1998) (Figure 5).

Is the clinical benefit of HAART solely due to reduction of virus replication? The reduction of HIV specific antiviral T and B cell immunity during HAART could also reduce autoimmune reactivities in analogy to the hypothesis of an autoreactive component to HIV pathogenesis (paper IV), (Harrer *et al*., 1993; Zinkernagel, 1995). Thus, although immune-based interventions aiming at restoring the HIV specific immunity are especially attractive during HAART, the approach should be cautious. Monitoring of autoimmune reactivities during immune-based therapy is important so that additional autoimmunity is not induced (Paper IV).

The process of mature CD4+ cell repopulation is impaired during HIV progression, largely because the chronic immune stimulation leads to defects in antigen-specific CD4+ T cell activation and proliferation. The initiation of highly active antiretroviral therapy (HAART) in advanced disease partly reverses the process of HIV-induced CD4+ T cell depletion. Autran has shown that T cell reconstitution after HAART occurs in three phases, with an early rise in CD4+ memory cells, a reduction in T cell activation, and a late rise in naïve CD4+ lymphocytes with decline of CD8+ cells. However, CD8+ counts still remain higher than normal and CD4+ counts remain below normal. Better reversibility should be achieved with therapeutic interventions initiated earlier in the disease and with optimal combination regimens (Autran *et al*., 1997). A rationale for early initiation of aggressive therapy has been presented. It is now recommended that HAART is initiated in all patients with a virus load exceeding 5-10000 copies/ml regardless of CD4+ counts or in patients with CD4+ counts below $500x10^6$/l (Carpenter *et al*., 1997). The prognosis and the effectiveness of therapy are monitored with plasma viral load during HAART treatment. The viral load is a better predictor of progression to AIDS and death than the number of CD4+ T cells (Mellors *et al*., 1996).

Immune-based therapy

Alteration of the immune response during HIV-1 infection is possible through a number of ways. Passive immunotherapy with HIV-1 neutralizing mouse monoclonal antibodies can stimulate T cell proliferative responses, decrease the level of p24 antigen in serum and improve CD4 counts (Hinkula *et al*., 1994). The addition of immunostimulatory drugs like interleukin 2 (IL-2) has been tested in clinical trials but the toxicity of the drugs and the induction of increased plasma viral load have been a major drawback in these studies (Kovacs *et al*., 1995). However, when the dose was optimized, adverse events decreased and IL-2 induced sustained increases in the amount of CD4+ cells with no increase in viremia. Still, the induction of new CD4+ lymphocytes needs to be further characterized with analysis of the CD4+ cell function during IL-2 therapy (Kovacs *et al*., 1996). Adoptive transfer by infusion of autologous HIV-specific CTL expanded in vitro to HIV+ patients resulted in no significant changes in CD4 and CD8 lymphocyte counts and virus load (Tan *et al*., 1999). This contrasts with the success of CTL therapy in treating or preventing Epstein-Barr virus and cytomegalovirus disease after bone marrow transplantation (BMT) and could be due to rapid apoptosis of transferred CTL clones.

Vaccines

The early history of the use of vaccines in different forms did not begin with Edward Jenner. Indian monks drank snake venom as early as the 7th century to become immune to its effects. Chinese medical texts from 1695 describe inoculations against smallpox in China. These Chinese texts describe both intranasal and oral routes for inoculation. However, the first

scientific work in the use of variolation was that of Edward Jenner (Jenner, 1798). Extensive efforts since Jenners discoveries have led to the effective control of these ten major infectious diseases: smallpox, diphtheria, tetanus, yellow fever, pertussis, *Haemophilus influenzae* type b disease, poliomyelitis, measles, mumps and rubella. Smallpox is now completely eradicated and WHO has targeted poliomyelitis for eradication by the year 2000. The development of effective preventive vaccines also seems to be the best hope of stopping the catastrophic HIV epidemic.

The hypothesis that an inadequate immune response generated by natural infection leads to viral persistence supports the rationale of using a therapeutic vaccine in HIV-1 infection. Several immunization strategies have met with rather disappointing results using subunit formulations. However, recently published results with subunit HIV-1 rgp160 immunization, showed an effective induction of T cell immune responses without severe adverse effects. The vaccine group had fewer deaths after two years but not at the end of the trial (Sandström and Wahren, 1999). Combining optimal antiretroviral therapy with immune-based therapy is now recommended for further studies.

DNA vaccines

Plasmid DNA genetic vaccination using expression vectors, leading to induction of an immune response, is a rapidly growing field of research. Protein is expressed in vivo following introduction of cDNA expression cassettes (Dubensky *et al*., 1984; Wolff *et al*., 1990), resulting in the development of specific immune responses to the expressed protein (Raz *et al*., 1993; Tang *et al*., 1992; Tighe *et al*., 1998; Ulmer *et al*., 1993).

Genetic vaccination has now been demonstrated to induce protective immune responses against viral (Bourne *et al*., 1996; Boyer *et al*., 1997; Cox *et al*., 1993; Donnelly *et al*., 1996; Kuhober *et al*., 1996; Ulmer *et al*., 1993; Xiang *et al*., 1994), bacterial (Huygen *et al*., 1996; Luke *et al*., 1997; Tascon *et al*., 1996) and parasitic (Sedegah *et al*., 1994) diseases in many different species. Moreover, DNA inoculation was considered for treatment of several conditions and diseases, including autoimmune disease (Ugen *et al*., 1994). The advantages of using DNA vaccines over both subunit vaccines and live attenuated virus vaccines include expression of the relevant antigen in vivo without the use of adjuvants or live agents and the simplicity and low cost of developing and producing the vaccine. This results in the production of an effective immunogen without the safety considerations associated with live attenuated vaccines. Moreover, sufficient quantities of plasmid DNA can be produced by simple bacterial fermentation and purification techniques, thereby avoiding many of the complications and expense associated with the production of purified recombinant proteins.

Studies on the fate of plasmid DNA after application *in vivo* and the origin of the immunogens have focused on systemic administration, especially the intramuscular (i.m.) route (Donnelly *et al*., 1997). Myocytes appear to be the predominant cell type transfected during i.m. injection of plasmid DNA and the resulting transcription and translation of the gene occur in the muscle cells. These myocytes act as an enduring source of plasmid encoded protein, but it appears that the cells may not be the primary inducers of immune responses. Removal of the injected sites after inoculation has little effect on the level or nature of immunity. Indeed, it seems that dendritic cells transfected at the injection site and migrating subsequently to draining lymph nodes (DLN) are the principal source of immunogenic materials (Akbari *et al*., 1999; Casares *et al*., 1997; Condon *et al*., 1996; Klinman *et al*., 1998; Porgador *et al*., 1998). The encoded protein is presented to the immune system by antigen-presenting cells (APCs) in the context of MHC class I and class II.

Thus, DNA immunization mimics live attenuated vaccines by producing the encoded protein endogenously and allowing for the proper processing and folding of the gene product into a native structure. However, the mechanism of antigen presentation during DNA vaccination is not fully understood. The induction of MHC class I–restricted CTL raises the question how the antigen is presented during i.m. injection of plasmid DNA. The ability of secreted proteins to be endocytosed and to enter the pathway of antigen processing that ultimately leads to presentation via MHC class II molecules is well known. The transfection of myocytes normally not expressing MHC class I should not elicit MHC class I–restricted CTL responses. Cooperative mechanisms of antigen processing may be of particular importance in immune responses induced by DNA immunization. There are several explanations for the phenomena by which MHC class I–restricted CTL might be induced after DNA vaccination (adopted from Donnelly *et al*., 1997):

shows that this form of plasmid administration results in good immune induction as well as immune modulation (Chun *et al*., 1998; Kuklin *et al*., 1997; Kuklin *et al*., 1998; Wang *et al*., 1997). Following mucosal inoculation, protein expression was observed in the lung and DLN, but it is not clear how the plasmid disseminates to such sites or how mucosal plasmid DNA delivery results in the systemic immune induction and the immunomodulatory effects observed. It is suggested that migrating APC, primed mucosally, subsequently reach DLN and there present the immunogenic materials with local and systemic immune induction as well as immunomodulatory effects (Akbari *et al*., 1999; Casares *et al*., 1997; Cohen *et al*., 1998; Condon *et al*., 1996; Klinman *et al*., 1998; Porgador *et al*., 1998).

DNA vaccine trials in humans have recently been initiated against HIV-1 and malaria. These preliminary studies have focused on safety and immunogenicity and specifically studied the

Suggested pathways for antigen presentation after DNA immunization

1. Antigen presentation mediated directly by transfected myocytes.

2. Professional APCs becoming transfected and serving as APCs. This is the situation in mucosal, subcutaneous and epidermal application of plasmid DNA.

3. Cross priming. Transfer of antigen from transfected myocytes to professional APCs. The ability of antigens produced by non-APCs to prime CTL in the context of MHC molecules present only on professional APCs. In this hypothesis, muscle cells act primarily as factories for the production of antigen, which is then transferred to a bone marrow–derived professional APC to be processed and presented to CTL precursors for the induction of CTL.

In contrast to the i.m. route for DNA vaccination, mucosal, subcutaneous and particle mediated gene gun immunizations directly transfect professional APCs that can present the antigen in both class I and II pathways. Recent work on mucosal delivery of plasmid DNA

induction of vaccine specific cellular immune responses (Calarota *et al*., 1998; Calarota *et al*., 1999; MacGregor *et al*., 1998; Wang *et al*., 1998a). In paper VI we extended these studies to evaluate mucosal vaccination in HIV-1 infected individuals.

Bacterial DNA differs from vertebrate DNA in the frequency and methylation of CpG dinucleotides. In the DNA plasmid, presence of CpG motifs that contain a dinucleotide in a particular base context have immunostimulatory activity (Klinman *et al.*, 1997; Pisetsky, 1996; Sato *et al.*, 1996). The stimulatory effect was mapped to the CpG motif flanked by two 5' purines and two 3' pyrimidines. This sequence was shown to induce polyclonal B cell activation (Klinman *et al.*, 1996; Krieg *et al.*, 1995). Induction of T helper-1 (Th1) responses has also been proposed for certain short bacterial immunostimulatory DNA sequences (Roman *et al.*, 1997; Tighe *et al.*, 1998).

The CpG motifs in bacterial DNA induce a variety of immune effects, including polyclonal activation of murine and human B cells and IL-6 secretion. It was also proposed that CpG motifs would contribute to the development of systemic lupus erythematosus (SLE) (Krieg, 1995). However, more recent work shows that bacterial CpG DNA does not trigger lupus in normal or lupus-predisposed animals. Moreover, CpGs could even decrease the disease severity (Gilkeson *et al.*, 1996; Mor *et al.*, 1997). Thus, it was concluded that DNA plasmid immunization neither triggers autoimmunity nor accelerates the development of systemic autoimmunity (Mor *et al.*, 1997).

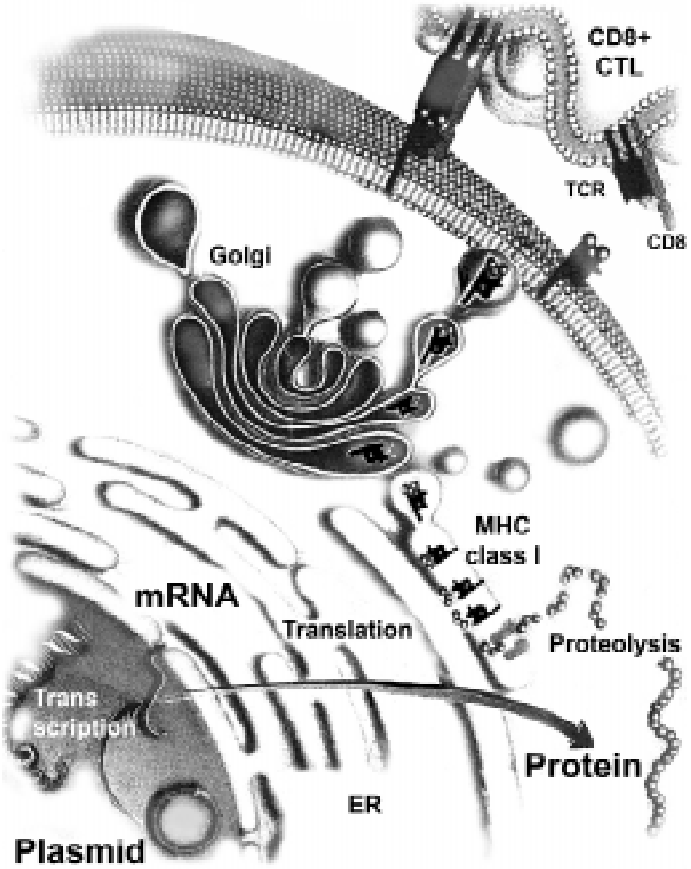


Figure 7. Plasmid DNA is transcribed in the nucleus and mRNA translated in the endoplasmatic reticulum (ER). Intracellular proteases cleave the protein and the antigen is associated with, and presented by, MHC class I on the surface of the cell. The events mimic the situation with an intracellular parasitic infection and induce a Th1 type of response.

Safety of DNA vaccines

The long-term safety concerns about plasmid DNA have become more significant as the uses for plasmid DNA have expanded from diseases such as HIV infection and cancer, to ordinary vaccination of the normal adult or pediatric population. DNA vaccines pose novel theoretical safety concerns apart for the normal pharmaceutical testing for toxicity associated with expression of the encoded protein or the plasmid itself. The key areas of concern include the potential integration of the plasmid DNA into the genome of host cells; the potential induction of immune tolerance or of autoimmunity; and the potential induction of anti-DNA antibodies.

Integration of injected plasmid DNA into the host cell genome may be silent. Potential mutagenic effects could arise if the integrations disrupt a functional cellular gene, or carcinogenic if they affect a regulatory gene for cell division, or activate an oncogene. Direct studies of integration in mice injected i.m. with plasmid DNA have not detected integration of the injected plasmid thus far. The calculated rate of mutation would be 3 orders of magnitude less (Nichols *et al*., 1995), or 3000 times less (Martin *et al*., 1999), than the spontaneous mutation rate for mammalian genomes. This level of integration, should it occur, is not considered to pose a significant safety concern.

Induction of immune tolerance or of autoimmunity has not generally been seen during DNA vaccination and it is not yet known whether DNA vaccines will induce anti-DNA antibodies in humans. An exception is the transient induction of anti-DNA antibodies by RT-DNA. These antibodies come and go, and their existence were ascribed to the nucleotide-binding properties of the RT-gene. However, preclinical animal studies suggest that induction of anti-DNA antibodies is unlikely (Donnelly *et al*., 1997).

AIMS OF THE STUDY

The aim of the study was to analyze immune and autoimmune responses in HIV-1 infection and during protein and genetic immunization and specifically to:

1. Analyse the efficacy of different mucosal routes for genetic immunization in mice and humans for induction of local mucosal immune responses

2. Study the IgG subclass dysfunction in HIV infection and HIV related lymphoma

MATERIALS

Papers I-III

Sample collections

Four- to twelve-week-old mice (C57BL/6 x DBA/2) F1 (H2b/d) (Hinkula *et al*., 1997), in groups of 8 to 14, were used. Serum was collected from blood at 7-10 days post-immunization by retro-orbital puncture. Intestinal secretions were collected from mice immediately after sacrifice by flushing a 10 cm piece of the small intestine with 2 ml of PBS containing 1 mg/ml trypsin-chymotrypsin inhibitor (Sigma). Vaginal samples were collected by washing the vaginal cavity with 50µl of phosphate-buffered saline (PBS) (Asakura *et al*., 1997). Fecal pellets were weighed and dissolved in PBS at a concentration of 10% w/v. Lung washings were collected by introducing plastic tubing into the trachea and washing the lung tissues with 1ml of PBS containing 1 mg ml-1 trypsin-chymotrypsin inhibitor (Lowell *et al*., 1997). All the samples were immediately centrifuged at 450 x g for 20 min.

Plasmid DNA

The vector backbones consisted of pUC8-derived plasmids controlled by the major immediate-early human cytomegalo virus (CMV) promoter (Boshart *et al*., 1985). Plasmid DNAs containing HIV-1_{HXB2} *tat* (544 nt, GenBank accession numbers K03455 and m19921), *rev* (356 nt, (Mermer *et al*., 1990)), $p37^{gag}$ (p24), HIV-1_{Bru} gp160, and HIV-1_{HXB3} *nef* (1244 nt including long terminal repeat (LTR), GenBank accession numbers m14100 and x03188) were used. The plasmids were purified in Qiagen Giga-Plasmid columns (Qiagen, Chatsworth, CA) and the stability of the DNA was evaluated by electrophoresis in 0.7% TBE-agarose gel prior to immunization. The constructs were not designed to secrete antigens into the extracellular space.

Papers IV-VI

Study subjects and immunizations, paper IV

Forty HIV-1 infected asymptomatic individuals were included in the study. All had a CD4 cell count above 400 ($x10^6$/l) and none had previously received antiviral treatment. Twenty-one individuals were tissue-typed as HLA A2 (Vartdal *et al*., 1987). HIV-1 IIIB/LAV derived recombinant gp160 (VaxSyn, MicroGeneSys, Meriden, CT), expressed in a baculovirus system and adsorbed to aluminum phosphate as adjuvant, was given in six 160 µg doses administered i.m. at 0, 1, 4, 8, 17 and 26 weeks.

Study subjects paper V

Serum samples from 12 HIV-1 infected patients diagnosed with lymphoma, 15 CDC group A (asymptomatic) patients and 11 patients in CDC groups B and C (symptomatic) were collected and stored frozen at -70°C prior to examination. Nine lymphomas were highly malignant, two were widespread subcutaneous lymphomas and one was of Hodgkin's mixed type. One was located intracerebrally, one rectally, two in peritoneal lymph nodes and the remaining were generalized or located in peripheral lymph nodes.

Study subjects paper VI

Eight HIV-1 infected individuals previously enrolled in a DNA vaccine trial with single plasmid immunizations were included in the study (Calarota *et al*., 1998). DNA immunization was initiated with intramuscular (i.m.) vaccination followed by intraoral (i.o.) jet-injection. The third immunization was i.m. in four individuals and i.o. in four.

Oral samples

One to three ml of resting saliva was collected on ice and immediately frozen and stored at –70°C as described in paper VI. The OraSure™ collector exerts osmotic pressure at the mucosal surface and thus collects oral mucosal transudate (OMT).

The Omni-SAL device collects resting saliva as described in paper VI (Hodinka *et al.*, 1998). The volume of the eluate (V_E) was measured and the content of oral fluid (V_{OMT} for the OraSure and V_{SALIVA} for the OmniSAL) was determined.

METHODS

Immunization

5-10 μg of each DNA plasmid dissolved in 50 μl PBS or distilled water was applied by different routes of delivery.

For intranasal application of DNA, non-anaesthetized animals were held by hand and the DNA solution (20 μl) was applied at the opening of the nostrils. The administered solution was inhaled spontaneously.

The technique of administering anaesthetic solutions by high-pressure jet-injection is a standard procedure in pediatric dentistry. Our jet injector (SyriJet markII, Mizzy Inc., NJ) was designed for the application of dental anaesthetic solutions in routine dental treatment. The plasmid solution (15 μl) was injected intraorally in a lateral direction, aiming at the buccal mucosa. The injection was confirmed by the observation of a distinct exterior swelling of the bucca.

For gene gun DNA immunization, plasmids were precipitated on gold beads with 2.5 M $CaCl_2$ and 100 μg/ml spermidine and coated into plastic tubes as previously described (Eisenbraun *et al.*, 1993; Fuller and Haynes, 1994; Fynan *et al.*, 1993). The particles were applied with the helium pulsed Accell device (Geniva Inc. Middletown, WI) to the area including the animal's oral cavity (paper II) or the epidermis of the abdominal skin (papers I and III).

Intramuscular injections were given to both quadriceps muscles by injecting a total of 20 μg per construct of DNA with a 28-gauge needle. Oral intramuscular injections were placed in the tongue of the mice (paper II).

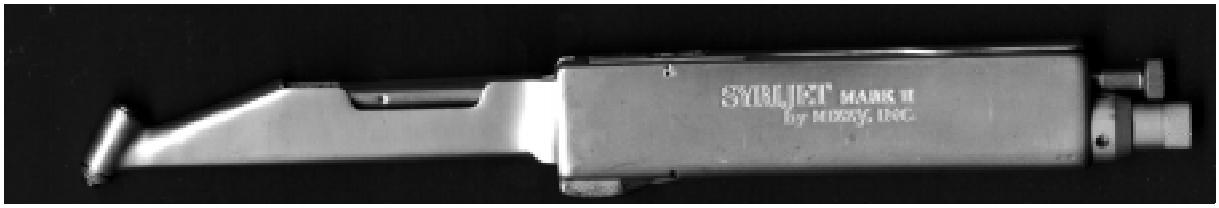


Figure 8. **The SyriJet MarkII Jet-injector.** The device is capable of delivering pharmaceuticals, i.e. local anaesthetics, in a volume of 0.05 to 0.2 ml. We evaluated the device for the delivery of mucosal plasmid DNA vaccination. The jet-injection technique elicited mucosal and systemic immune responses in preclinical (papers I and II) and human trials (paper VI).

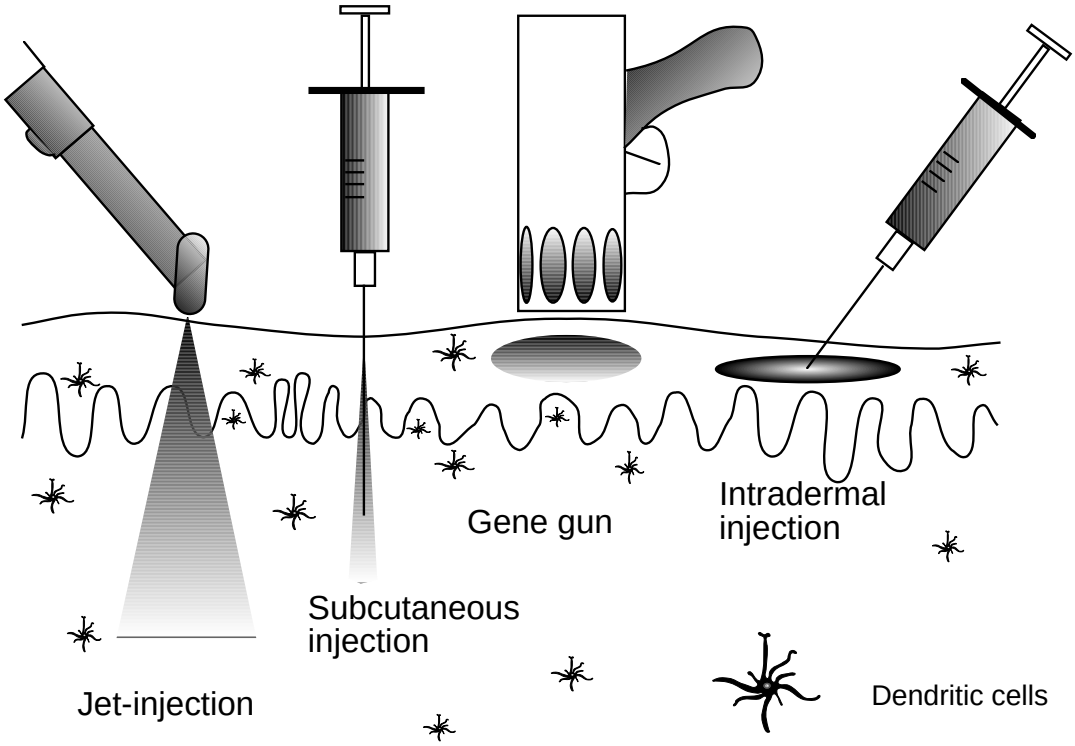


Figure 9. **Theoretical model of the different routes of plasmid DNA application in the skin and mucosa.** Depending on the device used for injection, the plasmid will penetrate into the tissues and transfect cells at different depths.

Purification of IgA

Mucosal and serum IgA and IgG antibodies were purified and analyzed for specific binding to recombinant antigens and peptides in enzyme linked immunoassay (ELISA). The IgA fraction was purified with Jaqualine (Vector Laboratories, Inc. Burlingame, CA, USA) and quantified by ELISA (Johansen and Svensson, 1997). It was used for a total IgA quantification as well as determination of specific IgA reactivity.

Lymphocyte proliferation assays and cytokines

Blood was collected at each visit and fresh PBMC were used for the proliferation assays as described (Leandersson *et al*., 1998; Wahren *et al*., 1994). The stimulation index (SI) was calculated for each sample by dividing the mean radioactivity (c. p. m.) of each antigen in triplicate by the mean medium c. p. m.

Mucosal cytokines in OMT were quantified in samples from both sides of the oral cavity, using commercial kits for Interferon-gamma (IFN-gamma), Interleukin-2 (IL-2), IL-4 and IL-10 (Quantikine™, R&D systems, Oxon, UK).

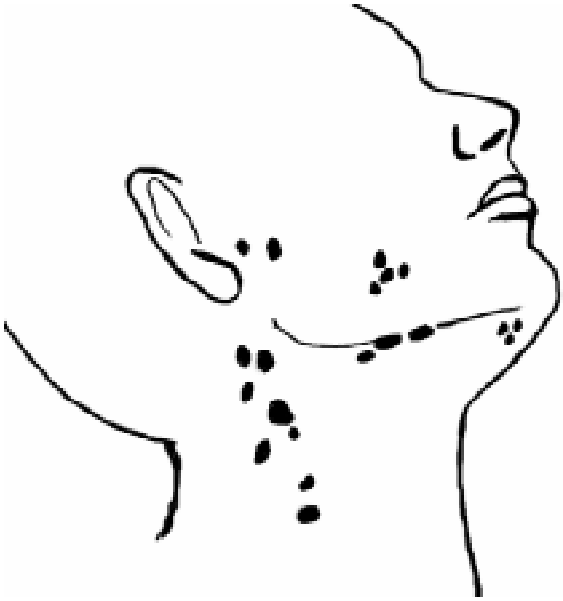
Oral biopsies

Oral mucosal biopsies were collected 48 hours after final oral jet-injection of plasmid DNA. The mucosa was anaesthetized by infiltration of 1.8 ml Xylocain/adrenalin (Lidocain 20 mg/ml formulated with adrenaline 12.5 µg/ml, Astra, Södertälje, Sweden) and 5mm punch biopsies with a depth of 3-5 mm were taken at the location of the jet-injection.

RESULTS AND DISCUSSION

Mucosal induction of immune responses by plasmid DNA

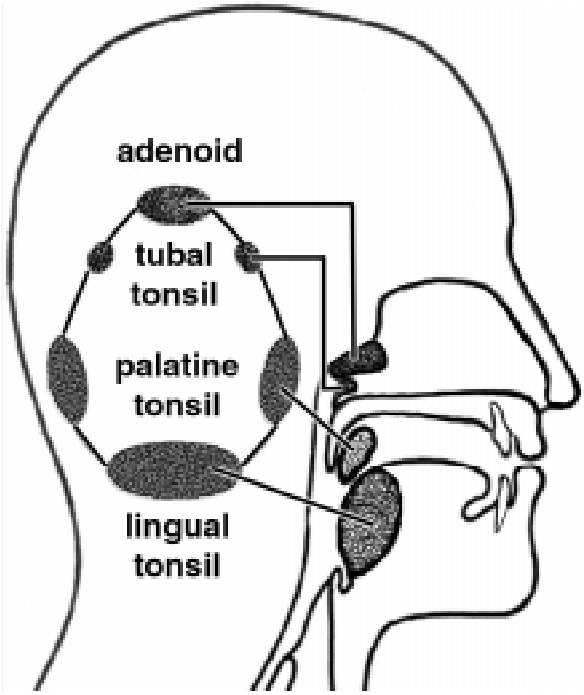
Mucosal infections of the vagina and rectum are two of the major pathways through which HIV currently disseminates. The architecture of the mucosal immune system includes the tonsils, adenoids and nasopharyngeal-associated lymphoreticular tissue (NALT) found in the oral cavity. NALT serves to provide inductive sites for the upper respiratory tract and the naso-oral cavity. The gastro-intestinal tract (GI; Peyer's patches and the appendix) and local, solitary lymph nodes build the gut-associated lymphoreticular tissue (GALT). Both NALT and GALT are inductive regions where foreign antigens derived from viruses, bacteria and yeast are encountered. In the salivary and other exocrine glands, the cells are stimulated to produce immunoglobulins of the IgA isotype (Ogra *et al*., 1999).



Regional draining lymph nodes (DLN)

Regional immunity

The rationale for measuring local mucosal responses during mucosal vaccinations is motivated by the concept of the common mucosal immune system. Infection at one site could lead to protection at that site and other mucosal sites (Rudzik *et al*., 1975). There are several characteristic features of the mucosal immune system. The most important one is the formation and secretion of secretory IgA (sIgA). Dimeric IgA from local plasma cells in the exocrine glands, binds to the polymeric Ig receptor (pIgR) on the basolateral surface of the epithelial cell in the mucous membrane. The dimeric IgA/pIgR complex is endocytosed and the pIgR cleaved off with a remaining portion left in the complex, now named the secretory component (sc). The dimeric IgA and sc form sIgA that is secreted into the lumen. This sIgA is the first line of defense against invading microorganisms. Viral resistance was shown to be mediated by sIgA on the surface of the mucous membranes (Renegar, 1992; Renegar and Small, 1991). Additionally, neutralization of viruses can be achieved during the transit of sIgA through the cells (Mazanec *et al*., 1995; Mazanec *et al*., 1992).



Nasopharyngeal-associated lymphoreticular tissue (NALT)

Figure 10. **Regional draining lymph nodes (DLN) for the oral cavity (top) and NALT tonsil tissue (bottom).** Systemic immune responses during mucosal immunization with jet-injection could arise by transfection of Langerhans cells in the mucosa and migration to NALT or DLN.

Route of vaccine application in preclinical studies

We have attempted to evaluate the effects of various devices and routes for application of plasmid DNA vaccines to induce local mucosal immune responses. By combining plasmids containing the HIV-1 genes rev, nef, tat, p24 and gp160 in a vaccine to elicit HIV-1 mucosal and systemic responses we found that the mixture of plasmids induced specific immune responses to a higher degree than previous studies utilizing single plasmid immunizations. The method of combining different plasmids was established in paper I, and applied for the further studies on plasmid immunizations.

Mucosal immunization of mice by jet-injection was efficient in inducing mucosal and systemic IgA antibodies and preferably systemic IgG2a, suggesting a Th1 type of immune response. Administration of the vaccines by the intranasal route efficiently induced high titres of systemic IgG. Gene gun immunization mediated induction of the Th2 isotype, IgG1. Responses after gene gun immunizations seldom induced systemic IgA and mucosal antibodies (Table 3, Papers I-III). Although previous reports suggested that intranasal application was less efficacious for induction of systemic responses than other routes (Fynan *et al*., 1993; Kuklin *et al*., 1997), our results indicate that intranasal instillation of plasmid DNA efficiently induces systemic and mucosal immune responses in mice. This discrepancy is unexplained but could reflect different techniques of administration of the immunogen. It remains to be determined where the uptake of DNA occurs and to what extent intranasal application delivers the DNA into the lower airways. When the DNA solution enters the nostrils of mice, it irritates the mucous membrane in the nasal cavity and provokes the animal to sneeze. The proportion of the DNA that enters the lungs of the animal has yet to be determined. Our preliminary experiments with India ink suggest that the major site of administration is local nasal-oral, not in the lower airways. When the localization was examined, no ink could be detected in the lung tissue (paper II).

In many respects, *in vivo* introduction of plasmids and the apparent production of the corresponding antigens, here demonstrated by the induction of specific immune responses, resemble the situation in natural infection. A particularly frequent IgG immune response with high titers was evident in the animals immunized intranasally, with almost all animals responding to the gene products of the plasmid preparations. Although the i.m. route elicited poor primary humoral responses (paper III), these evolved during boost immunizations (paper II). During the course of immunizations, the intranasal route gave strong and persistent IgG reactivities after a single dose for the tat and p24 DNA compared to gp160, where high levels developed after three immunizations. A substantial portion of the serum IgG may be derived from spleen cells during intranasal immunization, as a high frequency of these mice developed *in vitro* B cell responses. Thus, intra-nasal immunization not only triggers mucosal humoral responses but perhaps even stronger systemic antibody responses. Systemic IgA was induced by jet-injection, intranasal administration and intramuscular injection. Oral gene gun immunizations did not elicit systemic IgA.

In vitro antigen stimulation of spleen cells revealed additional reactive mice among the non-responders (Papers I-III). Tat, gp160 and p24 induced the major reactivity in the IgG B cell stimulation assay. Rev and p24 elicited measurable IgA secretion during B cell stimulation. Intranasal immunization exhibited the highest total B cell response frequency, followed by i.m. and local oral jet-injection immunization.

The demonstration of variations in the immune response following mucosal DNA immunization by different routes has implications in further vaccine experiments. The present work introduces a novel way of DNA immunization, i.e. jet-injection, with promising results for induction of mucosal IgA antibodies and Th1 type of immunity. The immunization procedure has several practical advantages over traditional vaccine delivery.

Jet-injection in human mucosa

The results from the preclinical research led us to move the jet-injector into clinical tests in HIV-1 infected individuals (Table 3). In these trials we found indications of a Th1 type of response following intraoral plasmid DNA vaccination with a combination of HIV-1 genes encoding the Nef, Rev and Tat gene products. Analysis of OMT revealed high levels of the cytokine IL-2 in the mucosa and immunohistochemical analysis indicated a DTH like picture. By jet-injection we also induced systemic T cell proliferative responses to the plasmid products comparable to that of i.m. vaccination. However, specific mucosal antibody production was not triggered to the same degree. No serious adverse effects were observed after jet-injection or i.m. injection. One of eight patients exhibited a clinically visible local reaction on one occasion and two patients had mild anamnestic symptoms that were not verified clinically. However, jet-injection was a fast and safe technique in preclinical and clinical tests. It can easily be applied in large-scale vaccination trials. There are several jet delivery devices on the market designed for various routes and injection procedures.

Further evaluation is in progress of the possible immunological effects that the high-pressure jet-injection might have on the recruitment of immunologically competent cells. The spread of DNA into different tissues after injection is also of interest (Figure 9 and 10). These results demonstrate that, in addition to the site, the inoculation method has implications for the mucosal immune response following DNA vaccination. The injected material spread in the tissues according to the law of least resistance. In humans (Paper VI), the orifice of the Syrijet is aimed towards the mandibular bone. Thus, we can expect a lateral spread of the injected material along the mandibular periosteum. Tonsil and regional lymph nodes are also not far from the injection site and an induction of HIV specific Th1 immunity in the lymphoid tissues could target the persisting pool of HIV infected cells within the lymphoid tissues during HAART (Andersson *et al*., 1998).

Table 3. Overview of HIV specific responses in DNA immunization

Major responses in non-infected individuals, preclinical studies ¶

Preclinical Naive mice	Mucosal IgA and IgG	Systemic IgA and IgG	T helper isotypes	Cell mediated responses
Mucosa, jet-injection	+++	++	Th1	++
Mucosa, intranasal	++	+++	Th1/Th2	+++
Intramuscular	+	+++	Th1/Th2	++
Intradermal, gene-gun	+/-	++	Th2	+

¶*From papers I, II, III*

Major responses in infected individuals ¶

Clinical trial HIV+ human	Mucosal and systemic IgA and IgG	Local response	Mucosal IL-2 cytokines	Cell mediated responses
Mucosa, jet-injection	+/-	DTH Th1	++++	+++
Intramuscular	+/-	NA	+	+++

¶*From paper VI* NA; not applicable

Jet-injection of healthy and infected individuals induces immune responses preferentially of a Th1 type. The antigen specificity of the response in humans will have to be further verified by studies of local reactivity in healthy and non-immunized HIV infected individuals. The HIV-1 specific systemic T cell responses observed following mucosal jet-injection could be due to efficient drainage to regional lymph nodes in the area of injection. Most notably, submandibular and cervical lymph nodes but also tonsils are in close proximity to the injection site (Figure 10).

The apparent recruitment of dendritic cells to the injection site would be a possible pathway for successful systemic immune responses. In future experiments, which are ongoing, we wish to further evaluate the jet-injection technique at mucosal sites of humans. The feasibility, with the Syrijet, of getting direct access to several mucosal tissues and the spread of the injected material in the tissues may be advantages over other delivery systems.

Local mucosal immunoglobulins in non-infected animals

The induction of HIV-1 specific mucosal IgA in secretions was measured from several sites: lungs, intestines and vaginal washings in mice (papers II and III). Primary responses with anti-p24 fecal and lung IgA antibodies, were efficiently induced by both intranasal and gene gun application but not by i.m. inoculation. Few mice responded with vaginal HIV-specific IgA secretions after one inoculation, irrespective of the route of DNA-delivery (paper III). Oral jet-injection of DNA induced the highest mucosal IgA content in both lung lavage and intestinal washings as well as good systemic IgA. Thus, the intraoral jet-injection might affect the type of immune response mounted. Gene gun immunizations with a poor systemic IgA response elicited p24 specific mucosal IgA in intestinal samples. The p24 plasmid mounted a strong IgA response in the lung lavage compared to the response in intestine. The same was true for the gp160 plasmid. IgA and IgG antibodies in mucosal washes were not correlated with serum antibody responses, suggesting the contribution of locally synthesized antibodies of these isotypes. This finding is supported by the concept of a common mucosal immune system (Ogra *et al*., 1999) and previously shown with bacterial protein antigen immunization (Russell *et al*., 1996).

Specific antibodies and IgG2a/ IgG1 ratios

Repeated preclinical observations indicate that both gene gun-based immunization and intranasal application induce primary systemic humoral immune responses significantly better than intramuscular (i.m.) injection. IgG subclass analysis of IgG2a/IgG1 ratios indicates a predominance for Th2 type of responses for these routes (Figure 11), although less prominent after intranasal application which also could induce Th1 subclass ratios. I.m. injection preferentially induced a Th1 type of response in the subclass analysis. These results were comparable in anti-p24 and anti-Nef specific subclass analysis. However, with a different antigen and by repeated DNA immunizations (paper II), i.m. injections induced a Th2-like subclass profile to the gp160 antigen (Figure 11). During repeated immunizations, i.m. injections induced increasing titers of antibodies in more animals, possibly indicating a switch from a Th1 to a Th2 dominated immune response. Intranasal application, on the other hand, developed to a more Th1 like response during repeated immunizations. Finally, the gene gun route consistently induced a Th2 type of response both after prime and boost immunizations. These discrepancies could result from the use of different plasmids and antigens, but could also be caused by a maturation of the immune responses during repeated DNA immunizations (Figure 11).

Given that neither of our DNA vaccine constructs were designed to secrete the antigens into the extracellular space, our observations concerning the immunoglobulin subclass profiles are compatible with previous findings (Pertmer *et al*., 1996). The Th1 dominated re-

sponse after jet-injection, compared to gene gun inoculation, is to some extent in concordance with previous findings (Feltquate *et al*., 1997). They showed that saline-DNA injection predominantly raised a Th1 type of response with mostly IgG2a anti-haemagglutin antibody, while gene gun DNA immunization elicited a predominantly Th2 type of response, with mostly IgG1 antibodies. Our gene gun-based immunization gave a Th2 type of response compared to other parenteral routes of DNA delivery and this has been demonstrated in at least five different antigen systems (Feltquate *et al*., 1997; Fuller and Haynes, 1994; Pertmer *et al*., 1996). In addition, IgG1 secretion was predominant when the DNA vaccine was delivered by intranasal application (Asakura *et al*., 1997). Our results during mucosal vaccination (paper II) have shown that intranasal immunization predominantly elicited IgG2a antibodies as well, and that intramuscular tongue injections gave systemic IgG1. Thus, we conclude that the site of application and the number of immunizations seem to influence the type of IgG subclass elicited after DNA immunization.

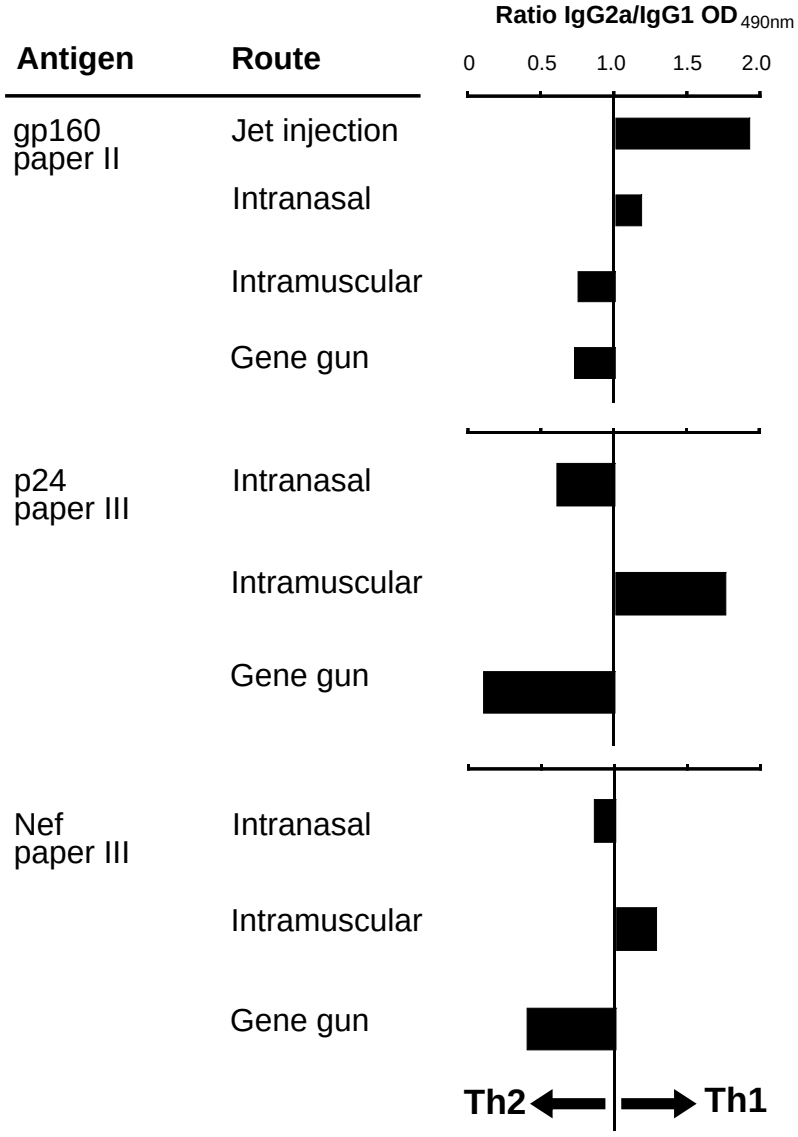


Figure 11. **Systemic IgG2a/IgG1 subclass ratios** to indicated antigens after plasmid DNA immunization by different routes. In paper II, gp160 subclass ratio was analyzed after three immunizations. In paper III, p24 and Nef specific IgG subclasses were measured after one immunization. A value above 1 indicates a Th1 response and a value below 1 indicates a Th2 response.

Oral cavity responses in infected individuals

Although transient specific salivary IgA and IgG antibodies reactive with the plasmid products were present throughout the study, the responses were not correlated to immunizations in humans (paper VI). Local mucosal responses induced by mucosal jet-injection in the oral cavity were further assessed by collecting oral mucosal transudate (OMT) at the site of the jet-injection. HIV infected controls as well as uninfected individuals exhibited significantly lower amounts of cytokines in mucosal transudate compared to immunized persons ($p<0.0001$).

Local production of cytokines was not induced by saline jet-injection alone. In contrast, high concentrations of IL-2 were found in OMT following i.m. prime and i.o. boost DNA vaccination. Intraoral jet-injection initiated a high concentration of IL-2 in the mucosal transudate that persisted for four months (Figure 12). The levels were higher on the right side compared to the control left side ($p<0.0001$). Low initial IL-2 production on the left side could be detected but did not persist to the end of the study. IFN-gamma, IL-4 and IL-10 were present at low concentrations, not correlated to immunizations or not detected at all in OMT samples.

Cell accumulation at the local site

Analysis of these biopsies taken two days post vaccination from the local site of immunization and the contralateral side was performed on formaline fixed tissue sections. The vaccinated side exhibited significantly higher frequencies of T cells, APCs and HLA DR expressing cells than the non-vaccinated side. However, the site could, theoretically, exhibit better responsiveness to additional immuni-zations, as indicated by a delayed type hyper-reactivity (DTH) like picture in microscopy analysis. The biopsies revealed a massive infiltration of mononucleated cells as well as granulocytes on the vaccinated side (right) compared to the control side (left) at various depths of the biopsy but most prominently just beneath the basal membrane.

The B cell marker CD20 and the plasma cell related antigen Syndecan 1 were expressed at low levels and comparably between the right and left side biopsies. Thus, immuno-histochemistry analysis points towards a strong vaccine induced, DTH-like inflammation dominated by both CD4+ and CD8+ T cells and granulocytes. Most of the T cells expressed the memory phenotype CD45 RO. HLA-DR expression and increased amounts of dendritic cells were also more frequent in the immunized tissue.

Cellular immune responses in DNA immunization

Repeated DNA immunizations have been suggested to influence the efficacy in inducing CTL responses (Fuller and Haynes, 1994; Pertmer *et al*., 1996). It seems that the intramuscular route of DNA delivery is an important determinant for the induction of CTL (Yasutomi *et al*., 1996). Intramuscular injection clearly induced the highest proportion of CTL responders with our plasmid expressing the HIV Nef protein. The magnitude of the CTL responses in paper III reflected the Th1 profile found by the subclass serology. However, as our findings indicate, further studies need to be conducted to elucidate the nature of the Th1/Th2 responses during mucosal DNA immunization and how these maturate during primary immune responses and boosting immunizations. Induction of CTL by plasmid DNA in humans was reported elsewhere (Calarota *et al*., 1998; Calarota *et al*., 1999).

In vitro stimulation of fresh PBMC with HIV Nef, Tat and Rev proteins was performed during immunizations in humans (paper VI). All patients had received immunizations with Nef, Tat or Rev alone and thus exhibited proliferative responses prior to the initiation of the immunizations with combined plasmids. All patients were negative before the first immunization with single plasmids (Calarota *et al*., 1998; Calarota *et al*., 1999). New specific T cell proliferative responses were induced by both i.m. needle injection and i.o. jet-injection. The level and timing of the mean peak of proliferative responses varied and the values were positive on >80% of the occasions.

T helper cell responses

Extracellular forms of pathogens, like bacterial infections, are often associated with humoral responses in which specific immunoglobulins are generated in an attempt to neutralize the foreign organism. Intracellular microorganisms, on the other hand, such as viruses and some types of bacteria and protozoans, elicit mostly cell-mediated cytolytic activity and the production of cytokines such as IFN-gamma and TNF.

Most pathogens are usually preferentially susceptible to one type of immune response. Thus, the response elicited during an infection will determine whether the host will be infected or resist the infectious agent as well as subsequent infections with the same pathogen. Ineffective response to the invading pathogen can lead to the spread of the infection, resulting in pathology and sometimes death. The challenge for new vaccines is to sensitize the hosts to respond with the correct type of immunity.

CD4+ T helper lymphocytes can be divided into two distinct effector cell subsets, based on functional capabilities and cytokine profiles. The introduced antigen influences the initial responses following infection by a pathogen. The mode of entry and tissue distribution will establish how the immunogen will encounter the relevant antigen-specific T cells.

T cell clones in mice with distinct functional abilities (Kim *et al*., 1985) and capacity to release unique profiles of cytokines associated either with inflammatory responses or with B cell help were identified in 1986 (Mosmann *et al*., 1986; Stout and Bottomly, 1989). Th1 CD4+ T cell clones were shown to secrete IL-2, IFNγ, and TNFα and mediate delayed-type hypersensitivity (DTH) responses (Cher and Mosmann, 1987). Th2 clones secreted the cytokines IL-4 and IL-5 and induced IgG1 and IgE production from B cells (Coffman *et al*., 1988; Killar *et al*., 1987). Cell-mediated and humoral responses tend not to overlap during infection and the two types of clones did not overlap either. Since the identification of Th1/Th2 clones, several additional cytokines have been defined. Th1 cells produce IFNγ and TNFβ, while Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13. The Th0 subset has also been defined with a mixed production of Th1 and Th2 cytokines (Firestein *et al*., 1989).

The cytokines promoting Th1 differentiation are IFNγ and IL-12. IL-4 is the dominant cytokine affecting priming for Th2 cells, although IL-10 also has been reported to promote the development of Th2 cells (Seder and Paul, 1994).

DNA vaccines support both Th1 and Th2 responses, depending on the route (i.m. gene gun, intradermal or mucosal) as well as on the vector protein (secreted or not). As opposed to classic vaccination, i.e. protein, the feasibility to modulate the immune response is a great advantage in DNA vaccine technology (Robinson and Torres, 1997).

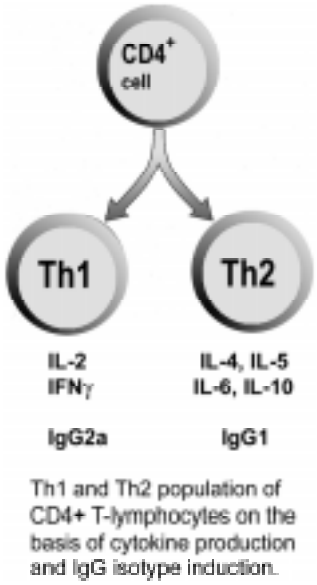


Figure 12. **Th1 and Th2 help**. The secretion of specific cytokines and the induction of IgG2a in mice characterize the Th1 type of immune responses. Th2 also has a specific pattern of cytokines and induces secretion of IgG1 in mice. It has been suggested that the Th1 type of help confers HIV specific immunity and that the shift to Th2 is a way for the virus to escape the immune response.

The fate of the plasmid

The plasmids were detected in mucosal biopsies two days post final i.o. jet-injection by PCR after digestion and DNA extraction. These studies were carried out in HIV infected humans (VI). Four individuals were PCR positive on the vaccine side. One individual was positive for the Rev plasmid also on the control side. The site of injection was primed with plasmid immunization four months earlier. Still, the probability of residual plasmid DNA in the tissue from the previous injection is unlikely (Paper VI).

IMMUNE DYSFUNCTION CONTRIBUTING TO AUTOIMMUNITY AND LYMPHOPROLIFERATIVE MALIGNANCIES

Lymphoproliferative disorders

In immunodeficiency, be it congenital, acquired or iatrogenic, malignancies and autoimmune disorders develop at a higher rate than expected in the immunocompetent host (Jonsson *et al*., 1999; Penn, 1994; Schaller, 1975).

Lymphoma associated with HIV-1 infection is most often a relatively late manifestation of disease (Beral *et al*., 1991; Feichtinger *et al*., 1992; Levine, 1992; Ziegler and McGrath, 1990). The lymphomas often carry the EBV genome and consist of high-grade, B cell tumors, with a predilection for extranodal involvement and short survival. In contrast to classical, or EBV negative, lymphomatous malignancies, HIV-1 associated lymphomas often have an aggressive clinical course and a rapidly fatal outcome (Knowles *et al*., 1988; Levine, 1994).

The role of cytokines

IL-10, produced in normal monocytes following *in vitro* gp120 treatment, could exert an inhibitory effect on CD4+ and CD8+ proliferation (Schols and De Clercq, 1996). In addition to its growth-promoting and differentiating activities on human B cells (Briere *et al*., 1994), IL-10 is reported to act as a switch factor for IgG1 and IgG3. The study (paper V) was undertaken to analyze possible immunological imbalances in HIV-infected individuals with lymphoma regarding the IgG subclass distribution to previously defined epitopes of HIV-1 and EBV proteins.

HIV specific IgG subclasses in HIV-related lymphoma

IgG1 is the dominant subclass in anti-HIV antibody responses and is elicited in response to protein antigens together with IgG3. In general, IgG3 was significantly decreased in individuals with low CD4 counts and lymphoma patients often had lower IgG3 compared to the symptomatic patients. Antibodies reactive to the V3 region have been correlated to neutralization of HIV-1 infectivity and probably interfer with coreceptor binding (Figure 4) (Broliden *et al*., 1992; Cocchi *et al*., 1996; Javaherian *et al*., 1990). For the lymphoma patients, subclass reactivity to the V3 peptide was restricted to subclasses IgG1 and IgG3. Compared with symptomatic patients, the lymphoma patients exhibited significantly lower titers of IgG3 (P=0.005 Mann-Whitney U-test). Additionally, IgG3 titers were significantly lower (P=0.01 Mann-Whitney U-test) in the lymphoma group compared to the symptomatic group to the gp41 conserved immunodominant epitope, previously reported to be IgG1 and IgG2 restricted (Chiodi *et al*., 1989). IgG4 typically follows repeated antigenic stimulation. HIV-specific IgG4 reactivity was seen almost exclusively in the symptomatic group compared to the asymptomatic and lymphoma groups; this might represent a late broadened subclass pattern to a high antigenic burden.

Among the anti-HIV IgG subclasses of sera from HIV-1 subjects, IgG1 was dominant regardless of the clinical stage of infection. The patients with lymphoma retained their responses to immunodominant antigens despite their generally low CD4 values. The low CD4 cell values may decrease the CD4 help, particularly to IgG3 responses. However, these patients had higher frequencies and titers of

EBV p107 antibodies. Low levels of p107 antibodies were found in lymphoma patients with EBV DNA present in the sera, a finding well in concordance with previous work where low levels of p107 antibodies in EBV DNA positive subjects reflected an impaired EBV T cell control (Barkholt *et al.*, 1996). This impairment may consist of an altered IL-10 expression with inhibitory effects of IL-10 or vIL-10 on T cells.

The lymphoma patients, compared to the symptomatic patients, had decreased responses of IgG1 and IgG3 to gp120 peptide and IgG3 to gp41 and V3 peptides. These findings could have implications for the development of lymphoma, since gp120 and gp41 recently have been reported to stimulate B cell growth and differentiation through IL-10, possibly towards a development of malignant B cell clones (Edelman *et al.*, 1996; Miyazaki *et al.*, 1993; Stuart *et al.*, 1995).

B cells and EBV

Several mechanisms have been suggested as contributing to HIV-1 related lymphomagenesis. Direct polyclonal activation of B-lymphocytes (Schnittman *et al.*, 1986), and the influence of Epstein-Barr virus infection are likely to have significant roles in the pathogenesis. EBV is associated with approximately 50% of the lymphomas in HIV-disease and those with demonstrable EBV DNA in serum have a poorly controlled lymphoma (Herndier *et al.*, 1994; Muller *et al.*, 1995; Pelicci *et al.*, 1986; Stuart *et al.*, 1995; Subar *et al.*, 1988). Recent work points towards EBV negative tumors having a less aggressive clinical course (Kaplan *et al.*, 1995). All patients were EBV antibody positive with both IgG1 and IgG3 responses to p107. The lymphoma patients with poor EBV p107 reactivities had detectable serum EBV DNA, which has previously been implicated as a marker for decreased T cell control of EBV-transformed cells (Barkholt *et al.*, 1996).

EBV specific IgG also appears to be important in inhibiting outgrowth of transformed EBV-carrying cells. Recent work (Abedi *et al.*, 1997) suggests a role for EBV specific antibodies, purified from EBV seropositive donors, in the prevention of EBV-related tumor development in SCID mice repopulated with human PBMCs. This might be related to antibody interactions with the EBV nuclear antigens (EBNAs) and/or EBV latent membrane proteins (LMPs) which occur at the tumor cell surface, since these antigens are implicated in B cell transformation (Kieff and Liebowitz, 1990).

IgG subclass disorders may develop as a manifestation of an impairment of the B cell function due to the malignancy per se, but more likely as a result of functional imbalance in immune regulation developing with declining CD4 cells. Previous findings point to a loss of HIV-1 specific minor IgG subclasses during disease progression (Sundqvist *et al.*, 1986). We interpret the observed loss of IgG subclass reactivities in the lymphoma group as a sign of disease progression, since the mean CD4 count of these patients is below that of even the symptomatic group.

Autoimmunity not induced during HIV-1 rgp160 immunizations

HIV-induced immune dysfunction contributing to autoimmunity and lymphoproliferative malignancies is complex and probably initiated by activation of virally infected macrophages (Morrow *et al.*, 1991).

Our study group (Paper V) consisted of relatively healthy individuals (CD4 counts > 400), which may account for the lack of more prominent autoreactivity. However, the study was initiated to define if vaccination would induce autoimmunity. Autoimmune antibodies were not induced during vaccination as defined by antibodies against CD8+ cells bearing class I A2 and peptides representing epitopes with suggested molecular mimicry between HLA and gp160.

Pathogenesis of HIV related autoimmunity

The question if autoantibodies directed towards self-components could arise during immunization with rgp160 was addressed in forty HIV-infected asymptomatic individuals. The patients were studied for autoreactive antibodies before, during and after 6 months of rgp160 immunization. The HLA class II DR beta-chain stretch comprising aa 17-81 contains four sites that have a certain homology with HIV gp160 sites, as shown in Table 1. These amino acids form an alpha helix. They are positioned so that antibodies directed to these epitopes could interfere with the antigen-presenting capacity of the class II groove. By mimicking the identified/described HLA class I and II homologous regions with synthetic peptides, we analyzed the IgG reactivity during HIV rgp160 immunization (Table 1). CD8+ cells and chemically synthesized peptides representing regions of homology between HLA and HIV-1 gp160 were used as antigens in cytotoxicity assays and in ELISA in order to investigate increasing evidence of an autoimmune component in the pathogenesis of HIV-1 infection and progression to AIDS (Habeshaw *et al*., 1992; Hoffmann *et al*., 1991; Moore *et al*., 1993).

Autoantibodies during HIV therapeutic immunization

Autoantibodies as defined by reactivity in ELISA to peptides representing regions of HLA class I and class II were indeed present in the HIV-1 infected individuals at a frequency ranging from 33 to 78 percent. IgG reactivity to these HLA homologous peptides was, however, lower than to HIV unique peptides. After vaccination with rgp160, these frequencies decreased to between 25 and 58 percent at 15 months after vaccination. A significant increase in titer was observed only against the HIV-peptide ml283. No evidence of significantly increased reactivity to the HLA peptides after rgp160 immunization could be found. Indeed, reactivity (OD 405nm) 15 months after initial vaccination decreased towards HLA-peptides p12, p13, p14 .

Relations to markers of autoimmune disease

For comparison, sera were also assayed for the occurrence of autoantibodies typical for patients with the autoimmune rheumatic disease Sjögren´s syndrome, as previously reported in HIV-infection (Argov *et al*., 1991; Muller *et al*., 1992). No autoantibodies reacting with the 60 kD SS-A/Ro antigen could be detected by ELISA or Western blot in any of the sera. Neither were reactions detected by ELISA towards the La/SS-B antigen.

Decreasing levels of autoantibodies during immunizations

The frequency of reactivity to the homologous HLA DR peptide p12 decreased from 65 percent to 45 percent after vaccination, which is slightly above the 33 percent previously reported (Golding *et al*., 1988). The presence of crossreactive antibodies to this region has been correlated with lack of T cell responsiveness to influenza or tetanus (Blackburn *et al*., 1991). A significantly decreased reactivity ($p < 0.001$) to HLA peptide p12 after vaccination was related to increased T cell proliferative responses to a number of HIV and other antigens, including tetanus toxoid and HIV proteins (Wahren *et al*., 1994).

Several autoantigens have been suggested to contribute to the pathogenesis of AIDS. Anti-lymphocyte antibodies deserve special note since it has been suggested that such antibodies might be one mechanism involved in T cell depletion. Indeed, the specificity of these antibodies remains to be fully characterized but could in part be directed to HLA class I and class II, as described in paper V.

CONCLUSIONS

These results demonstrate the feasibility of jet-injection of plasmid DNA for induction of specific immune responses in mice and humans. The injection into the mucosal tissues of the oral cavity was well tolerated and easily monitored for adverse events.

The site and mode of application and the number of immunizations influence the type of immune response elicited following plasmid DNA immunization.

The combination of plasmids induces specific mucosal and systemic humoral responses to a higher degree than previous studies utilizing single plasmid immunizations.

Exposure to rgp160 favors the HIV-specific antibody response instead of the autoimmune responses in vaccinated patients.

HIV patients with lymphoma had low specific IgG subclass reactivities as a sign of disease progression, with low CD4 counts indicating the effects of CD4 control on IgG3 induction

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