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Modulation of HIV Immune Responses in Natural Infection and after Genetic Immunization

by

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CONTENTS

ORIGINAL PAPERS

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

I. Calarota S., Liberatore D., Rabinovich R., Libonatti O., Martinez Peralta L. Quantitative indirect immunofluorescence as an alternative to Western blot for the diagnosis and follow-up of pediatric AIDS.
Pediat AIDS HIV Infect 1994; 5:97-100.

II. Liberatore D., Avila M. M., Calarota S., Libonatti O., Martinez Peralta L. Diagnosis of perinatally acquired HIV-1 infection using an IgA ELISA test.
Pediat AIDS HIV Infect 1996; 7:164-167.

III. Calarota S., Jansson M., Levi M., Broliden K., Libonatti O., Wigzell H., Wahren B. Immunodominant glycoprotein 41 epitope identified by seroreactivity in HIV type 1-infected individuals.
AIDS Res Hum Retroviruses 1996; 12:705-713.

IV. Pampuro S. E., Calarota S. A., Marquina S. A., Rabinovich R. D., Libonatti O. V. Reactivity of Argentine serum samples against synthetic V3-based HIV-1 peptides.
J Acquir Immune Defic Syndr Hum Retrovirol 1996; 12:527-528.

V. Calarota S., Bratt G., Nordlund S., Hinkula J., Leandersson A-C., Sandström E, Wahren B. Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients.
Lancet 1998; 351:1320-1325.

VI. Calarota S. A., Leandersson A-C., Bratt G., Hinkula J., Klinman D. M., Weinhold K. J., Sandström E., Wahren B. Immune responses in asymptomatic HIV-1-infected patients after HIV-DNA immunization followed by highly active antiretroviral treatment.
J Immunol 1999; 163:2330-2338.

VII. Calarota S. A., Kjerrström A., Wahren B. A gene combination raises broad HIV-specific cytotoxicity.
Manuscript

ABBREVIATIONS

aa	amino acid
AIDS	acquired immunodeficiency syndrome
ADCC	antibody dependent cellular cytotoxicity
APC	antigen presenting cell
CCR5	cystein-cystein linked chemokine receptor 5
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CMV	cytomegalovirus
CXCR4	cystein-x-cystein linked chemokine receptor 4
CTL	cytotoxic T lymphocyte
CTLp	cytotoxic T lymphocyte precursors
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunoabsorbent assay
env	HIV-1 envelope gene
gag	HIV-1 group antigen gene
gp	glycoprotein
HAART	highly active antiretroviral treatment
HIV-1/2	human immunodeficiency virus type 1/2
HLA	human leukocyte antigen
IF, IFA	immunofluorescence assay
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LTNP	long term nonprogressors
LTR	long terminal repeat
Mab	monoclonal antibody
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
mRNA	messenger ribonucleic acid
MuLV	murine leukemia virus
NK	natural killer
NSI	non syncytium induced
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
pol	HIV-1 polymerase gene
RANTES	a chemokine that is regulated upon activation of normal T-cell, expressed and secreted
RNA	ribonucleic acid
RP	rapid progressors
RT	reverse transcriptase
SI	syncytium induced; stimulation index
Th	T helper
WB	Western blot

Amino acid single letter code

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophane
Y	Tyrosine

INTRODUCTION

DISCOVERY OF AIDS

In 1981 several cases of *Pneumocystis carinii* pneumonia and Kaposi's sarcoma, among young homosexual men and intravenous drug users, were reported to the Centers for Disease Control (CDC) in the United States (45, 48, 110, 128). Such diseases are normally associated with immune deficiencies (127, 208). The patients had decreased counts of CD4+ T cells (97, 127, 208). Soon after, the CDC began to get reports describing an identical immunosuppressive syndrome among hemophiliacs (40), blood transfusion recipients (44), Haitian immigrants to the United States (43), sexual partners of individuals with this syndrome (46), and, eventually, infants of mothers with the disease (42). Such findings suggested the presence of a new transmissible disease possibly caused by a virus, and it was named acquired immunodeficiency syndrome, AIDS. Since that time, the spread of AIDS worldwide has been dramatic.

IDENTIFICATION OF THE HUMAN IMMUNODEFICIENCY VIRUS

In 1983, French researchers isolated a new retrovirus from the lymph node of a man with persistent lymphadenopathy syndrome (10); at that time, the physicians suspected that the syndrome was associated with AIDS. Soon after, the virus was isolated from the peripheral blood mononuclear cells (PBMC) of adult and pediatric AIDS patients in the United States (114, 192, 246). For a while, three names were used for the same virus; then, in 1986, the new retrovirus was renamed human immunodeficiency virus, HIV (61). Following the discovery of an antigenic variant in 1986 in Western Africa, the original virus was named HIV-1 and the variant designated as HIV-2 (55). HIV-2 appears to spread more slowly and to be less pathogenic than HIV-1 (327). HIV-2 is closely related to the simian immunodeficiency virus (SIV), which infects many African primates, and is thought to have been transmitted from the sooty mangaby monkey to man (281). Recently, it has been reported that the African chimpanzee *P. t. troglodytes* is the primary reservoir for HIV-1 and the source of viral introduction into the human population (115).

EPIDEMIOLOGY OF HIV-1

The Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) estimated that as of December 1998 some 33.4 million adults and 1.2 million children had been infected with HIV-1, with the largest burden in sub-Saharan Africa and in Asia (310). This virus has already taken the lives of nearly 14 million adults and children. In Latin America, 1.4 million people had been infected with HIV-1 up to the end of 1998. Argentina reported the second highest incidence of AIDS cases in South America, exceeded by Brazil. Today, the highest rate of HIV-1 infection in Argentina occurs among intravenous drug users (18). In the last few years, the number of AIDS cases reported among individuals aged 20-34 years has risen significantly. Considering the long period of asymptomatic infection induced by HIV, it is likely that new infections are being acquired comparatively early in life.

CHARACTERISTICS OF HIV-1

CLASSIFICATION

Lentiviruses constitute a genus of the *retroviridae* family, which includes several viruses infecting diverse species. HIV-1 and HIV-2 are lentiviruses, as are SIV, equine infectious anemia virus (EIAV), visna virus, caprine arthritis-encephalitits virus (CAEV), bovine immunodeficiency virus (BIV), and feline immunodeficiency virus (FIV). Lentiviruses are host-specific viruses which cause slowly progressive diseases in their hosts and are characterized by cytopathic changes in host cells.

HIV-1 VIRION STRUCTURE

The viral particle, with a diameter of about 110 nm, has a cone-shaped core composed of the viral p24 (Figure 1). Inside this capsid are two identical single-stranded RNA molecules of approximately 9.2 kb each with which the viral reverse transcriptase (RT) and the nucleocapsid proteins are closely associated. The replicative enzymes ribonuclease, integrase and protease are also contained in the nucleocapsid. The inner portion of the viral membrane is surrounded by the p17 protein, wich provides the matrix of the viral structure. The viral surface is made up of 72 spikes representing trimers or tetramers of glycoprotein complexes. Each spike consists of an external surface envelope protein, gp120, interacting non-covalently with the transmembrane protein, gp41, that crosses the lipid bilayer of the envelope. The envelope also contains cellular proteins acquired during virus budding, including ICAM (intracellular adhesion molecule), β2-microglobulin, and the human major histocompatibility complex (MHC) class I and II molecules (106, 200, 258).

GENOMIC ORGANIZATION AND GENE PRODUCTS

The genome of HIV-1 is similar to the other retroviruses and contains three major genes, flanked by long terminal repeats (LTR) that are essential for the replication mechanism (292) (Figure 1).

1. The *gag* gene encodes the precursor p55, which is further cleaved by the viral protease to the structural proteins p24, p17, p7 and p6.
2. The *pol* gene codes for a precursor protein which, after proteolytic clevage, results in three viral enzymes: p11 protease, p66/51 RT, and p32 integrase.
3. The *env* gene codes for the precursor gp160, later proteolytically cleaved into the two envelope proteins gp120 and gp41.

In addition to these genes, the HIV-1 genome encodes accessory proteins with important functions for viral replication and infection (70, 91). The **Tat** (transcriptional transactivator) protein initiates and/or stabilizes elongation of viral mRNA transcripts by binding to a special site called TAR (Tat responsive region) located in the LTR. Tat is the major protein involved in upregulating HIV replication. The **Rev** protein (regulation of viral expression) interacts with a structure called RRE (Rev responsive element) located in the *env* gene. This interaction permits unspliced mRNA to enter the cytoplasm from the nucleus and to

give rise to full-length viral proteins needed for progeny production. The **Nef** protein is one of the first HIV proteins to be produced in infected cells and is the most immunogenic of the accessory proteins. Nef has at least three distinct activities in infected cells: down-regulation of cell surface CD4 and MHC class I molecules and enhancement of virion infectivity (139). The **Vif** protein seems to be important for the cell-cell transmission of virus. It has been reported to be crucial for proviral DNA synthesis and involved in the final stages of the nucleoprotein core packing (147, 313). The **Vpr** protein affects the nuclear localization of viral nucleic acids in non dividing cells and induces cell differentiation (141, 194, 335), while the **Vpu** protein enhances virion release and degrades CD4 in the endoplasmatic reticulum (91, 170, 328). Vpr, Vif and Nef are found in the viral particle (108). The **Tev** protein, which exhibits both Tat and Rev activities in fuctional assays, is produced very early after infection and is not found in the virion (14).

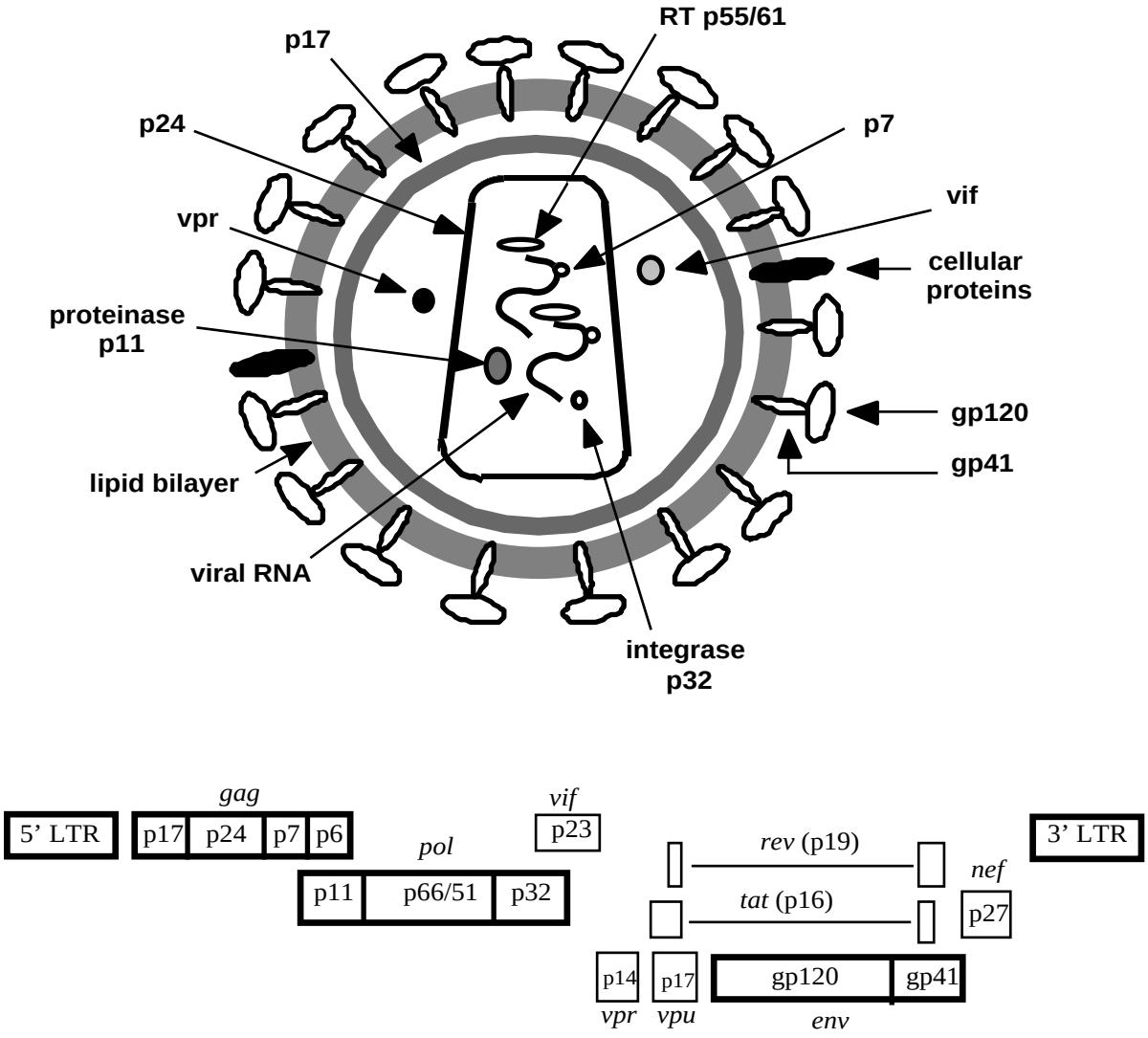

Figure 1. Structure of HIV-1 particle and genomic organization. The boxes represent the localization of the viral genes. Lines indicate the connected exons of the *rev* and *tat* genes. Below the virion, the proteins are indicated by size in kD.

REPLICATION CYCLE

Virus replication begins with virus attachment to the target cell. The major targets for HIV-1 infection are cells expressing CD4, such as T-helper cells (165, 166), monocytes, macrophages, dendritic cells and brain microglial cells (116, 144, 235). Infection of a target cell is initiated by binding of the viral envelope protein gp120 to the CD4 receptor on the surface of the cell membrane (73, 165). This binding is mediated by the CD4 binding domain (located in the C-terminal half of gp120) by interaction with the third variable region of gp120, the V3 loop (229). The CD4 molecule consists of immunoglobulin-like loop structures, of which the first is involved in gp120 binding (23). Subsequently, the gp120-CD4 interaction causes conformational changes in the viral protein gp120 that contribute to the exposure of binding sites for the cellular coreceptors. These coreceptors have been identified to be members of the chemokine receptor family and are necessary in order to induce fusion between the viral and cellular membranes (79, 85, 86, 98). The α-chemokine receptor CXCR4 and the β-chemokine receptor CCR5 serve as entry cofactors for T-cell tropic and macrophage tropic HIV-1 strains, respectively (149). These alterations of the gp120 molecule allow the fusogenic domain of gp41 to be exposed to the cell surface which, in turn, leads to fusion of viral and host cell membranes (109, 325) and entry of the virus into the cell.

After entry, the viral genome is released into the cytoplasm by the uncoating of the viral core, followed by activation of RT to initiate the synthesis of a DNA strand of negative polarity from the genomic viral RNA template. The RNA strand is then degraded by RNaseH in the DNA/RNA hybrid and a second strand of DNA is synthesised with plus strand polarity. The resulting double-stranded DNA is the proviral form of the viral genome which migrates into the nucleus and is then inserted into the host cell genome by the viral integrase. The integrated viral DNA can remain latent until the host cell is activated. At that point the viral DNA is transcribed by cellular RNA polymerase. Proviral transcriptional activity is regulated by several cellular transcription factors as well as various viral-encoded factors. First, the multiply-spliced mRNAs are transcribed and produce the regulatory proteins Tat, Rev and Nef. Transcription is increased by Tat activity on the TAR in the LTR region. As the level of Rev increases, transport of unspliced viral RNA to the cytoplasm is accelerated. Nef makes up to 80% of the early viral transcripts, although it is produced at all stages of viral gene expression.

Expression of late transcripts gives rise to structural proteins. During the post-translation period, the envelope proteins are glycosylated and cleaved by cellular proteases into gp120 and gp41. The envelope proteins, gag polyproteins, pol polyproteins and the new viral genomes are assembled into new viral particles at the cell membrane. The virus progeny particles are released by budding through the cell membrane. The maturation of the virus particles is completed during and after budding from the host cell when the viral protease cleaves the gag and pol polyproteins into functional proteins.

GENETIC VARIATION

The DNA sequence diversity seen in HIV is generated by its RT enzyme, which has been shown to be extremely error-prone and thus gives rise to nucleotide substitutions, insertions, deletions, repetitions and recombinations. The turnover of the viral population within an infected individual is remarkably rapid. Early during infection, the patients harbor a relatively homogeneous virus population; over time, the population becomes heterogeneous and then, in the late stages of the disease, becomes more homogeneous again (215). HIV-1 genes present different degrees of variation; for instance, the *pol* gene shows considerably lower variability than the *env* gene. The *env* gene is divided into five variable regions, designated V1 to V5, and five constant regions, denoted C1 to C5 (295). The level of variation of the *gag* gene lies between *pol* and *env*. Mutations in the Gag and Pol proteins more often result in non-viable viruses, while the Env proteins accept extensive variation, allowing escape from the host immune system.

HIV-1 can be divided into two groups, based on the genomic analysis of viral sequences from both *env* and *gag* genes, called major (M) and outlier (O) (174). A third group N (for novel or non-M-non-O) was presented recently after the analysis of an HIV-1 variant from a Cameroonian individual (288). Eight genetic subtypes (A through H) have been defined within the M group and two additional new subtypes (I and J) were further described (175, 188). The encountered prevalence of group O viruses is currently low. Subtype B represented the vast majority of viruses circulating in North America and Europe, while it was rarely found in Africa. Instead, all the other subtypes were found in Africa, as well as group O and group N viruses. Furthermore, subtype B was found to be spreading in Thailand and South America. As a consequence of immigration, the original geographical distribution of HIV-1 subtypes is gradually changing. Today, in no country of the world is just a single clade of virus circulating.

VIRAL PHENOTYPES

Primary HIV-1 isolates have been classified into different phenotypic groups according to distinct *in vitro* properties. On the basis of virus replication rate, syncytium-induction and ability to infect CD4+ cell lines, HIV-1 isolates have been classified as slow/low nonsyncytium-inducing (NSI) macrophage-tropic or rapid/high syncytium-inducing (SI) T-cell-tropic viruses (6, 99, 173, 280). The finding that chemokine receptors have a critical role in the cellular entry of HIV-1 has led to a new classification for HIV-1 according to coreceptor usage (16). Isolates with the ability to enter cells using CXCR4 as coreceptor correspond to the previously described rapid/high SI isolates (renamed X4) and isolates which use CCR5 comprise the slow/low NSI isolates (renamed R5).

THE IMMUNE SYSTEM: GENERAL ASPECTS

The immune system defends the host against infection by means of both nonspecific and specific components. The first line of defense is the innate (nonspecific) immunity, while the adaptive immune response displays specificity, diversity,

memory and self/nonself recognition. Adaptive immunity can be divided into humoral and cell-mediated responses. The humoral response involves the interaction of B-cells with the antigen and their differentiation into antibody-secreting plasma cells. The cell-mediated response involves T-helper cells (Th), which respond to antigen by producing cytokines, and cytotoxic T-lymphocytes (CTL), which mediate killing of virus infected cells. Antigen-specific activation and proliferation of Th cells is required for the generation of both pathways. Th cells can become activated by recognition of the antigen associated with MHC molecules on the membrane of antigen-presenting cells (APC), which include macrophages, B-lymphocytes, and dendritic cells. APC digest the antigens into small peptides and present them in association with MHC class I or II to the T-lymphocytes. Antigen presentation by B-cells is mediated by the binding of the antigen to the immunoglobulin receptor, internalization, degradation into peptide fragments and association to MHC class II molecules.

MHC class II molecules, expressed primarily on cells of the immune system, bind peptides from exogenous antigens which are ingested by endocytosis or phagocytosis and degraded into small peptides in intracellular vesicles. CD4+ cells recognize antigen associated with MHC class II molecules. This interaction leads to activation as well as proliferation of Th cells, which are required for generation of humoral and cellular immune responses.

Upon activation, CD4+ T helper lymphocytes differentiate from precursor Th0 cells into two subsets, Th1 and Th2, which can be distinguished by the profile of cytokines produced. Th1 cells predominantly activate macrophages and upregulate cell-mediated immunity; they secrete IFN-γ, IL-2, IL-12, IL-15, and IL-18. Th2 cells mainly induce humoral responses and secrete IL-4, IL-5, IL-6, IL-10, and IL-13.

The cytokines secreted by activated Th cells also regulate the proliferation and differentiation of nonspecific effector cells that play roles in cell-mediated responses. IL-2 and IFN-γ in particular have been shown to activate macrophages and to enhance both their ability to kill ingested pathogens and the cytolytic activity of natural killer cells (NK).

MHC class I molecules, found on the membrane of nearly all nucleated cells in association with β2-microglobulin, bind peptides which are produced within the cell. Generation of peptides involves protein degradation in the cytosol, transport of peptides across the endoplasmatic reticulum, loading of peptides into MHC class I molecules, and transport of the peptide-MHC complex to the cell surface. The CD8+ cells recognize the complex of peptide-MHC class I molecules and, in the presence of IL-2 secretion by Th cells, become cytotoxic cells.

This clear dichotomy between an exogenous processing pathway for MHC class II-restricted response and an endogenous pathway for MHC class I-restricted response is supported by extensive experimental data. However, recent data suggest

that various exogenous antigens may efficiently prime MHC class I-restricted CTL responses (151, 160, 207, 253, 278).

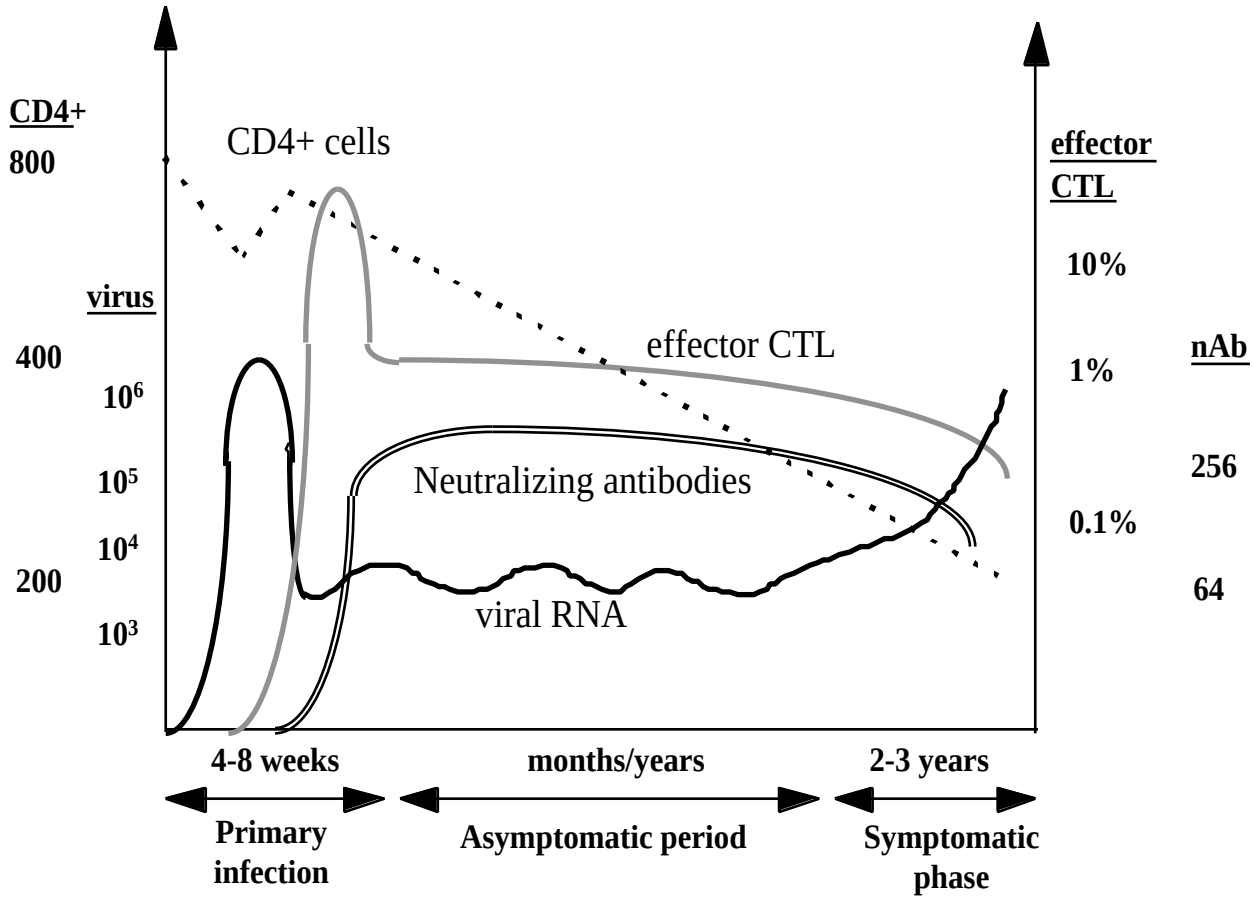

Figure 2. Clinical, immunological and virological course of HIV-1 infection. Shown are CD4+ counts/µl blood, viral RNA as copies/ml plasma, effector CTL as % of CD8 T cells in PBMC and neutralizing antibody titers (nAb). Adapted from (153, 267 and own observations).

PATHOGENESIS OF HIV-1 INFECTION

HIV is transmitted during sexual intercourse, parenteral exposure to HIV contaminated blood or blood products, and vertically from mother to child. Studies of persons with acute HIV-1 infection demonstrate selective infection by certain populations of HIV-1 variants. While both NSI and SI viruses can be transmitted, the former make up 90-95% of sexually transmitted viruses (336, 337).

Immediately after infection, high levels of viremia occur in peripheral blood, allowing systemic dissemination of the virus (Figure 2). Generally, within 4 weeks the level of virus in blood is reduced substantially, probably as a result of immune reactions against the virus, and virus remains low during the persistent period. Virus-specific CTL may play a critical role; these cells could mediated clearance of HIV-infected cells. Many years later, at the time of symptoms, a high level of viremia is observed which remains during disease progression (64).

The CD4+ cell counts decrease during primary infection but may remain close to normal levels for 3-4 months following primary infection. During the persistent period there is a steady drop in the CD4+ counts and in some cases a rapid decrease can be observed at the time of increased virus production.

The number of CD8+ cells rises during primary infection, after which the level returns to just above the normal and remains elevated until the final stages of disease. When the individual develops symptoms, CD4+ cells are usually below 200 cells/μl and the blood levels of HIV are high compared with those during the asymptomatic stage. The biological properties of the virus at this point differ from those observed soon after primary infection, such as enhanced cellular host range, rapid kinetics of replication, increased syncytium induction, and efficient cell killing; all properties of an SI, X4 virus (53, 300).

CLINICAL FEATURES AND TREATMENT

The signs and symptoms of acute HIV-1 infection appear within days to weeks after initial exposure (162). The most common signs and symptoms include fever, fatigue, rash, headache, lymphadenopathy, pharyngitis, and myalgia. Seroconversion to HIV-1 antibody positivity occurs within 3-12 weeks. The patients may begin to develop a spectrum of clinical conditions, after a variable period of years, as a result of the deteriorated immune system. The clinical manifestations of AIDS can include systemic, neurological, gastrointestinal, infectious and malignant complications. Some infected individuals progress after less than three years of infection (rapid progressors, RP), while others can remain asymptomatic for 10 years or more (long-term nonprogressors, LTNP) (34, 231). Intermediate progressors, the majority of cases, develop AIDS in approximately 10 years. Once a patient develops AIDS, survival is about 2 years.

Most of the antiretroviral drugs used against HIV have been nucleoside analogues, which are incorporated by the RT into the growing viral DNA, leading to its termination. The most used anti-HIV agent in clinical trials is AZT (3'-azido-3'-deoxythymidine). It has been reported that the treatment of infected individuals with AZT decreases mortality rate, reduces frequency of opportunistic infections and increases the number of CD4+ cells (103). However, the prolonged use of this drug leads to the induction of toxicity and the emergence of resistant viral strains (78, 143).

It has been shown that AZT in combination with another nucleoside analogue, 3TC (2'-deoxy-3'-thiacytidine), reduces the risk of progression and death by 50% (296). Current therapeutic strategies include the use of a combination of drugs directed at different steps in the viral life cycle to maximize the magnitude and durability of virus suppression and minimize the emergence of escape mutants. Protease inhibitors are potent drugs that prevent the maturation of viral particles.

The combination of two nucleoside analogues and one protease inhibitor, also known as highly active antiretroviral treatment (HAART), has been shown to dramatically decrease viral load, increase CD4+ counts, delay disease progression and prolong survival (25, 36, 133, 137). However, the recognition of long-term toxicity associated with protease inhibitors has raised concern about the complications, such as hyperlipidemia and lipodystrophy (erroneous fat distribution), and the overall long-term consequences. Currently, the benefits of these agents clearly outweigh the toxicities that are becoming apparent with long-term protease inhibitor exposure.

Combination therapy offers the advantage of a greater reduction of viral load and a greater chance of preventing the emergence of drug-resistant mutants when compared to monotherapy. However, HIV-1 persists in a latent form in resting CD4+ T cells. The persistence of latently infected cells is a major obstacle to virus eradication (102).

FACTORS INVOLVED IN DISEASE PROGRESSION

Several reports have demonstrated that the viral biological phenotype is associated with disease progression. NSI virus isolates were found in individuals who had a lower prevalence of AIDS (24). It has been reported that individuals infected via injection of drugs exhibit longer survival than those infected through sexual contacts, suggesting that the transmission route may influence disease course (237). Furthermore, deletions in the *nef* gene have been associated with a reduced rate of disease progression (77, 164, 217). However, *nef* defective viruses can only account for a fraction of LTNP.

Furthermore, several host factors have been found to influence the rate of disease progression among infected individuals. Strong specific immune responses have been described in LTNP, while these responses decreased in RP (34, 134). There have been several attempts to demonstrate HLA associations with disease progression. The HLA A1/B8/DR3 haplotype is associated with rapid disease progression and HLA A25, A32, B18, B27, B51 and B57 with slow progression (326). Resistance to HIV infection among prostitutes in Nairobi was linked to HLA A2 and HLA-DR13. These associations are weak, but imply that in some patients presentation of and immune response to certain viral epitope is of importance.

CCR5 is the major coreceptor for NSI strains. It has been shown that a 32-base pair deletion within the CCR5 gene results in premature termination of the protein, which is not transported to the cell surface (86). Individuals with homozygous

alleles appear to be resistant to HIV infection. However, this resistance is not absolute (19, 230, 301). Individuals with a genotype heterozygous for the 32-base pair deletion show a delay in disease progression (26, 271). The primary infection therefore usually takes place with an R5 virus, which is strongly dependent on this coreceptor. Once the virus mutates to X4, the progression may become rapid (24, 26).

THE HOST IMMUNE RESPONSES AGAINST HIV-1 INFECTION

HUMORAL IMMUNE RESPONSES

Antibody responses to HIV-1 appear within days to weeks after infection. HIV-infected individuals can produce antibodies to several HIV-1 gene products, including both envelope (gp160, gp120, gp41) and core proteins (p55, p24, p17) (209). At seroconversion, HIV-specific IgM antibodies can be detected. Within a few weeks these antibodies decline and HIV-specific IgG antibodies appear. These specificities are used for diagnostic purposes or as indirect measures of viral replication (113, 297).

Correlations between humoral responses and disease progression have been established. When high anti-p24 antibody levels develop after infection, they correlate with slower progression. However, the correlation between response to gp120 and rate of disease progression has been controversial. High titers of anti-gp120 antibodies have been correlated with rapid progression. On the other hand, strong reactivity to the C-terminal region of gp120 correlates with slow progression. A recent study has shown that sera from rapid and slow progressors can be differentiated by reactivity to a panel of envelope peptides (198).

The vast majority of antibodies generated during natural HIV-1 infection do not bind well and are probably of limited efficacy in controlling the virus. Different factors influence the antibody recognition of an antigenic epitope, such as variation in the amino acid sequence, alteration of conformation and masking by other protein domains, and carbohydrate groups (329).

Neutralizing antibodies

A conventional response to viral infection is the production of antibodies that attach to the virus and inactivate or neutralize it. Two mechanisms of HIV neutralization have been described: inhibition of virus binding to the target cell and interference with post-binding events, including fusion of the virus and cell membranes (243). Some studies, but not all, have found a correlation between strong neutralization to laboratory HIV-1 isolates and improved disease outcome (219). Slow progressors are able to neutralize primary isolates more frequently than rapid progressors (240, 334). A longitudinal study of neutralizing antibodies and disease progression, reported recently, indicated, however, that the level of neutralizing antibodies is not predictive of disease progression (38).

In infected individuals, anti-HIV neutralizing antibodies are detectable in the serum within 1-2 months after infection, when the viremic peak has already passed (Figure 2). The major target for humoral antibody responses is the HIV-1 envelope. The gp120 V3 loop has long been considered the principal neutralizing domain of HIV-1, evoking type-specific neutralizing antibodies (156, 186), but has also been described to elicit cross-neutralizing antibodies (2, 123). A second cluster of neutralizing epitopes is located in the V1/V2 region of gp120 (111, 212). Furthermore, the CD4-binding domain of gp120 has been described to elicit neutralizing antibodies against different isolates (145, 303). HIV-specific neutralizing antibodies against other regions on the gp120 and gp41 have been reported (30). The major neutralizing epitope described on gp41 is located in the conserved ectodomain and induces cross-neutralizing antibodies (222, 223). Other epitopes on gp41 have also been described (49).

Antibodies participating in cellular reactions

The ADCC (antibody-dependent cell-mediated cytotoxicity) and NK cells are important effector mechanisms for the elimination of tumors and virus-infected cells. The non-specific NK activity is mediated by CD16+ cells, whereas ADCC is a target-cell-specific effector function that requires the presence of target-specific antibodies. In HIV-1 infection, both NK and ADCC effector functions are adversely affected. In ADCC, HIV-specific antibodies bind to HIV antigens on the surface of infected cells, and then induce killing mediated by MHC-unrestricted CD16+, Fc-receptor-bearing, effector cells. It has been shown that ADCC is mediated by antibodies of the IgG1 subclass and that the envelope constitutes a target for this reaction; multiple epitopes on gp120 and gp41 proteins have been identified (4, 225). This activity is present in early stages of disease and declines markedly during disease progression (177, 305). In HIV-infected adults, ADCC antibodies have been detected as early as 3 months after primary infection and are often detected before neutralizing antibodies (130). The development and maintenance of higher ADCC correlated with slower progression in adults and children (11, 28). However, other investigators have been unable to confirm these observations (72, 228). In addition, NK cells have been found to have a decreased function when the infected individuals progress to disease (32, 105).

CELL-MEDIATED IMMUNE RESPONSES

Helper T-cell responses to HIV-1

CD4+ helper T-cell responses are known to be essential for the maintenance of effective immunity in chronic viral infections. The reduction in the number of CD4+ lymphocytes is the immunological deficit that characterizes HIV infection. In HIV-infected individuals, early studies documented defects in the CD4+ cell responses that were observed early in the infection (284, 315). However, subsequent studies showed that the T-cell function, as measured by lymphocyte proliferation in response to HIV-1 antigens, is weak or absent in all stages of disease (244, 264, 316). A model has been presented in which the Th1 and Th2

cytokines play an immunoregulatory role in HIV infection and where resistance to HIV-1 infection and/or progression to AIDS is dependent on a Th1>Th2 dominance (58).

CTL as antiviral host defense

Virus-specific CTL play a central role as protective host defense by eliminating the infectious agent. This T-cell population expresses CD8 molecules and recognizes peptide antigen associated with MHC class I molecules. The contact with the antigen by their specific T-cell receptor triggers the activation of these cells to divide, differentiate and mediate the lysis of infected cells. The lytic process is caused by the secretion of soluble lytic proteins (perforin and granzymes); and the expression of membrane-bound Fas ligand inducing apoptosis in Fas expressing cells (161). CTL can also release cytokines and chemokines when they encounter the antigen. The CTL that initially expand on antigen contact can persist as memory cells.

CTL responses to HIV-1

Table 1 summarizes the cellular immune responses in HIV-1 infection. In 1987, HIV-specific CTL activity by CD8+ cells was reported in infected individuals, using fresh PBMC and bronchoalveolar lavage (242, 321). Since then, intense research has contributed to a more comprehensive understanding of this response. Compared with most acute viral infections, HIV is unusual in that circulating CTL effector cells are present in the peripheral blood of many patients and CTL activity can be measured in fresh blood cells even without *in vitro* stimulation (321). In infected individuals, specific CTL have also been detected in lymph nodes, spleen, genital tract and cerebrospinal fluid (54, 135, 154, 220). The most frequently recognized proteins are Gag, Pol, Env and Nef (213). CTL responses to Tat, Rev and Vif proteins have also been detected but are present at a lower frequency (183, 257). The precursor frequencies of HIV-1-specific CTL, as determined in limiting dilution assays, typically range from 10^{-3} to 10^{-5}, and represent memory CTL (35, 168, 179). On the other hand, MHC class II-restricted CD4+ CTL have been described in blood of some infected patients, but their role in HIV-1 infection is not clear (195).

CTL and suppression of HIV-1 replication

The functions of HIV-specific CTL and their action on viral replication are summarized in Figure 3. In addition to their MHC class I-restricted antigen-specific CTL effector function, an important activity of CD8+ cells is the noncytolytic suppression of HIV-1 (319). Efforts to identify the nature and role of this inhibitory activity have been ongoing since the late 1980s. This activity was primarily exhibited by CD28+, HLA DR+, CD8+ cells and partly mediated by a soluble factor (optimal activity may require cell-cell contact) that arrested viral replication at the transcriptional level. The CD8+ antiviral factor (CAF) is not related to any known cytokine (193) and can suppress many different virus isolates, including HIV strains that are both NSI and SI (9). Recent reports suggest that the β-chemokines MIP-1α, MIP-1β (macrophage inflammatory protein-1α/β) and RANTES (regulated upon activation of normal T-cell, expressed and secreted)

and IL-16 have suppressive effects (8, 60). Although the β-chemokines can block HIV replication, these cellular factors are not CAF. Evidence of this conclusion includes the following: the anti-HIV-1 activity of β-chemokines appears to be limited to NSI strains that use the CCR5 coreceptor, whereas CAF can block the replication of all types of HIV; β-chemokines can block the entry of HIV into the cell, whereas CAF blocks virus production at the level of transcription.

Role of HIV-specific CTL in natural HIV-1 infection

Most HIV-infected individuals rapidly develop HIV-specific CTL responses after infection, suggesting that these cells are at least partly responsible for the fall in viremia (20, 178) (Figure 2). In addition, the soluble factors produced by CD8+ cells have been shown to inhibit viral replication in the early stages of acute infection and may therefore contribute to the reduction of the viral load (203).

Generally, HIV-specific CTL response is maintained throughout the asymptomatic stage but often declines with disease progression (7). The noncytotoxic anti-HIV response of CD8+ cells decreases concomitant with the development of disease (184). The persistent replication of HIV-1 probably sustains permanent CTL activation, although the need for the antigen's presence to maintain CTL memory is still unclear. In RP the viral load generally seems to increase despite strong HIV-specific CTL responses, suggesting that HIV has escaped antiviral responses. Several studies of LTNP have shown robust and persistent HIV-specific CTL responses, and an inverse correlation between CTLp frequencies and cell-associated viremia has been associated with LTNP (168, 256, 312).

Some LTNP seem to have rather low levels of HIV-specific CTL (100). In addition, no clear relationship has been found in HIV-infected children between CTLp frequencies and viral load or disease progression (201). Despite such controversies, the majority of studies to date seem to support the concept that HIV-specific CTL responses contribute to the control of viral replication and thus, delay the onset of disease.

At present no satisfactory explanation exists for the decline in HIV-specific CTL response in late disease. At least two reasons could be proposed. First, exhaustion of HIV-specific CTL due to prolonged high-level antigen exposure. Second, depletion of CTL responses due to lack of sufficient levels of CD4+ Th cells (267). Loss of HIV-specific CTL may be a reflection of progressive immune deficiency induced by HIV-1 infection. The HIV-induced immune deficiency may include both direct and indirect mechanisms, such as a direct cytopathic effect on CD4+ T cells, and indirect effects in which immunopathogenesis may be the major driving force. Suggested models include: anti-CD4+ cytotoxic activity, apoptosis, cell destruction via circulating gp120 attachment to uninfected CD4+ cells (bystander effect), immunosuppressive effects of immune complexes and/or viral proteins, CD8+ suppressor factors, anti-CD4 autoantibodies, and cytokine destruction of CD4+ cells (191, 285).

Table 1. Cellular immune responses in HIV-1 infection

• **Mediated by CTL**	References
In natural infection	
Class I-restricted CTL (CD8+)	320
* unusually high frequency	146,321
* directed at multiple epitopes	183,213,257
* initial control of viral replication during primary infection	20,178
* deterioration during disease progression	7
Possible reasons	
- CTL exhaustion	267
- lack of CD4+ Th cells	267
- HIV infection of CTL	196
- suppression of CTL activity by a soluble factor	159,270
* frequent cross-reactivity between different HIV-1 clades	126
* contribution to clearence of HIV-1 infection in HIV-1 exposed seronegative subjects	269,283
CTL evasion mechanisms used by HIV-1	
* MHC class I downregulation	210,279
* amino acid sequence variation:	
- affecting MHC binding	176
- affecting TCR recognition	176
* sequestration (in the brain)	214
* viral latency (provirus integrates but is not expressed)	214
* CD4 downregulation	
- affecting class II-restricted CTL	own suggestion
Class II-restricted CTL (CD4+)	
* in HIV-infected and gp160-immunized subjects	195,294
CD8+ anti-HIV	
* inhibition of viral replication by soluble factors	9,319
Therapeutic implications of HIV-specific CTL	
* adoptive transfer of CTL	27,172
* vaccines that induce CTL	
- recombinant proteins	181,252
- naked DNA (in this thesis)	V-VII 22,202
Induction by prophylactic HIV-1 vaccines	
* recombinant vaccinia alone or boosting with protein	65,90,238
* recombinant canarypox alone or boosting with protein	13,57,96,104

Table 1. (cont.)

• **Mediated by NK cells**	References
In natural infection	
* lysis of target cells by ADCC	197,306
* early after infection and declining in progression	177,305
During therapeutic vaccination	
* ADCC induced during recombinant gp160 vaccination	29
* NK lysis during DNA vaccination (under evaluation, A.L. Hultström, KI and SMI)	
• **Mediated by Th cells**	
In natural infection	
* qualitative impairment early in infection	282,315
* weak or absent in most HIV-infected individuals	244,316
* loss with disease progression	58,284
* significant proliferative responses:	
- in LTNP	264
- in acutely infected individuals during HAART	264
* demonstrated in HIV-1 exposed seronegative subjects	59
* directed at multiple epitopes	209,225, 287,314
Induction by therapeutic HIV-1 vaccines	
* recombinant protein	181,187,252, 272,318
* naked DNA (in this thesis)	V, VI 22,202
Induction by prophylactic HIV-1 vaccines	
* gp120 V3 branched peptides	125
* recombinant canarypox alone or boosting with protein	57,90,239
* recombinant protein	12,124,132
* recombinant vaccinia	131

CTL among high risk seronegative subjects

Indirect evidence for the role of CTL in controlling HIV infection comes from observations that HIV-specific CTL responses are present in persons exposed to the virus but presenting no evidence of infection. These include sexual partners of infected individuals, children born to infected mothers, healthcare workers exposed to infectious body fluids and female prostitutes in Africa (269, 283). Based upon

the assumption that the induction of CTL requires endogenous synthesis of viral proteins, these findings suggest that transient infection and virus clearance by CTL are indeed posible.

Recently, two studies reported that a significant proportion of HIV-exposed seronegative subjects had HIV-specific precursor CTL and none (17) or only one subject (120) had the Δ32 CCR5/Δ32 CCR5 genotype associated with HIV resistance. This indicated that inheritance of the Δ32 CCR5 mutation does not account for the majority of non-infected individuals. The presence of cellular immunity in these persons suggests either protective immunity or a low level of detectable infection.

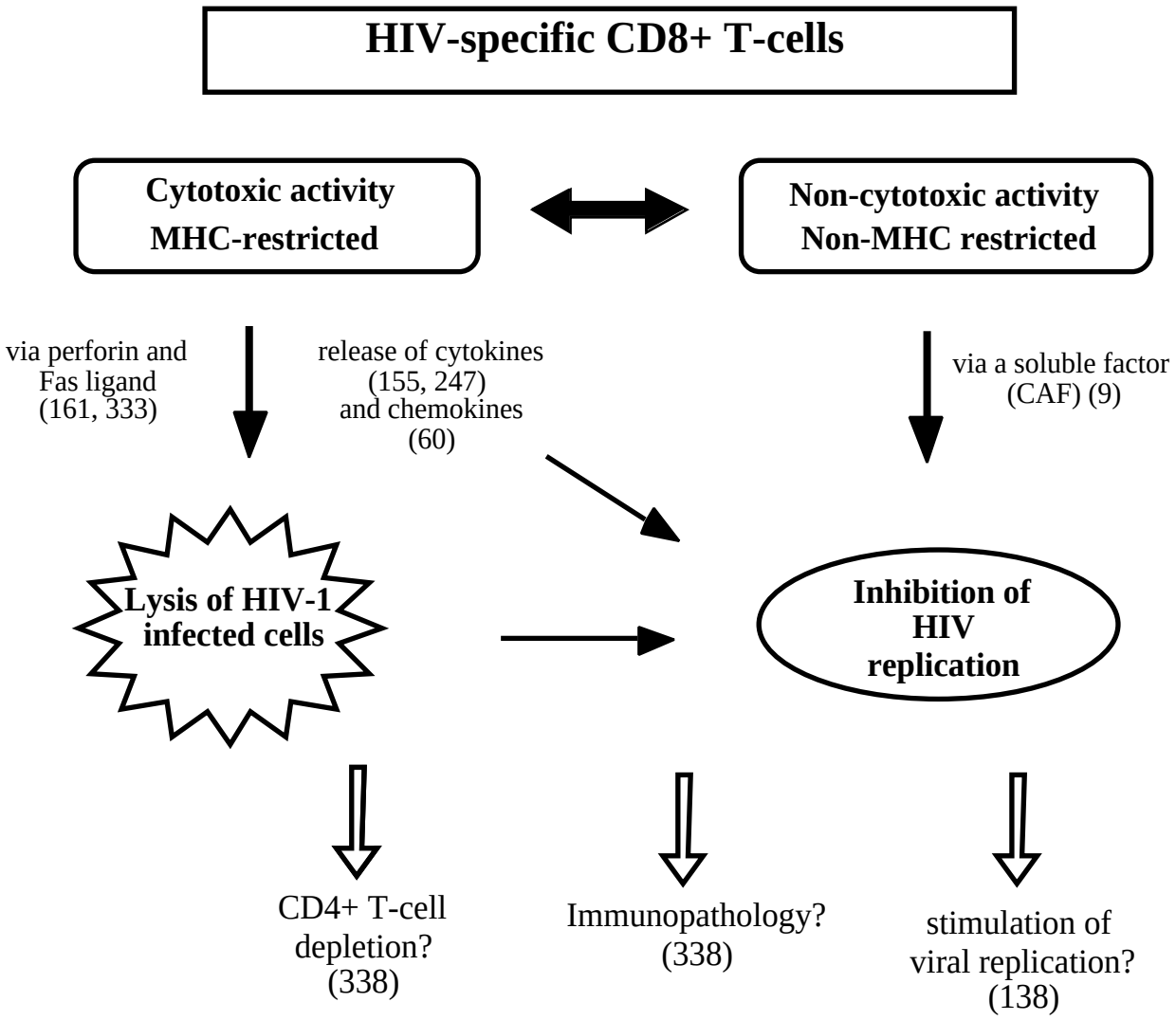

Figure 3. HIV-specific CD8+ T-cells functions. CTL lyse HIV-1 infected cells and also release cytokines and chemokines upon contact with antigen-expressing cells. Both direct cell lysis and cytokines inhibit viral replication. The non-cytotoxic activity is mediated by a soluble factor (CAF) which suppresses HIV replication. The potential adverse effects of CTL activity listed below are still debated.

Viral escape from CTL recognition

HIV appears to affect immune activation or processing and recognition of viral antigens and therefore avoids the host immune response (107). For instance, certain viral proteins, such as Tat and Nef, can alter MHC expression (210, 279). Rapid evolution of viral sequences is believed to represent a major mechanism of viral escape (176, 214). Viral variation can result in nonrecognition of epitopes either by affecting MHC binding or TCR interaction (69). Some HIV Gag or RT variants have been found to induce antagonistic effects by inhibition of CTL recognition of wild-type peptides (169, 216). The ability of the virus to escape from CTL recognition and the broad cell tropism of HIV-1 are both considered to confer viral persistence despite vigorous HIV-specific CTL responses (167).

Other escape mechanisms have been suggested, such as sequestration of the virus in sites where CTLs do not access effectively. Viral latency is another possibility, since latent provirus can not be detected by the immune system (214).

CTL as therapy

Augmentation of CTL activity could be a therapeutic strategy for HIV-infected patients. In view of the increasing evidence that CTL contributes to the control of the virus in infected individuals and that highly exposed but HIV seronegative persons make CTL responses, a major effort is being made to design vaccines for the induction or enhancement of HIV-specific CTL responses. From the data available, however, the adoptive transfer of autologous CTL clones to HIV-infected individuals does not appear to provide a significant benefit (27, 172).

HIV-1 VACCINES

There are obstacles to the development of effective vaccines against HIV. Nevertheless, several types of vaccines have been designed and results from studies in infected individuals and uninfected volunteers have been reviewed (56, 83, 140, 190). Early vaccine trials focused on a subunit of HIV-1, the envelope protein. Subunit vaccines were considered much safer than inactivated vaccines because they carry no risk of infection from the vaccine itself. Immunization with recombinant envelope subunits (gp160 or gp120) has been shown to be safe and to induce neutralizing antibodies. The low HIV-specific CTL response was mainly mediated by CD4+ cells. Live recombinant vectors, such as vaccinia, were well tolerated and induced CTL responses. The combination of recombinant HIV-1/vaccinia virus and boosting with recombinant gp160 was proven to be more effective in inducing CTL responses and cross-neutralizing antibodies than immunization by either immunogen alone. However, the disadvantage of the vaccinia vector is that it appeared to be ineffective in persons with prior vaccinia exposure, being useful only for vaccinia-naive persons. To address the safety concern, a canarypox vector (ALVAC) which does not replicate in human cells has been studied, alone or in combination with subunit boosting. It was safe and induced both humoral and cell-mediated immune responses. Live attenuated HIV-1 vaccines have also been discussed for human use, following the relatively good protection by such vaccines

in macaques (80, 158). However, live attenuated vaccines involve a risk of reversion to a pathogenic form.

This thesis will focus on a novel vaccine candidate approach: immunization with plasmid DNA, which does not have replicative properties.

AIMS OF THE STUDY

The aims of this study were:

- to evaluate methods for the diagnosis and follow-up of children born to HIV-infected mothers.

- to analyze the antibody reactivity of HIV-1 infected individuals to HIV-1 envelope glycoprotein.

- to evaluate the efficacy of HIV-DNA immunization in inducing immune responses in asymptomatic HIV-1-infected patients.

- to develop efficient methods to evaluate cytotoxicity during genetic immunization.

MATERIALS

The studies with children born to HIV-1 infected mothers took place at the National Reference Centre for AIDS in Buenos Aires, Argentina. These children were monitored at the Pedro Elizalde Children's Hospital, a reference pediatric hospital in Buenos Aires. Each child's infection status was determined according to the CDC classification (39, 47). Seroreversion was considered in any child who had two or more negative tests at least 3 months apart performed at 6-18 months of age and who remained without symptoms that could be attributed to HIV disease.
In **Paper I**, a total of 102 sera sequentially obtained from 23 infants born to HIV-1 infected mothers were analyzed. Patients' age ranged from 5 days to 40 months after birth. Of the 23 infants, 8 were HIV-1 infected (CDC status P2) and 15 seroreverted, showing no serological or clinical evidence of HIV-1 infection.

Paper II. A total of 117 serum samples were obtained periodically from 86 children born to HIV-1 infected mothers, with ages ranging from 1 to 12 months. Forty-six infants were HIV-infected and 40 uninfected. Infants were followed-up until at least 20 months of age to ensure accuracy of the classification of infection status.

Paper III. Sixty-three HIV-1 infected individuals, in clinical stages ranging from asymptomatic infection to AIDS, were included in this study. Serum samples were obtained from 20 Argentinian intravenous drug-abusing patients and 43 Swedish patients (40 homosexual men, 2 intravenous drug abusers and 1 heterosexual man).

In **Paper IV,** we examined sera from 93 HIV-1-seropositive patients resident in Buenos Aires, Argentina.

Papers V and VI. Nine asymptomatic HIV-1 infected inividuals from Sweden were enrolled in these studies. They belong to a cohort of 40 patients who have participated since 1991 in a study of recombinant gp160 immunization in asymptomatic HIV-1 infected individuals with CD4+ counts above 400 X 10^6/L. They received HIV-1 recombinant gp160 vaccine regularly over a total period of 5 years, initially combined with either AZT or placebo. The 9 patients were selected for having no or low antibody reactivities to the HIV-1 Nef, Rev, or Tat. Thus, since 1996, three individuals were immunized with HIV-1 *nef* DNA, three with HIV-1 *rev* DNA and three with HIV-1 *tat* DNA. DNA constructs (100µg in distilled water) were administered by intramuscular injection into the right deltoid muscle at days 0, 60 and 180. Five patients are naive to antiretroviral treatment. One patient was already receiving antiretroviral treatment, one started HAART on the day of the second DNA immunization, and two patients did so after the third DNA immunization.

Paper VII. Eight of the nine asymptomatic HIV-infected patients described above participated in this study, while one *rev* DNA immunized patient moved abroad in 1997 and was lost to follow-up. After 13-18 months from the last single DNA

immunization, the eight patients were immunized with a combination of all three plasmids encoding the *nef*, *rev* and *tat* HIV-1 genes, mixed together in a single formulation (100μg of each). Immunizations were given at days 0 (intramuscular injection), 60 (intraoral jet injection) and 180 (four patients receiving intramuscular injection and four receiving intraoral jet injection). Intramuscular injection was given into the right deltoid muscle and jet injection in the junction of the buccal mucosa and the mandibular bone on the right side.

In **Papers V, VI and VII**, asymptomatic HIV-1 infected individuals, who also belong to the cohort of 40 patients repeatedly vaccinated with rgp160, who had not received HIV-DNA immunization, were recruited as controls.

HIV-1 TRANSMISSION FROM MOTHER TO CHILD

Infants born to HIV-1 infected mothers account for the great majority of pediatric cases of AIDS. The rate of mother to child transmission varies from 13% to 42% (94, 289). The lowest rates are reported in Europe, the highest in Africa. Reasons for this difference are still unclear, but concomitant infections of the mother, differences in virulence of the virus strains, and frequency of breastfeeding were proposed as contributing factors.

There is evidence that HIV infection can occur in utero as well as intrapartum and postpartum (31, 89, 93). A working definition has been proposed: the detection of virus by PCR within 48 hours of birth indicates that infection of the child occurred during gestation, whereas delayed appearence of virus implies intrapartum transmission (31). Postpartum transmission can occur via breastfeeding (87, 94).

Vertical transmission of HIV-1 is influenced by several factors. There is a correlation between low maternal CD4 T-cell counts at delivery and increased risk of transmission (93). A high viral load in the maternal circulation has been associated with increased transmission (93, 275). Effects of the administration of zidovudine (a nucleoside analogue) to pregnant HIV-infected women as well as their babies during 6 weeks after birth, suggested that the viral load has a strong impact on the likelihood of transmission; the rate was reduced by 67% (41). In addition, the administration of a single dose of nevirapine (a non-nucleoside RT inhibitor) to women during labor and to their newborns during the first week of life, showed a potent antiretroviral activity. This regimen is promising as a potential alternative for interrupting HIV-1 transmission in the intrapartum and early postpartum period (221). It has reduced transmission of HIV infection to newborns dramatically in most developed countries.

The viral phenotype may influence transmission of virus to the child. Rapid/high SI viruses are isolated more frequently from transmitting mothers than from those who do not transmit the virus (274). Several studies have suggested that maternal antibodies to the V3 region of gp120 may be associated with a lower rate of vertical transmission (81, 119, 265). However, other studies have not confirmed this observation (136, 233, 260, 293, 308; Calarota S, unpublished results). In addition, a correlation between maternal antibody binding to epitopes within the carboxy region of gp41 and lack of vertical transmission was demonstrated (307).

Mothers with neutralizing antibodies against primary HIV-1 isolates have a reduced risk of infecting their children (277). In a recent study, the strength and breadth of CTL response and its correlation with HIV-1 vertical transmission were determined in infected women during and after pregnancy (157). Variable levels of HIV-specific CTL response were present and Pol and Nef specific-CTL precursor frequencies were higher during pregnancy in nontransmitters than in transmitter mothers. Plaeger et al. reported an increased risk of vertical transmission in

mothers with low CD8 cell-mediated viral suppression function during the third trimester of pregnancy. They also showed that transmitting mothers had a higher viral burden (241).

Table 2. Comparison of the 1987 and 1994 classification systems for HIV infection in children under 13 years of age (39, 47)

1987 Classification			1994 Classification
P0	**Indetermined infection** Perinatally exposed children less than 15 months of age who have antibodies to HIV		**Prefix "E"**
P1	**Asymptomatic infection**		**N**
	A	Normal immune function	
	B	Abnormal immune function	
	C	Immune function not tested	
P2	**Symptomatic infection**		
	A	Non specific findings	**A, B, and C***
	B	Progressive neurological disease	**C**
	C	Lymphoid interstitial pneumonitis	**B**
	D	Secondary infectious diseases	
		D1 Typical of AIDS	**C**
		D2 Recurrent serious bacterial infections	**C**
		D3 Others (oral candidiasis, recurrent herpes stomatitis, multidermatomal or disseminated herpes zoster)	**B**
	E	Secondary cancers	
		E1 Typical of AIDS	**C**
		E2 Others	**D**
	F	Other diseases possibly related to HIV infection (hepatitis, cardiopathy, nephropathy, anemia, trombocytopenia, dermatologic disease)	**B**

*Category A, mildly symptomatic; category B, moderately symptomatic; category C, severely symptomatic

Most infants born to HIV-infected mothers do not acquire the infection. Protective immunity has been suggested by the presence of specific cytotoxic T-cell activity in uninfected children born to HIV-1-infected mothers (76, 268). About a quarter of infected children progress rapidly to AIDS within the first year of life, while the others develop AIDS slowly over several years (95, 304). As also observed in adults, disease in children correlates with the presence of rapid/high SI viruses (75).

HIV-1 infection in children has a wide spectrum of clinical manifestations (236, 276). Some infants present severe immunodeficiency, whereas others have non-specific findings, such as hepatosplenomegaly, persistent fever, parotitis, and recurrent gastroenteritis. *Pneumocystis carinii* pneumonia has a peak incidence between 3-6 months of age and is associated with a high mortality rate (95). Neurological manifestations are common in children with rapidly progressive

disease. Recurrent bacterial infections and lymphocytic interstitial pneumonitis are important manifestations in older children. Infants rarely develop Kaposi's sarcoma or other HIV-associated tumours.

The CDC has established a classification system for HIV-infected children under 13 years of age (39, 47) (Table 2). This system represents one tool for defining infection in children.

DIAGNOSIS AND FOLLOW-UP OF CHILDREN BORN TO HIV-1 INFECTED MOTHERS

We have focused our research on serological methods for the diagnosis and follow-up of children born to infected mothers. Easy and inexpensive tests were explored, which may facilitate the diagnosis of HIV infection in developing countries. We replaced Western blot (WB) with a quantitative IFA, which is significantly less costly (Paper I). In addition, we showed that IgA ELISA test is an effective and simple method for the early diagnosis of perinatally acquired HIV-1 infection (Paper II).

The diagnosis of HIV-1 infection in children born to HIV-1 infected mothers is of essential importance for identifying infants who may benefit from early treatment. Diagnosis of HIV infection in these children is complicated by the presence of maternal anti-HIV IgG antibodies, which cross the placenta to the fetus and may persist through the first 15-18 months in sera of newborns (262). Standard anti-HIV IgG antibody tests suggest that all these children are HIV-antibody positive at birth, whereas only 13-42% are actually infected. Alternative tools for the diagnosis have been developed, including virus culture, PCR (polymerase chain reaction), and detection of p24 antigen. The detection of anti-HIV-1 IgM and IgA and *in vitro* antibody production (291) are more specific for infection, since they are produced by the newborn. PCR and virus culture are the most sensitive and specific assays for detecting HIV infection in children born to infected mothers.

However, not all of these tests are easy to perform. PCR and virus isolation require laboratories with high biosafety standards and special equipment, which are not available in many parts of the world; these techniques are also very expensive. Our IgA ELISA method provided a very good alternative.

Children born to HIV-1 seropositive mothers are followed up in many cases at 3-month intervals for at least 15 months after birth. The loss of maternal anti-HIV-1 antibodies in noninfected children or the permanence of a positive reaction, which suggests infection, can then be observed. WB is the most commonly used test for confirmation of HIV antibodies and IFA is an alternative confirmatory assay used in many countries of Central and Latin America.

We found that, in infected children, IFA IgG titers increased with the appearance of new bands or with an increase in the intensity of previously reactive bands in the WB.

The noninfected children presented decreasing IgG titers, corresponding to the loss of WB reactivity. In 4 out of 15 noninfected infants, negative IFA results appeared on average 6 months earlier than negative WB results. Table 3 shows a comparison of IFA IgG titers with WB in HIV-infected and noninfected infants born to HIV-1-infected mothers. Two infected children presented decreasing IFA IgG titers, corresponding to the worsening of their clinical status. This decrease is possibly related to severe hypogammaglobulinemia as a result of immunodeficiency and low antibody production.

Although IFA is less sensitive for antibody detection to, for example *gag* or *pol* derived antigens, it appears to have the same sensitivity and specificity as the WB in determining HIV infection in newborn children. The possibility of titrating the antibodies (IFA is a quantitative measure rather than a conventional scale), means that infected and noninfected children can be distinguished at an early time point.

The noninfected children according to the first IFA negative result, remained seronegative during follow-up. Similar results were reported by the European Collaborative Study involving the follow-up of 600 children; none developed AIDS or persistent immunodeficiency after loss of antibodies (94). However, we recommend a follow-up to 24 months in any child in whom HIV infection can not be ruled out by earlier diagnostic tests. We suggest this because exceptional cases have been reported where clinical HIV infection appeared after antibody tests had become negative (189).

Table 3. Comparison of IFA titers with WB bands in A) HIV-1 infected and B) noninfected infants born to HIV-1 infected mothers

	Infant and age (months)	CDC clinical status	IFA IgG titers	WB bands								
				160	120	65	55	51	41	31	24	18
A)	9	P0	2560	3	3	3	2	3	2	2	3	1
	10	P0	640	3	3	3	2	3	2	2	3	1
	14	P1B	10240	3	3	3	2	2	2	2	2	—
	20	P2A	2560	3	3	3	3	3	3	3	3	1
	32	P2A	2560	3	3	3	1	1	3	2	1	3
	39	P2A	640	3	3	3	1	1	3	1	1	1
B)	3	P0	160	3	3	3	3	3	3	3	3	—
	7	P0	—	1	1	1	1	—	1	1	1	—
	12	Neg.	—	—	—	—	—	—	—	—	—	—
	24	Neg.	—	—	—	—	—	—	—	—	—	—
	31	Neg.	—	—	—	—	—	—	—	—	—	—
	38	Neg.	—	—	—	—	—	—	—	—	—	—

Children under 18 months of age born to mothers with HIV infection are definied as HIV infected when positive results are obtained on two separate determinations

from one of the following HIV detection tests: presence of p24 antigen, positive PCR or viral isolation (39, 47). However, the p24-antigen assay is less sensitive than viral isolation or PCR (262). Once again, PCR and viral isolation are expensive and technically complex.

Alternative serological methods applied to the early diagnosis of HIV infection in infants include the detection of HIV-specific IgM or IgA antibodies. The detection of HIV-specific IgA seems to be a more sensitive assay than detection of HIV-specific IgM, probably because IgM production is transient.

We evaluated an ELISA test for the detection of anti-HIV-1 IgA antibodies for the early diagnosis of perinatally acquired HIV-1 infection. All samples from all noninfected children were IgA negative (Table 1, Paper II). IgA anti-HIV-1 was detected in 83% of sera from all infected children, with a significant and high detection in children who were over 6 months of age when compared with the younger group.

The detection of anti-HIV IgA by specific assays has been described for diagnosis of perinatally acquired HIV infection using WB and dotblots assays (185, 206, 234, 249, 324). The negative results for our HIV-infected children under 6 months of age agree with other studies where the sensitivity of IgA assays was shown to be lower in children less than 3 months old (185, 249, 324). Our study confirmed the validity and clinical utility of anti-HIV IgA antibodies with an ELISA test for the diagnosis of HIV infection in children over 6 months of age born to HIV-1-infected mothers.

ANTIBODY REACTIVITY OF HIV-1 INFECTED INDIVIDUALS TO HIV-1 ENVELOPE

NEW IMMUNODOMINANT REGION ON gp41

A new immunodominant epitope on gp41 adjacent to the ELDKWA sequence was identified to which the majority of the sera from both Argentinian (70%) and Swedish (65%) HIV-1 infected individuals showed reactivity (Paper III). The number of patients with seroreactivity against the central region, which includes the ELDKWA sequence, was low (Table 4). In addition, the capacity of sera to neutralize HIV-1 was analyzed by attempting to correlate peptide binding with neutralization. Although the identified new immunodominant gp41 region did not appear to be directly involved in neutralization, it may be useful as a diagnostic tool in combination with previously identified immunodominant gp41 peptides (118).

The transmembrane envelope glycoprotein gp41 is relatively conserved and has not shown the regions of hypervariability that are seen in gp120 (218). The epitope 598-609 of gp41, which contains two cysteine residues critical for antigenic conformation due to the creation of a disulfide loop between residues 603-609, has

been reported to be immunodominant in HIV-infected individuals (148). Antibodies to this epitope are not neutralizing and do not block infection by HIV. Other gp41 epitopes were reported: one was found between residues 640-672 and the other was identified by peptides from the intracytoplasmatic region of gp41 (129).

Several human monoclonal antibodies to gp41 have been described; only a few were found to have neutralizing activity (67, 68, 248). To date, there is a potently neutralizing human Mab to gp41, called 2F5. This antibody has been shown to recognize the highly conserved gp41 epitope with the amino acid sequence ELDKWA (aa663-668) and to neutralize a broad variety of HIV-1 strains (88, 222, 223). The mechanism of neutralization is probably a post-binding conformational event, which was suggested as an effect of binding the Mab 2F5 to the virion (224).

We analyzed sera from HIV-1 infected patients against synthetic peptides from this gp41 region to determine its immunogenicity in natural infection. Sera obtained from two different populations of HIV-1-infected individuals were tested against a panel of overlapping peptides covering aa647-684 of HIV-1 gp41 (Paper III).

The identified epitope was mapped more closely in order to investigate the amino acids essential for antibody binding in this region. Sera were tested against a set of substitution peptides representing aa667-680 in which each residue was sequentially substituted with alanine. The carboxyl amino acids WNWFDI close to the ELDKWA sequence were the most important for antibody binding.

Differences in reactivity were noted between Argentinian and Swedish samples with peptides covering the central region, where moderate to strong reactivities of Argentinian sera were seen only to aa661-670 peptide, while Swedish sera displayed moderate to strong reactivity to the peptides spanning aa655-668. These observations indicate that residues near the N-terminal of the ELDKWA epitope contributed to the binding of antibodies with this region in some of the sera. These differences in reactivities of Argentinian and Swedish sera to this region might be due to different virus strains appearing in these geographical regions. The coexistence of subtypes B, F and an *env* recombinant B/F has been reported in Argentina (205), whereas in Sweden less of subtype B recombinants are found (3).

Table 4. Peptide ELISA seroreactivity of HIV-1 infected patients to aa647-684 of gp41

Serologically reactive protein regions	***Argentinian sera (n = 20)***	***Swedish sera (n = 43)***
Central (aa655-672)	20%	7%
C-terminal (aa665-684)	45%	42%
Combined (aa647-684)	25%	23%
No reactivity	10%	28%

SEROREACTIVITY AND gp120 V3 SEQUENCE VARIABILITY

The characterization of genetic variability is of great importance for the development of efficient vaccines and for the design of diagnostic reagents. Antibody-binding assays with synthetic peptides representing the HIV-1 gp120 V3 loop, have been used as a screening tool to predict genotypes (51). In Paper IV we analyzed the specificity of antibodies to the V3 domain of gp120 in sera from Argentinian HIV-1-infected patients. The peptides chosen corresponded to seven well-characterized viral isolates from different geographic areas and one Argentinian consensus sequence. A previous study performed in 1992 reported the Argentinian V3 loop sequences (121). Subtype characterization was not performed at that time but the amino acid patterns were found to resemble those of subtype B.

The highest percentage of reactivity (91.4%) was obtained with the Argentine consensus sequence, followed by the MN V3 sequence (82.8%), both corresponding to subtype B. Peptide MAL (subtype D) gave the lowest reactivity. It has been reported worldwide that peptides based on sequences of the HIV-1 MN strain react with the majority of the sera tested (37). Nevertheless, eight Argentinian sera included in this study reacted with the local consensus peptide but not with the one corresponding to HIV-1 MN.

The presence of subtype B in Argentina has been suggested by other reports (33, 82). However, in our study, some other peptides representing HIV-1 strains corresponding to subtype B showed low reactivity (SF2, LAI, RF and WMJ2), indicating the need to identify and characterize of local strains in order to design adequate diagnostic and vaccine strategies.

The HIV-1 gp120 contains highly variable regions. Within the V3 region a loop containing 35-36 amino acids is formed by a disulphide bridge between two cysteine residues. The loop is composed of a mixture of variable and conserved residues, the apex motif of this loop being GPGR or GPGQ (174). The V3 domain contains important determinants for neutralizing antibodies (2, 123, 156, 186), CTL (225), viral infectivity (152) and participates in the interaction between gp120 and chemokine receptors for viral cell entry. There has been a great deal of interest in this region due to the immunogenicity and functional importance of the V3 loop. The result is a worldwide compilation of V3 region sequences which provides an overview of the variation of this sequence stretch (174).

DNA IMMUNIZATION

DNA immunization involves the direct inoculation into tissues of genetic material. The gene of interest is carried on plasmid, which is then administered to a host. The DNA is taken up by host cells and travels to the cell nucleus, where it is transcribed and then translated in the cytoplasm of the cell. Endogenous protein synthesis mimics viral infection in allowing presentation of the foreign antigenic peptides by MHC class I. Proteins may also be secreted. It is believed that exogenously released antigen primes the induction of humoral response, and that

the uptake of soluble protein or peptide complexes by APC allows presentation by MHC class II to Th lymphocytes. Thus, both humoral and cellular immune response may therefore be induced (50, 302, 317).

DNA-based immunization has been shown in animal models to be effective for the induction of humoral as well as cellular immune responses. The raised responses have good longevity. In addition, studies involving virus challenges have shown protective immunity (21, 112, 309).

Early studies of DNA immunization demonstrated that DNA vaccines could generate immune responses against influenza (309), HIV-1 (322) and hepatitis B (74). Soon after, the use of DNA vaccinates was extended and explored in many other infectious disease models, including HIV-2 (1), hepatitis C (117), rabies (330), herpes simplex virus type 1 and 2 (266, 290), tuberculosis (299), malaria (84), cytomegalovirus (CMV) (122), rotavirus (52), *Leishmania major* (331), *Toxoplasma gondii* (5), Ebola (332); and also in cancer experimental model systems (15).

VECTORS FOR VACCINE USE

In most of the studies, plasmids DNA deriving from *E. coli* were used. They were modified to carry the early promoter from the human CMV, which is known to induce expression at high levels in many eukaryotic cell types. Other components of a DNA vaccine are: a cloning site to insert the gene of interest, a polyadenylation termination sequence, a selectable marker such as an ampicillin-resistance gene, and a prokaryotic origin of replication to permit production in bacteria.

ADJUVANTICITY OF DNA

It has been demonstrated that certain DNA sequences in the plasmid backbone act as an adjuvant and may control the nature of the subsequent immune response to the gene product (171, 180). The DNA motifs (5'-Purine-Purine-CG-Pyrimidine-Pyrimidine-3'), "CpG motifs" or "immunostimulatory sequences", are 20 times more common in bacterial than mammalian DNA. In eukaryotes, these dinucleotides contain a methylated cytosine, whereas in bacteria they are unmethylated. Recent studies indicate that CpG motifs in the plasmid backbone of a DNA vaccine can influence the nature and magnitude of the immune response (171, 263, 273). All these findings suggest that the plasmid DNA contains two units: a transcription unit that directs antigen synthesis, providing the specificity of the immune response, and the CpG motifs that directly stimulate B-cells, induce Th1 cytokines and co-stimulatory molecule expression on APCs. Both these forces drive the response to the encoded antigen.

ROUTES OF IMMUNIZATION

Gene vaccines have been administered by several routes. Initially, vector DNA was coated on gold beads and shot directly into the cells of the epidermis using a gene gun (298). In subsequent experiments, DNA constructs were applied by a syringe

into muscle (309). Intranasal, intradermal, subcutaneous, intravaginal and intravenous routes have also given rise to immune responses, although with larger variations. In general, gene gun delivery of DNA to the skin results in a Th2 response, while vectors injected intramuscularly induce a preferential Th1 response (62).

MECHANISMS OF IMMUNITY

The mechanism of initiation of immune responses by DNA vaccines has not yet been fully elucidated. It was originally proposed that the CTL priming occurs via endogenous processing and presentation of plasmid-derived antigen within the transfected myocytes, whereas the soluble protein (secreted from myocytes or released upon myocyte death) could generate the antibody and T-helper responses (Figure 4A). However, muscle is not considered the best site for antigen presentation because it contains few, if any, dendritic cells and macrophages and because myocytes lack important costimulatory molecules. Bone marrow-derived cells are assumed to serve as APC; such cells are dendritic cells or monocyte/macrophage. This takes place either upon direct transfection or upon reprocessing of antigen provided by other cells, such as muscle cells (66, 261).

Two other proposed mechanisms both involve bone marrow-derived APC (232). These cells express high levels of MHC class I and II molecules and costimulatory molecules. One mechanism proposes, in the case of intramuscular immunization, that the antigen produced by transfected myocytes is tranferred to bone marrow-derived APC that have infiltrated the muscle as part of an inflammatory response to the immunization procedure. The transferred protein would then cross-over into the MCH class I-processing pathway, allowing the APC to prime CTL response (Figure 4B). The second mechanism proposes the direct transfection of a small number of APC infiltrating at the site of the infected DNA. These cells can then activate the CD8+ T-cells via the classical MHC class I-processing pathway. CD4+ T-cells and antibody responses are generated by soluble antigen produced by either transfected myocytes/keratinocytes or transfected APC (Figure 4C). This mechanism appears to be likely in epidermal immunization because the skin has a high proportion of Langerhans' cells, while in muscle dendritic cells and macrophages are scarce.

MODULATION OF IMMUNE RESPONSES

Several factors have been proposed to modulate the magnitude as well as the orientation of the immune responses. Ways of manipulating the immune response to genetic immunization include changing the route of immunization, modifying the number of CpG motifs in the plasmid, and altering the immunization regimen or coadministering genes for cytokines or costimulatory molecules (62).

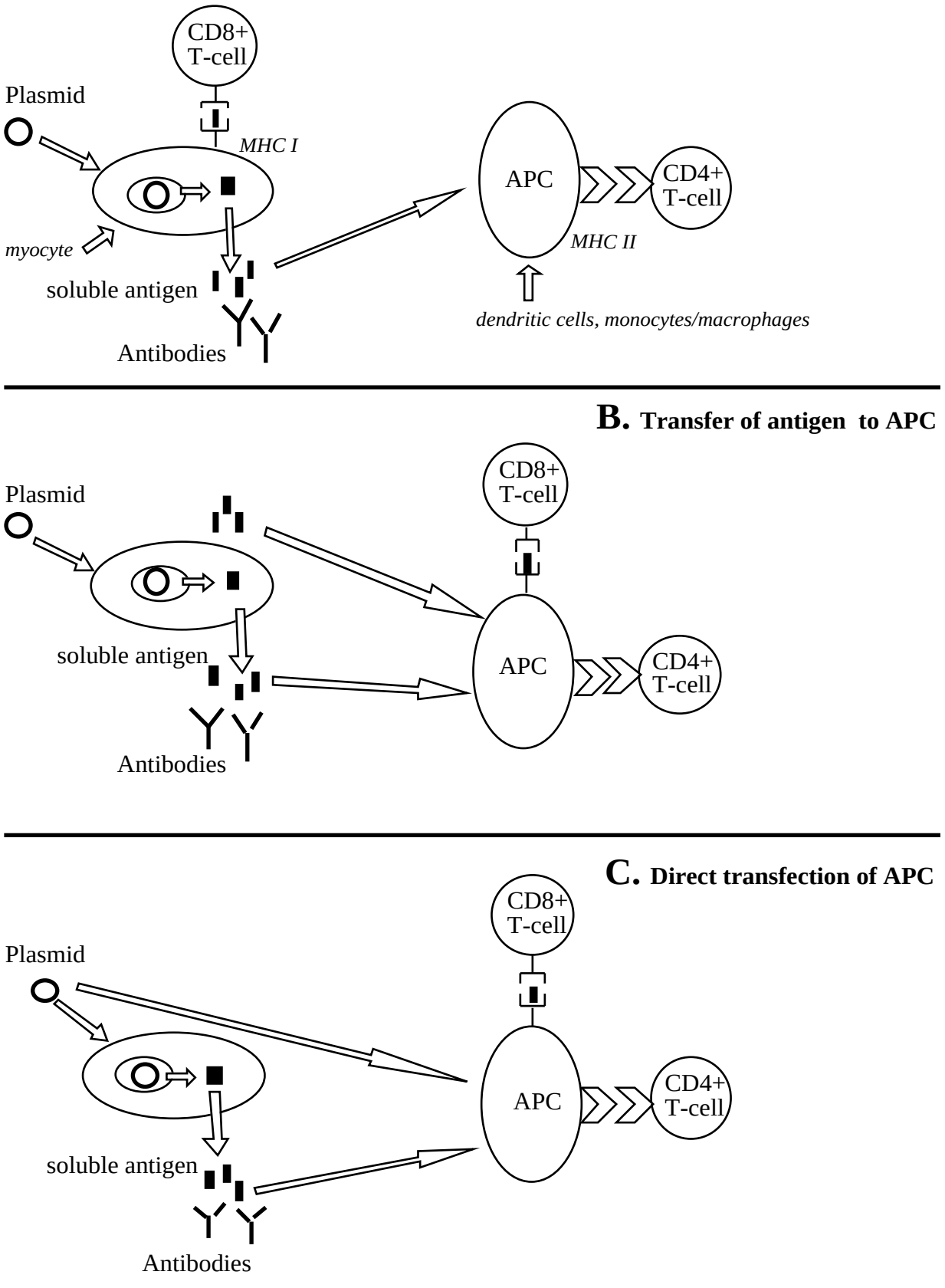

Figure 4. Possible models for generation of immune responses by DNA immunization with an emphasis on the priming of MHC class I-restricted CTL response.
A. Direct presentation by transfected tissue-specific cells
B. Transfer of antigen to APC with crossover into the MHC class I-processing pathway
C. Direct transfection of APC infiltrating the target tissue, activating CD8+ T-cells

THERAPEUTIC DNA IMMUNIZATION AGAINST HIV-1

We decided to evaluate the safety and immunogenicity of DNA immunization encoding the regulatory HIV-1 genes in already HIV-1 infected individuals (Papers V, VI, and VII). We focused on the *nef*, *rev* and *tat* HIV-1 genes since they encode proteins which are essential for virus replication, are synthesized early during infection and are conserved among different viral isolates.

Both Tat and Rev have important effects on viral gene expression. Tat increases the production of viral mRNA and is released from the cells. Virus with defective Tat does not replicate efficiently. In addition, extracellular Tat induces expression of the HIV-1 coreceptors on the target cells, promoting virus spreading (150). Rev is involved in the transport of late mRNA from the nucleus to the cytoplasm, allowing the production of the structural proteins. Nef is a strong immunogen. It down-regulates both the CD4 receptor and MHC class I molecules from the surface of infected cells. This downregulation prevents both superinfection and premature cell death of the infected cells by reducing the epitope density on their surface. It possibly limits CTL function (63). It still needs to be determined whether the decrease in MHC class I expression is associated with a decreased ability of CTL to inhibit virus replication.

It has been shown that CTL responses to Rev are less frequent and that Tat is seldom recognized by CTL in HIV-infected individuals. On the other hand, low concentrations of Rev- and Tat-specific CTL have been associated with rapid progression to AIDS (312). CTL against Nef may have several advantages in that Nef is one of the first proteins to be produced in HIV-infected cells. Several regions of this molecule are conserved between different HIV-1 isolates, Nef peptides are well represented in infected cells and several of them are highly immunogenic (259, 286). Altogether, these properties suggested to us that the induction of immune responses against Tat, Rev and Nef might contribute to the elimination of infected cells before the release of new viral particles. This effect might extend the asymptomatic period of the disease.

Nine asymptomatic HIV-1 infected patients were included in our studies. They were selected from a well-studied group of 40 HIV-infected indviduals who have been vaccinated with rgp160 (187, 318). Sera from these 40 patients were repeatedly tested by ELISA, using peptides and proteins representing the HIV-1 *nef*, *rev* and *tat* genes. They were selected for having no or low antibody reactivities to the protein encoded by the gene used for immunization. Our selection criterion was intended to enable us to measure HIV-specific immune responses.

Our first goal was to analyze the immunogenicity of a single construct and its gene product. Thus, three patients were immunized with HIV-1 *nef* DNA, three with HIV-1 *rev* DNA and three with HIV-1 *tat* DNA. The second goal was to evaluate the efficacy in the induction of humoral and cellular responses of the combination of all three constructs, given in a single formulation. One of the patients moved

abroad and was lost to follow-up, so eight patients were further immunized with the combination of plasmids (HIV-1 *nef*, *rev* and *tat* DNA). The study design is presented in Paper VII (Table 1) and the DNA plasmids used for immunizations are shown in Figure 5.

ALTERNATIVE METHODS FOR DETECTION OF HIV-SPECIFIC CTL

Several approaches have been employed to detect HIV-specific CTL (267). The method we used for the *in vitro* expansion of HIV-specific CTL is based on stimulation with paraformaldehyde-fixed autologous B-LCL (B lymphoblastoid cell lines immortalized with EBV) infected with recombinant vaccinia vectors encoding HIV-1 proteins (311). Recombinant vaccinia vectors containing Nef, Rev and Tat HIV-1 proteins as well as the HIV-1/MuLV virus were used in our studies, with the addition of IL-2 and IL-7, which are effective in enhancing the expansion of CTLs from stimulated cultures (101). Compared with non-specific methods of stimulation, this approach enhances CTL detection by inducing selective CTL expansion, with reduced interference from background lysis. We used limiting dilution analysis to estimate CTLp frequencies. CTL responses were measured against autologous B-LCL infected with recombinant vaccinia vectors encoding Nef, Rev and Tat HIV-1 proteins. These cells express endogenously processed antigen in the context of MHC class I molecules. Alternatively, we were able to infect B-LCL with HIV-1 by using the HIV-1/MuLV pseudotype virus. Since multiple antigens are presented in such infected cells, this targeting system probably is a more natural representation of an *in vivo* cellular target than any other available test system.

Since all patients included in our studies had had vaccinia vaccination in childhood, we added nonradiolabeled B-LCL infected with the wild type vaccinia virus as cold targets in all experiments in which the cultures were stimulated with APC infected with recombinant vaccinia (199). This allows us to distinguish HIV-specific CTL from vaccinia-specific CTL. It also permitted monitoring of a non-HIV related CTL. The vaccinia-specific CTL was not influenced by HIV-DNA immunization.

HIV-SPECIFIC CTL RESPONSES INDUCED BY SINGLE DNA IMMUNIZATION

We showed that vaccination with plasmid DNA expressing the HIV-1 regulatory genes *nef*, *rev* and *tat* induced HIV-specific CTL responses in eight out of nine asymptomatic HIV-1-infected individuals. Antigen-specific CTLp were also increased after single DNA immunizations, which indicates that even immunodeficient individuals have the capacity to respond to immunization by forming new HIV-specific memory cells (Papers V and VI).

Cytotoxic activity was quite marked and endurable in patients immunized with *nef* DNA. Of the three individuals immunized with *rev* DNA, one was a non-responder and two were consider to have a transient CTL response. After *tat* DNA immunization, moderate HIV-specific CTL responses were detected in the three patients, and considered transient in one of them. Cytolysis was mediated by CD8+ MHC class I-restricted CTL. MHC class-I mismatched targets were not recognized by the CTL.

In populations of HIV-infected individuals, 50-70% have detectable Nef-specific CTL (182, 257). Based on our selection criterion, and on the possibility of a pre-existing response in already infected individuals, it is reasonable to argue that one can boost that response. The lytic activity detected after immunization was considered to be associated with the DNA immunization since CTL assays from all nine patients were negative before DNA immunization.

The initiation of HAART appeared not to contribute to the induction of new HIV-specific CTL responses. During the follow-up of patients with both DNA vaccination and antiretroviral treatment, no HIV-specifc CTL activity was detected in two of them, one patient was continuously a non-responder and one showed a positive response when viral load started to increase. It has been suggested that these responses depend on continued viral replication (226). A correlation has also been shown between the decrease in plasma HIV RNA to undetectable levels and the disappearance of CTL activity in individuals with acute HIV primary infection treated with HAART (71, 204).

Ogg et al. have reported a reduction in HIV-specific CTL after the initiation of HAART in both newly and chronically HIV-infected persons (227). However, most of our non-HAART treated and DNA immunized patients also appeared to lose their CTL reactivity, indicating that the DNA-induced CTL activity is short lived in the immunodeficient individuals. In one patient (no. 39), this activity remained up to 17 months after the last *nef* DNA immunization. This patient is still naive to antiretroviral treatment.

By means of DNA immunization we were thus able to induce CTL responses against Nef, Rev and Tat in infected individuals. Recently, increases in HIV-specific CTL responses in HIV-1-infected individuals were reported after vaccination with plasmid DNA encoding the *env* and *rev* HIV-1 genes (22, 202). In addition, the induction of CTL in healthy individuals by a malaria DNA vaccine was shown (323).

The profile of IFN-γ secretion observed when human PBMC were transfected with the *nef*, *rev* and *tat* DNA parallelled the CTL activity. Increased IFN-γ production was elicited by the DNA vaccines, with the *nef* encoding gene being the most active followed by *tat* and *rev* (Paper VI).

DNA IMMUNIZATION INDUCES ANTIGEN-SPECIFIC T-CELL PROLIFERATIVE RESPONSES

Many HIV-infected individuals with chronic infection lack a specific T-cell proliferative response to HIV-1 proteins (315). However, these responses can be detected in LTNP (264). It has been shown that during infection, improved HIV-specific T-cell responses may be induced by vaccination with subcomponents of HIV-1, such as gp160 (187, 250, 272).

We explored the possibility that proliferative responses might be induced by DNA

immunization and found an increased antigen-specific proliferative response in all except one of our patients (patient 37). Before the first DNA immunization, low or no proliferations were seen to Nef, Rev or Tat antigens in all nine patients.

A correlation between increased cytolytic activities and antigen-specific proliferative responses was observed at several, but not all, time points. It is clear that these responses were induced in the majority of the DNA immunized patients, that they can be seen in a higher frequency than in non-DNA immunized individuals, and may persist for many months after the last plasmid immunization. HIV-specific proliferative T-cell responses remarkably remained or improved in the four patients treated by HAART (Paper VI).

Figure 6 presents the lymphocyte proliferative responses to Nef, Rev and Tat antigens, on the day of and 14 days after each immunization, in the nine DNA immunized HIV-infected patients. During the first part of the study (single DNA immunization), five patients evaluated in this manner showed increases in SI values after the first immunization (the three patients immunized with *nef* DNA, one with *rev* DNA and one with *tat* DNA). In general, these responses appeared to peak and decline during the follow-up, except for patient 7 who showed a positive response to Nef antigen up to day 194 (two weeks after the third *nef* DNA immunization).

Beside viral load reduction, HAART induces a significant and rapid increase of CD4+ T-cells, interpreted either as proliferation or redistribution of T-cells compartmentalized in the lymphoid tissue. However, it is important to know whether the improvement is coupled to restored immunocompetence. We found that HAART alone did not improve Th cell activity in chronic infection (187). The initiation of HAART during primary infection results in the generation and maintenance of strong HIV-specific Th responses. This is similar to the Th cell activity of LTNP, which control viral replication without antiretroviral treatment (264). Pontesilli et al. reported a weak lymphoproliferative response to HIV-1 gag 17 antigen, but not to p24, after the initiation of HAART treatment, soon after viral load reduction (245).

Figure 6 shows the T-cell proliferative responses after immunization with the combined vaccine. During this part of the study, patients 7 and 37 exhibited positive responses at day 0 to Nef, Rev and Tat antigens. However, these responses appeared to decrease during the follow-up period, but were still positive to Nef and Rev in patient 7 up to day 194. Patients 39 and 12 showed negative proliferative responses to the three antigens tested at day 0; these responses showed a peak at days 14 and 74, respectively, and then decreased to low values, except to Nef antigen in patient 12. Three patients were found negative to Rev antigen at day 0 (nos. 3, 29 and 6), being detectable at least once after immunizations with the combined vaccine. Patient 25 was negative to Rev and Tat at day 0; these responses increased after the first immunization and were still positive to Tat antigen at day 194.

In summary, and compared to baseline (day 0 before any immunization), all eight patients improved their specific T-cell response to at least one of the proteins encoded by the plasmids. Three patients improved their specific T-cell response to all three proteins.

T-cell proliferative responses to HIV-1 antigens have been reported by some authors to be minimally modified by post-exposure immunization with recombinant antigen (163), while others reported improved HIV-specific T-cell responses after vaccination with rgp160. These responses were not influenced by the addition of antiviral monochemotherapy (187).

Our results thus indicate that the T-cell proliferative responses to Nef, Rev or Tat antigens are related to DNA immunizations. Continued high levels were observed after the initiation of HAART and further increases were seen in some patients who did not receive antiretroviral treatment. Altogether, all these data suggest that the combination of DNA vaccination with HAART might result both in the induction of immune responses and in viral load reduction.

ANTIBODY RESPONSES TO HIV-1 ANTIGENS FOLLOWING DNA IMMUNIZATION

The patients were initially selected for having no or low initial antibody reactivities to Nef (3 patients), Rev (3 patients) and Tat (3 patients) HIV-1 antigens. The *nef* and *rev* DNA immunized patients developed IgG serum antibodies to the respective peptides, which were of a low and variable magnitude. In general, the IgG antibody titers after combined immunizations were still of a low magnitude. However, some sera showed moderately high reactivity against the Rev peptide covering aa1-20.

In natural infection, antibodies to Nef, Rev and Tat HIV-1 proteins have been reported in variable numbers of patients. In general, antibody levels to these antigens decrease as HIV infection progresses to AIDS (251, 254, 255). In our study, the highest antibody titers were observed in sera from patients immunized with *nef* DNA. They were directed to peptides covering the C-terminal region of HIV-1 Nef. These results confirm that Nef is the most immunogenic of the HIV-1 regulatory proteins and are also in accordance with the IL-6 inducing capacity of the *nef* plasmid (Paper VI).

Rev antibody titers were low, which may be due to the low antigenicity of the small HIV-1 Rev protein (255). Very low IgG reactivity was found before immunization to HIV-1 Tat peptides in patients immunized with *tat* DNA. No increase in antibody titers was observed in the three patients after immunizations. Probably a combination of DNA immunization with, for instance, protein boosting might help to enhance the humoral responses.

IMMUNIZATION WITH A COMBINATION OF DNA PLASMIDS RAISES BROAD HIV-SPECIFIC CTL RESPONSES

The efficacy of a combination of the DNA plasmids encoding the HIV-1 *nef*, *rev* and *tat* regulatory genes was then evaluated (Paper VII). We found an interesting

effect after immunization with the combined vaccine; a high frequency of CTLp was detected against target cells infected with HIV-1/MuLV virus. We have used this HIV-1 pseudotype virus in order to obtain an alternative targeting strategy. Theoretically, infection by the HIV-1/MuLV virus should result in several HIV-1 proteins being displayed on the target cells; thus, this model may better represent the natural situation.

When single DNA plasmid immunizations were given, B-cells infected with a single HIV-1 gene expressed in recombinant vaccinia vectors were recognized more efficiently by the induced CTL (Paper V). These targets may process and present the HIV-1 peptides without competition from other peptides. However, the cytolytic activity induced by vaccination with all three plasmids combined was more efficient against targets theoretically presenting more than one specific HIV-1 peptide. It is likely that competition for peptide presentation takes place in the targets infected with the whole virus genome.

One important advantage of using DNA immunization over the traditional vaccines is the possibility of combining genes in a single vaccine. Our data suggest that the combination of plasmids was more effective than the individual components in inducing CTL responses to the whole HIV-1 gene products in humans. In addition, the combination of plasmids did not appear to drastically change the CTL responses to the single regulatory HIV-1 proteins.

It has been suggested that the responses induced by immunizations with a combination of plasmid DNAs might be reduced, due to interference at the level of antigen production or antigen competition. However, immunizations with a combination of five HIV-1 DNA plasmids (gp160, p24, *nef*, *rev* and *tat*) gave immune responses in mice that were strong compared with those obtained using each plasmid in a single injection (142). In addition, protective immunity in guinea pigs from primary genital disease was induced after immunizations with a combination of plasmids DNA expressing the herpes simplex virus-2 glycoproteins D and B (211).

CpG EFFECTS

The DNA was effective without the need for external adjuvants. Part of this effectiveness may be due to an immunomodulatory effect of the DNA itself. It has been shown that the unmetylated CpG dinucleotides in the carrier bacterial DNA can enhance the Th1 immune response (263). In our study, and as expected, CpG motifs presented in the *E.coli* DNA but absent from mammalian DNA stimulated human PBMC to produce IFN-γ and IL-6 (Paper VI). Increased IFN-γ production was also elicited by the DNA vaccines, with the *nef* encoding gene being the most active. The same profile was observed when IL-6 production was monitored. These findings indicate that the DNA plasmids used in our studies contained motifs that would be immunostimulatory *in vivo* in man.

It is apparent that the increased reactivities, after immunizations with the combined

vaccine, may be due to an additional unspecific stimulation. Again, the presence of CpG motifs in the plasmid DNA vaccine may contribute to the vaccine immunogenicity. These immunostimulatory sequences may be activators that permit generally increased memory cell reactivity also to other HIV-1 gene products.

SUMMARY OF CELLULAR AND HUMORAL IMMUNE RESPONSES INDUCED BY HIV-DNA IMMUNIZATION IN HIV-INFECTED INDIVIDUALS

Table 5 presents the summary of HIV-specific immune responses in asymptomatic HIV-1 infected patients immunized with HIV-1 *nef*, *rev* and *tat* expressing DNA. HIV-specific immune responses in asymptomatic non-DNA immunized HIV-infected patients (control patients) are also shown.

Table 5. Asymptomatic HIV-1 infected patients immunized with HIV-1 *nef*, *rev* and *tat* expressing DNA vaccine

Immunological endpoint	Response before immunization	Measurable response[a]	No response[b]
CTLp[c]	> 1 X 10^{-6}	> 1 X 10^{-6}	
DNA immunized patients	4/9	9/9	0/9
control patients		6/6	0/6
CTL activity[d]	>10% specific lysis	>10% specific lysis	
DNA immunized patients	0/9	9/9	0/9
control patients		5/6	1/6
Th proliferative response[e]	SI > 3	SI > 3	
DNA immunized patients	0/9	9/9	0/9
control patients		2/4	2/4
Antibody responses[f]	IgG titer > 20	IgG titer > 20	
DNA immunized patients	3/9[g]	9/9	0/9
control patients		11/31[h]	20/31

[a] twice or more after immunizations. Analysis was performed up to 14 days after the third immunization with the combined vaccine for eight patients and up to 14 days after the third single immunization for one patient. Control patients were not selected and not DNA immunized.

[b] during all time points tested.

[c, d] to at least one of four types of targets infected with pseudotype HIV-1/MuLV or Nef, Rev or Tat carried by vaccinia virus.

[e] to at least one of three antigens (Nef, Rev or Tat).

[f] to any peptide covering the C-terminal region of Nef or the whole Rev or Tat proteins.

[g] three patients initially *tat* immunized had detectable antibodies to Tat peptides in pre-immunization sera.

[h] sera from control patients were tested on one occasion.

SAFETY CONSIDERATIONS

A number of concerns have been expressed, such as the formation of anti-DNA antibodies, the integration of plasmid DNA into the host genome and induction of tolerance rather than immunity. Present information indicates that plasmid DNA does not integrate and that the risk of integration is very low. The duration of plasmid expression differs for different plasmids. Despite the long persistence times, the induction of tolerance to the expressed antigen has not been demonstrated (92). This, however, is dependent on age at inoculation; very early after birth, tolerance might be induced. So far, there is no evidence of any detrimental systemic effect associated with DNA immunization in animal models as well as in humans.

All eight patients included in our study have been monitored after the last plasmid injection and none has developed an AIDS complication. One patient, who only received three single DNA immunizations, moved abroad in a healthy state. The immunizations were well tolerated, without significant local or systemic reactions. The safety of DNA immunization in humans has also been reported by others (202, 325).

The four patients who received both DNA immunizations and HAART showed increased CD4+ counts and significantly decreased viral loads. Probably, these changes were influenced by HAART. We found no evidence that HIV-DNA immunizations alone caused any significant decrease in viral load and/or increase in the CD4+ counts.

The DNA immunization studies described in this thesis were open-label and also have drawbacks such as a small number of patients, no blinded controls, and a selected group of patients. We have already started a new study, which is double-blind, in which patients on HAART are immunized with the combination of DNA constructs that encode the HIV-1 regulatory genes. The patients have a low viral load at entry and we wish to stimulate antigen-specific immunity. The future will include non-infected persons, and we hope that the studies described in this thesis will contribute to the development of such a prophylactic DNA vaccine.

PERSPECTIVE

Several DNA vaccines have been developed during the last 5 or 6 years. These developments have occurred despite the questions which remain to be elucidated in this field. This new approach has several advantages over traditional vaccines. DNA vaccines offer a simple alternative for generating both antibody and cell-mediated (CTL and proliferation) immunity. In contrast to live attenuated vaccines, there is no risk of infection. The DNA sequences can be manipulated to present part or all of the genome of the microbe of interest, allowing removal of the genes that might lead to adverse effects. Alternatively, cytokine genes or immunostimulatory sequences can be added to the plasmid in order to increase the immune responses specific to the encoded gene(s).

The studies presented in this thesis demonstrated that the therapeutic HIV-1 *nef*, *rev* and *tat* DNA vaccine is capable of inducing cellular and humoral immune responses in humans. Other investigators have used other HIV-1 genes which were given in other doses and schedules. Further work is needed to optimize the constructs that encode the gene(s), as well as the immunization strategies. Soon, our ideas and those from other groups of investigators will contribute to elucidate the optimal vaccine strategy against HIV-1.

CONCLUSIONS

We developed easy and inexpensive serological methods for the diagnosis and follow-up of children born to HIV-1-infected mothers. A quantitative immunofluorescence method replaced the cumbersome Western blot. The possibility of titrating antibodies made discrimination between HIV-infected and noninfected children feasible. In addition, we have shown that an anti-HIV-1 IgA assay is effective for early diagnosis of HIV-1-infected children, with a significant and high detection in children over 6 months of age.

Epitope reactivity might discriminate between different HIV-1 infected populations. Specificity of antibodies directed to conserved regions of HIV-1 was compared between patients from Argentina and Sweden. A new immunodominant region of gp41 was identified, against which the majority of Argentinian and Swedish patients showed reactivity. Although the epitope did not appear to be involved in functional reactivity, it may be useful as a diagnostic tool.

Reactivity against peptides representing the immunodominant third variable region of HIV-1 gp120 was analyzed in sera from Argentinian patients. Some peptides representing the subtype B showed low reactivities, indicating the need to identify and characterize local strains in order to design adequate diagnostic and vaccine strategies.

The efficacy of HIV-DNA, a novel class of vaccines, was evaluated for induction of immune responses in humans. Asymptomatic HIV-1-infected patients were immunized with DNA constructs encoding either HIV-1 *nef*, *rev* or *tat* regulatory proteins. Patients were selected for having no or low antibody reactivities to the antigen encoded by the plasmid DNA used for immunization. The DNA immunization induced HIV-specific cytotoxic and proliferative cellular responses. Increased levels of cytotoxic memory cells were induced in all DNA-immunized patients. Antibody induction was of a low magnitude. This demonstrates for the first time that HIV-DNA vaccination may be capable of inducing or re-inducing immune responses in HIV-infected humans.

Intensive chemotherapy is capable of reducing the viral load in HIV-1 infected individuals while infected cells are still present. HIV-specific cellular immune responses were evaluated in patients who started highly active antiretroviral treatment (HAART) during or after DNA immunizations. Significant reductions in viral load and increases in CD4+ counts were observed in those patients. DNA immunization by itself did not reduce viral load. The initiation of HAART therefore appears to contribute to the induction of HIV-specific CTL responses but by itself did not cause obvious re-induction of these activities.

The efficacy of a combination of plasmids encoding the HIV-1 regulatory genes (*nef*, *rev* and *tat)* was evaluated. The most remarkable change observed after immunization with the gene combination was the appearance of memory CTL against autologous targets infected with HIV-1. Autologous cells infected with HIV-1 will present all viral peptides and represent the *in vivo* situation. The possibility of enhancing HIV-specific immune responses in immunodeficient individuals is promising for continued genetic vaccine development.

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