Capacity of cytotoxic T lymphocytes to control the reproduction of human immunodeficiency virus

Het vermogen van cytotoxische T lymfocyten om de vermenigvuldiging van humaan immunodeficiëntie virus te beperken

Proefschrift

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aan Lidy en mijn ouders
voor Max en Coen

Contents

CHAPTER 3

Antiviral activity of HIV-1-specific cytotoxic T lymphocytes

CHAPTER 4

Vaccination with regulatory versus structural viral proteins

CHAPTER 5

Discussion

CHAPTER 6

Summary

CHAPTER 1

Introduction

Partially based on:

Van Baalen, C.A., M.R. Klein, A.M. Geretti, I.P.M. Keet, F. Miedema, C.A.C.M. Van Els, and A.D.M.E. Osterhaus. Selective in vitro expansion of HLA class I-restricted HIV-1 gag-specific CD8+ T cells from seropositive individuals: Identification of CTL epitopes and precursor frequencies. *AIDS 7:781-786 (1993)*

Geretti, A.M., C.A. van Baalen, J.C.C. Borleffs, C.A.C.M. Van Els, and A.D.M.E. Osterhaus. Kinetics and specificities of the T helper cell response to GP120 in the asymptomatic stage of HIV-1 infection. *Scand.J.Immunol. 39:355-362 (1994)*

1.1. AIDS

1.1.1. History

In 1981 and 1982, the incidence of unusual immunodeficiency-associated conditions increased among homosexual men and intravenous drugusers (1). Cases of *Pneumocystis carinii* pneumonia (PCP), Kaposi's sarcoma, mucosal candidiasis, disseminated cytomegalovirus infection, and chronic perianal herpes simplex ulcers were associated with evidence of T-lymphocyte dysfunction. The populations afflicted by this acquired immunodeficiency syndrome (AIDS) expanded to include hemophiliacs, transfusion recipients, sex-partners of risk-group members and children born to mothers at risk. These observations pointed to a transmissible agent that spread through genital secretions and blood. The search for such an agent led in 1983 to the discovery of a new retrovirus called lymphadenopathy-associated virus (LAV) (2), later renamed human immunodeficiency virus (HIV) (3). Following detection of HIV infection in clusters of AIDS patients (4-6), further epidemiological data and the fulfillment of Koch's postulates have established that HIV causes AIDS (7). By the end of 2001, more than 60 million people had become infected with HIV (8). An estimated 20 million people have already died, 40 million are incubating the virus, and five million became newly infected in 2001. About three million people have died from AIDS in 2001, mostly in sub-Saharan Africa (8;9).

1.1.2. How does HIV cause AIDS?

Virions infect cells after binding to CD4 molecules and chemokine receptors on the cell surface (10), by fusion of the viral and cellular membranes. Initial target cells in rectum, genital tract and bloodstream include CD4 bearing cells of the monocyte-macrophage lineage and lymphocytes (1). Infected cells and progeny virus bound to DC-SIGN on dendritic cells (11) disseminate to lymphoid organs and brain (12). Resident cells susceptible to infection sustain reproduction of the virus and may die in the process, either by cytopathic effects of high-level virus production or by antiviral immunity. A fraction of infected cells produce little or no virus and thus facilitate persistence of virus partly as integrated provirus (see below). Viruses can also persist as extracellular particles in the network of follicular dendritic cells (FDC) (13;14).

Because $CD4^+$ cells are central to immune responses against pathogens in general, ongoing HIV replication and associated direct and indirect mechanisms of $CD4^+$ cell loss (15;16) result in progressive immunodeficiency, until individuals die from AIDS (12). Fig. 1 shows a typical course of HIV infection in absence of therapy. Mean and median periods from time of infection to onset of AIDS are between 7 and 10 years in individuals over 12, and range from one to more than 20 years (1;12). Many studies distinguish rapid progressors (<3 years) from typical progressors (8-10 years), long-term asymptomatics or survivors and slow to non-progressors (>12-15 years) (17;18). Although without therapy relatively few individuals are in latter category, the reasons for their long-term survival may provide insights into possible preventive and therapeutic interventions for others. Multiple factors affecting rates of disease progression in infected individuals have been described, including genetic characteristics of the infecting virus and the recipient, as well as qualitative and quantitative aspects of HIV specific immune responses (12;17-20).

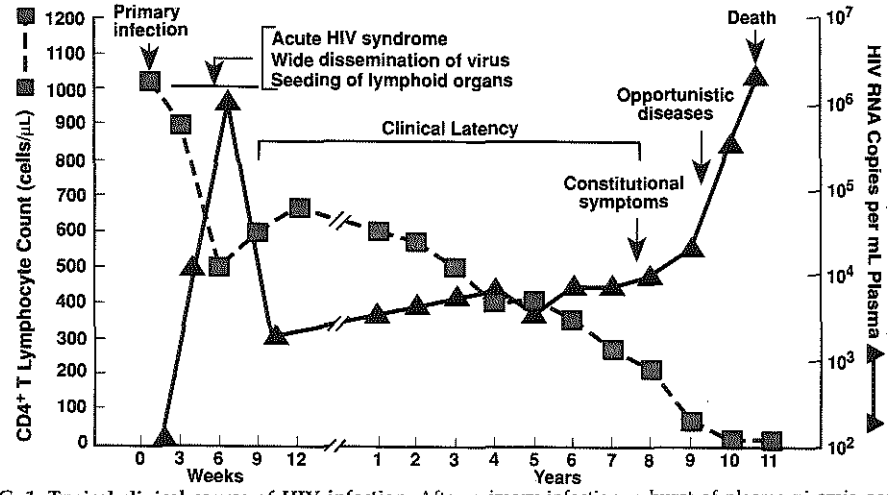

FIG. 1. Typical clinical course of HIV infection. After primary infection, a burst of plasma viremia occurs in concert with a transient decline in CD4⁺ T cell numbers. Partial immune control over viral replication ensues, resulting in a variable period of clinical latency. As the number of CD4⁺ T cells declines, the risk of developing constitutional symptoms and opportunistic diseases increases. (Reprinted from ref. 12, with permission.)

1.2. HIV

1.2.1. Classification, genome organisation and proteins

HIV is a member of the genus *lentivirus* of the *Retroviridae* family (21) on basis of their ability to reverse transcribe RNA into DNA (Latin *retro*: backward), morphological and biochemical characteristics, genome organization and gene homology. Lentiviruses are not oncogenic like other retroviruses such as human T cell leukaemia virus type I and II, but cause persistent infections resulting in a variety of chronic disorders and slow disease progression (Latin *lentus*: slow). On basis of serologic and genetic characteristics two types of human lentiviruses are distinguished, HIV-1 and HIV-2 (22). The relationship to lentiviruses of other primates and non-primates is shown in Fig. 2. Phylogenetic analysis indicates that HIV-1 originated from a simian immundeficiency virus lineage, SIV_{CPZ}, in chimpanzees (23;24), while HIV-2 arose from SIV lineages in other non-human primates (25), following multiple zoonotic transmissions of lentiviruses that originated from a common ancestor.

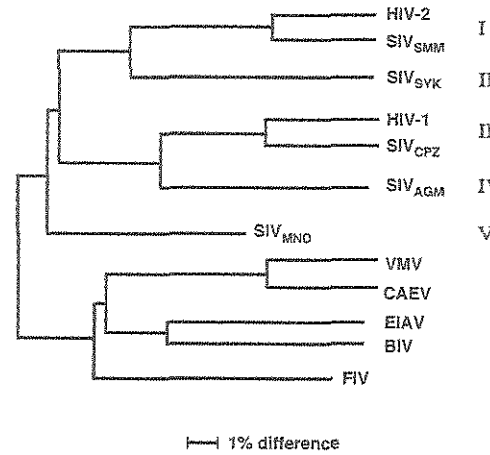

FIG. 2. Phylogenetic relationship of lentiviruses. Representative lentiviruses are compared using *pol* gene sequences. Five groups of primate lentiviruses are shown: HIV-1, HIV-2, SIV from sooty mangabey monkey (SIV_{SMM}), Sykes monkey (SIV_{SYK}), chimpanzee (SIV_{CPZ}), African green monkey (SIV_{AGM}), and mandrill (SIV_{MND}). Nonprimate lentiviruses are VMV: Visna-Maedi virus (sheep); CAEV: caprine arthritis-encephalitis virus (goat); EIAV: equine infectious anaemia virus (horse); BIV: bovine immunodeficiency virus (cow); and FIV: feline immunodeficiency virus (cat). The scale indicates percentage difference in nucleotide sequences in the pol gene. (Adapted from (22) in (21), reprinted with permission.)

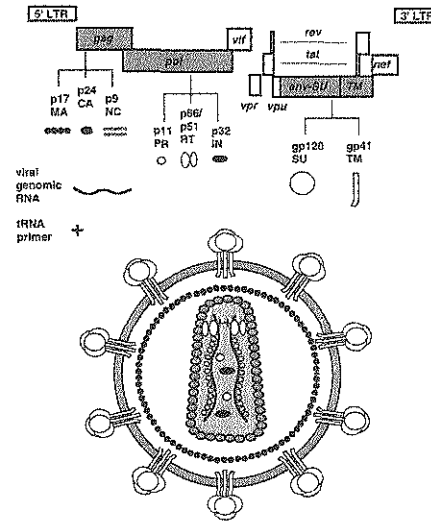

FIG. 3. Genome organisation and virion structure. The top portion of the figure shows the HIV-1 genome, with virion structural genes heavily shaded and accessory genes lightly shaded. Precursor polyproteins Gag-Pol, Gag, and Env are enzymatically processed to yield mature virion proteins. Gag-Pol and Gag undergo several cleavage steps mediated by the viral aspartic PR to produce eight smaller proteins. Env is cleaved once by a cellular PR, producing the SU gp120 and TM gp41. Env gp are represented as trimers. Exact positions of the proteins PR, RT, and IN in the viral core have not been elucidated. (Reprinted from ref. 21, with permission.)

Typical lentivirus particles are spherical, about 110 nm in diameter, and consist of a lipid bilayer membrane surrounding a conical nucleocapsid (Fig. 3) (21). Infectious virions contain two copies of single-stranded RNA, about 9.2 kb long, that have positive polarity with respect to translation. In the early stages of infection, the virion RNA genome is converted into double-stranded linear DNA by the process of reverse transcription, via viral-encoded reverse transcriptase (RT) (Fig. 4). The linear viral DNA is integrated into the host cell genome to produce the provirus. Genomic viral RNA is synthesized by cellular RNA polymerase II from proviral DNA. HIV encodes precursor polypeptides for virion proteins as well as several

TABLE 1. *Genes and proteins of primate lentiviruses*

Gene	Dispensable for Replication	Protein	Function	Localization
gag	No	$Pr55^{gag}$	Polyprotein precursor for virion core proteins MA (p17), CA (p24), NC (p9), p7	Virion nucleocapsid
pol	No	$Pr160^{gag\text{-}pol}$	Polyprotein precursor for virion enzymes PR: p10, RT and RNAse-H: p51/66, IN: p32	Virion (nucleocapsid?)
vif	Yes	p23	Viral infectivity factor, function unresolved	Cell cytoplasm
vpx[a]	Yes	p16	Virion protein, function unresolved	Virion
vpr	Yes	p15	Virion protein, function unresolved	Virion
tat	No	p14	Transcriptional transactivator, binds TAR and cell factor(s) (initiation and elongation of viral transcripts)	Primarily in cell nucleus
rev	No	P19	Posttranscriptional transactivator, binds RRE and cell factor(s) (splicing and/or transport and translation of viral mRNA)	Primarily in cell nucleus
vpu[b]	Yes	p16	Influences virus release, augments turnover of CD4 antigen	Integral cell membrane protein
env	No	gp160	Precursor for envelope glycoprotein: SU (gp120): CD4 receptor binding, TM (gp41): membrane fusion	Virion envelope, plasma membrane
nef	Yes	p27	Negative effector? , downregulates CD4 receptor and MHC, influences T-cell activation, enhances virion infectivity	Cell cytoplasm, plasma membrane

[a]Encoded only by HIV-2 and several SIV strains. [b]Encoded only by HIV-1 and SIVcpz. MA, matrix protein; CA, capsid protein; NC, nucleocapsid protein; PR, protease; RT, reverse transcriptase; IN, integrase; SU, surface glycoprotein; TM, transmembrane protein; TAR, tat-responsive element; RRE, rev-responsive element; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus. Table adapted from reference 21.

additional open reading frames (Table 1)(Fig. 3). The *gag* gene encodes the precursor for virion capsid and matrix proteins, the *pol* gene encodes the precursor for several virion enzymes [protease (PR), RT, RNase H, and integrase (IN)], and the *env* gene encodes the precursor for envelope glycoprotein (Env). The transcriptional activator (*tat*) and regulator of viral expression (*rev*) genes are each encoded by two overlapping exons and produce small non-virion proteins that are essential for viral replication. HIV also encodes several genes which are non-essential (i.e., dispensable) for viral replication in tissue culture cells. Non-essential genes, also designated "accessory" or "auxiliary" genes, encoded by HIV- I are *vif, vpr, vpu,* and *nef.*

1.2.2. Intracellular steps in viral replication

Following entry, reverse transcription and integration, proviral DNA is transcribed by cellular RNA polymerase II in the nucleus (Fig. 4) (21). Initially the level of viral-specific transcription is low in the absence of the transcriptional transactivator Tat. Viral transcripts in the nucleus may follow one of two pathways: A Rev-independent transport pathway, in which association with spliceosome components leads to removal of introns prior to export; or a Rev-dependent transport pathway, which leads to direct export of unspliced mRNA to the cytoplasm. The latter pathway requires a Rev responsive element (RRE) in the transcripts. Early in infection, before a significant amount of Rev protein has accumulated, the splicing pathway predominates. Multiply spliced messages, about 2 kb in size, are translated in the cytoplasm, and virion regulatory proteins Tat and Rev shuttle back to the nucleus as directed by basic nuclear localization domains in each protein. Later in the infectious cycle, increased levels of Tat in the nucleus lead to increased transcription of viral mRNAs. As Rev accumulates in the nucleus, more RRE-containing viral transcripts associate with Rev and are shuttled via the Rev-dependent pathway out of the nucleus. In the cytoplasm, these unspliced (9 kb) and singly spliced (4 kb) messages are translated to produce virion structural polyproteins (Gag, Gag-Pol, and Env) and accessory proteins (Vpu, Vif, Vpr, and Vpx). The $Pr55^{gag}$, $Pr160^{gag\text{-}pol}$ and viral RNA assemble to form the viral core that

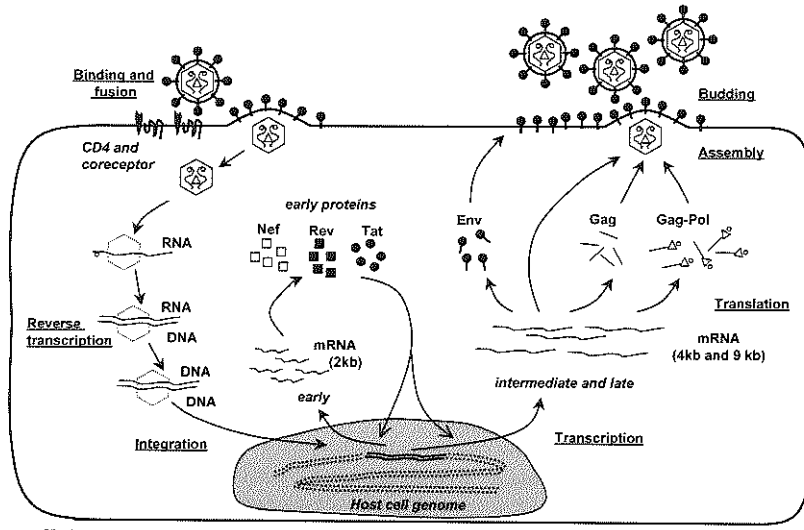

Fig. 4. Intracellular steps in viral replication. See main text for detailed description.

buds through the host cell membrane to form new virions. In addition to viral Env proteins, a select set of host cell proteins assemble into the membrane of mature virions, including HLA molecules. These may influence virion attachment and entry into T-lymphocytes, macrophages and other cell types.

1.2.3. Kinetics of transcription, protein expression, replication and reproduction

Multiple spliced transcripts are the first to be induced (26;27), and become detectable between 6-8 hours after infection of T cells with HIV-1 (28). Unspliced mRNA levels remain low until 24 hours after infection in T cells, followed by a sharp increase which coincides with onset of virion release (26) (Fig. 5). A similar pattern of viral gene expression has been observed in macrophages acutely infected with HIV and in chronically infected T cells and promonocytes following activation by phorbol esters (27-30). Following transmission by fusion of chronically non-productively infected cells to uninfected cells, *gag* and *env* transcripts do not increase significantly for at least 16 hours, and by 24 hours 20-fold and 7-fold higher levels were observed (31). In line with the ordered appearance of mRNAs, detection of Rev, Tat and Nef proteins can be as early as 6 hours following acute infection in T cells, while Env remained undetectable up to 12-16 hours and was first detected at 24 hours in these cells (32).

Productively infected cells were estimated to have an average life span of about two days (33;34). Estimates for the average generation time - defined as the time from release of a virion until it infects another cell and causes the release of a new generation of viral particles - range from 1.8 to 3 days in *in vivo* (34) and *in vitro* (35) studies, respectively. Another important measure for viral replication and reproduction dynamics is the number of cells infected by the viral progeny of an infected cell during its lifetime (if susceptible cells are not limiting), the basic reproduction ratio R_0 (36). If R_0 becomes smaller than 1, i.e. each HIV infected cell produces on average less than one newly infected cell, ongoing viral replication will be extinghuished. During the logarithmic growth phase of primary HIV-1 infection, a mean R_0 of 19.3 (range 7.4 to 34.0) was found in eight patients, suggesting that interventions of at least 95% efficacy would be needed to extinghuish productive viral infection (37).

The kinetics and dynamics described relate to active HIV-1 replication and

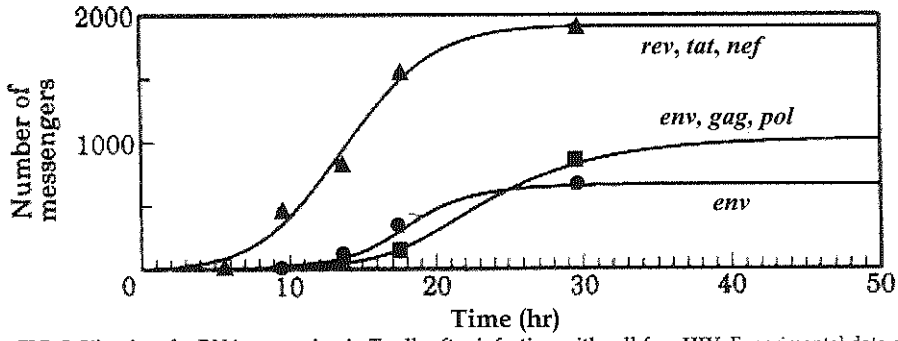

FIG. 5. Kinetics of mRNA expression in T cells after infection with cell-free HIV. Experimental data of Kim et al. [26], where levels of multiple spliced (early), singly spliced (intermediate) and unspliced (late) mRNA are represented by triangles, circles and squares, respectively. The curves show the results of a mathematical model that describes the biochemical processes that together determine proviral expression in this system [83].

reproduction *in vivo* or *in vitro* or both. Virus that persists *in vivo* as provirus in resting latently infected and long-lived cells, or as infectious particle in the FDC network, is not expected to contribute significantly to the virus produced at any one time. However, its potential to become re-activated can contribute to the chronic nature of HIV-1 infection and poses the most difficult challenge to any intervention, immune-mediated or otherwise.

1.3. Immune responses and correlates of protection

Both innate and adaptive immune systems respond to HIV infection, and many studies support their contribution to establishing and maintaining the clinically asymptomatic state (12;38). However, certain responses may become directly or indirectly harmful to the host (12), and the lack of clearly defined correlates of protective immunity to HIV infection still presents a major obstacle to the development of effective immunotherapies and vaccines (12;17;39).

1.3.1. Innate immune response

The soluble and cellular components of the innate immune system form the first line of defence against invading pathogens, including HIV (reviewed in refs. 38;40). Among the soluble components with anti-HIV activity are mannose-binding lectins (MBLs), complement, chemokines and other cytokines, e.g. tumor necrosis factor-α and interferons. In addition to directly affecting the extent of HIV replication, chemokines and cytokines can influence adaptive immune responses by driving T helper 1- or T helper 2-type responses, by recruiting natural killer (NK) cells, T cells and macrophages to sites of HIV infection, and by increasing the cytotoxic function of these cells. The cellular components of innate anti-HIV responses can involve neutrophils, dendritic cells, NK cells, γδ T cells, certain IFN-producing cells, and $CD8^+$ T cells that suppress viral transcription but lack most characteristics of HIV-specific cytotoxic T lymphocytes. Even if protection by innate immunity is not complete, the rapid nature of this system can provide time and conditions for adaptive immune responses to develop (38).

1.3.2. Humoral immune response

In primary HIV infection, a humoral immune response to HIV can usually be detected within 2 weeks of the onset of symptoms associated with acute infection (15;41). Clearance of HIV from the peripheral blood following primary HIV infection is generally thought to result, at least in part, from the activity of neutralizing antibodies and formation of antigen-antibody complexes (42). Complement-activating antibodies may be involved in virus neutralisation, but also in enhancement of infection (41;43). Induction of antibodies that could paradoxically facilitate HIV spread *in vivo*, and induction of inadequate levels of neutralizing antibodies, may explain in part the failure of neutralizing antibodies to prevent progressive HIV infection. Another explanation may be the high frequency of genetic mutations that occur during viral replication and lead to escape from neutralizing antibodies. This allows outgrowth of variant HIV strains that are not neutralized by existing strain-specific antibody responses (15).

1.3.3. Antibody-dependent Cellular Cytotoxicity (ADCC)

Some HIV-specific antibodies bind to IgG Fc-receptor-positive cells and sensitize them to mediate cytotoxic activity against infected or virus-coated cells (reviewed in (12)). These antibodies develop soon after primary infection and are detectable throughout the subsequent course of HIV infection, with some decrease in titres with the onset of AIDS. They have been shown to be directed primarily towards the HIV gp120 region, but ADCC against gp41 expressed on the cell surface has also been reported. Although $CD16^+$ NK-cells are important mediators for ADCC, monocytes may also mediate ADCC against HIV.

1.3.4. Cytotoxic T lymphocyte (CTL) response

The acute response and initial control phase in HIV infection is dominated by $CD8^+$ CTL (44;45), and many studies indicate that they are an important component of an effective host defence (see for recent reviews (46;47)). With their T cell receptor (TCR), these cells can recognize fragments of viral proteins that are presented by molecules of the class I major histocompatibility complex (MHC), in humans known as the human leucocyte antigen (HLA) system, on the surface of antigen presenting or infected cells (48) (Fig. 6). If CTL receive an appropriate signal, they can reduce HIV production by releasing factors that lyse infected cells or inhibit virus production non-lytically (49). However, spreading of virus will be controlled effectively only if inhibition of virus production occurs before a significant number of virions is released (50). The effectiveness of CTL, i.e. their capacity to reduce viral reproduction, has been directly addressed in relatively few studies (49;51-54), and is an important focus of the studies presented in this thesis.

Most studies on HIV-specific CTL have addressed the fine-specificity and magnitude of the response at different stages of infection (reviewed in (46;49;55)). Initially, these data have been obtained by examining the ability of cells from infected individuals to lyse autologous immortalized B lymphoblastoïd cell lines (B-LCL) that present HIV-1 epitopes, either following infection with recombinant vaccinia virus (rVV) containing genes encoding HIV-1 proteins or after addition of peptides that correspond to HIV-1 protein sequences. These cell lines are chromium labeled, allowing an indirect measure of cytolysis by measuring chromium release after incubation with PBMC or CTL clones. With this technology, PBMC from many infected individuals have been found to contain CTL directed against a variety of viral proteins including Gag, Pol, Env, Nef, Vif, Tat, and Rev (49;56).

Frequencies of circulating CTL were initially determined by limiting dilution cultures of PBMC after stimulation with antigen or non-specific agents. New techniques to address the quantity and specificity of CTL include *ex vivo* staining of

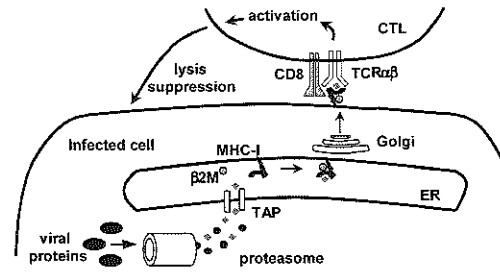

Fig. 6. Presentation of antigen to a $CD8^+$ T cell. Viral proteins are degraded by proteasomes yielding peptides that are transported to the lumen of the endoplasmatic reticulum (ER) by TAP molecules. Peptides associate with MHC molecules and migrate to the cell surface through the Golgi complex. Interactions between MHC-peptide complexes on antigen presenting or infected cells, and T cell receptor and CD8 molecules on T cells, can trigger activation signals and effector functions of T cells.

PBMC with fluorescent HLA-peptide complexes to detect cells with HIV-specific TCRs, and Elispot or flowcytometric assays to detect cytokine production by individual virus-specific cells (46).

The *in vivo* dynamics of CTL responses, viral loads and emergence of viral escape variants indicate that CTL exert considerable pressure on HIV replication during the primary and chronic stages of infection (19;47;49;57). In macaque models for AIDS, depletion of $CD8^+$ cells, before or after infection, resulted in increased viremia, prolonged depletion of $CD4^+$ T cells, and accelerated disease progression (58-60). Evasion of HIV from CTL pressure during the different stages of infection has been attributed to escape by mutation (61;62), persistence of virus in latently infected cells, immune-privelidged sites (63) or FDC-networks (13), and impairment of immune responses (19;20).

1.3.5. Helper T lymphocyte response

Central to induction and maintenance of both humoral and CTL responses is the helper T-cell response, mediated by T cells that express CD4. Proliferative responses of $CD4^+$ T cells to several viral antigens, including Gag, Pol, and variable and constant domains of gp120, are generally low and tend to decline over time (64;65). Production of IL-2 in response to gp120 peptide stimulation can be detected in individuals without proliferative responses and appears a longer preserved parameter of T helper function (64). Only in individuals who maintain low to undetectable viral loads and high CD4 cell counts for many years without treatment, robust HIV-1-specific proliferative responses can be demonstrated, directed predominantly at Gag (66).

One likely factor that can explain impairment of $CD4^+$ T cell help, and as a consequence the quality of humoral and CTL responses, is that HIV-specific $CD4^+$ T cells may be exceptionally susceptible to attack and destruction by HIV (16;47). HIV-specific T cells will become activated in the proximity of HIV-bearing or HIV-infected dendritic cells. Because activation of $CD4^+$ T cells facilitates productive infection, they are likely to be specifically eliminated.

1.4. Intervention strategies

The immunological and viral dynamics of HIV infection indicate that virus induced immune activation results in an active balance between viral replication and other mechanisms facilitating viral persistence on the one hand, and effective antiviral control on the other. Events early in infection are likely critical to the extent of virus dissemination, the level at which virus persists and the rate of disease progression (12;67). Multiple distinct intervention strategies are required to reduce the present and future impact of HIV on individuals and societies throughout the world.

1.4.1. Social strategies

The scale of the HIV/AIDS epidemic has exceeded all predictions since its identification, and the impact on social capital, population structure and economic growth, continues to increase worldwide (9). However, an ever-growing AIDS pandemic is not inevitable. Even within severely affected regions, some nations or

communities within nations have managed to sustain low incidence, and others with large established epidemics have managed to achieve reduced incidence in key populations. Three universal principles underlie successful responses to HIV: i) inclusion of people living with HIV centrally in the response, as a source of creativity in devising solutions and to focus on the realities of the epidemic; ii) integration of education, prevention and care strategies; iii) concerted efforts against stigma associated with HIV that continues to constitute a major barier to effective action (9). Although critically important, these approaches alone will not suffice.

Additional biomedical strategies in the form of antiretroviral drug therapy have reduced the morbidity and mortality associated with HIV infection as well as the risk of mother-to-child transmission, but only in a small proportion of the world's population affected by HIV, and with several other serious restrictions. Therefore, efforts to develop effective vaccines and vaccination strategies remain essential in the fight against AIDS. Recent developments in drug therapy and vaccine design are outlined below.

1.4.2. Antiretroviral drug therapy

Treatment of HIV infected individuals with antiretroviral viral drugs became particularly successful when combinations of drugs that inhibit the activity of HIV protease and reverse transcriptase were introduced (12). Virus in plasma typically declines to undetectable levels within two to four weeks and a degree of immune reconstitution is often observed (12;68). Despite clear beneficial effects of these therapies for infected individuals, the need for alternatives is emphasized by several complicating factors. These include continued low-level virus replication, toxicities of long-term therapy, difficulties with long-term compliance to therapy, development of drug resistant virus variants and resting $CD4^+$ T cells that provide a reservoir for HIV-1 with a predicted decay rate of approximately 60 years (69-71).

Following controlled treatment interruption, $CD4^+$ and $CD8^+$ T cell responses against HIV-1 can be immediately enhanced concurrently with increased viral replication, without a loss in $CD4^+$ T cell count or loss of a suppressive response to reinitiated treatment (68;72;73). Indeed, structured treatment interruption (STI) has been proposed to be comparable to an "attenuated-live vaccine" approach, where viral antigens are autologous and the "attenuation" is provided by re-initiation of a suppressive treatment following the period of viral replication. If and how an STI protocol might achieve prolonged periods of suppressive immunity in different patients setting is not known, but it is likely that the use of additional immuno-therapeutics such as HIV vaccines would be complementary in enhancing STI-induced immune-mediated control (68).

1.4.3. Vaccination

There are few examples of natural protective immunity to HIV-1 infection and no clearly defined immune correlates to guide the development of an AIDS vaccine (39). As outlined above, lack of complete immune control of HIV-1 may result from several factors, including early infection and depletion of helper T cells, persistence of virus in long-lived cells, resting cells and FDC network, rapid genetic escape from CTL and failure of envelop glycoproteins to elicit potent neutralizing antibody responses from which no escape is possible. Complete protection against HIV infection may therefore be difficult to achieve. However, recent new insights

regarding the basic immunology of B- and T-cell responses to epitopes from HIV-1 and other pathogens are leading to new strategies to induce more effective immune responses than does the virus itself (74;75). These new approaches can increase immunogenicity, influence the type of response, induce local immunity and avoid inhibitory immune mechanisms (76).

To date, HIV vaccines have been evaluated in over 70 documented phase I (dose-escalation safety and toxicity), five phase II (expanded safety and dose optimization) and two phase III (efficacy) clinical trials (77). No significant safety concerns have arisen, but data on the efficacy of any of these vaccines in human population are not yet available. Because no single most promising AIDS vaccine approach has been identified, multiple vaccine concepts are being persued, including synthetic or highly purified subunit antigens and viral genes delivered by direct injection of plasmid DNA or by infection with recombinant viral vectors (39;78;79). Subunit vaccines are designed to be safer than whole-inactivated or live-attenuated vaccines, however, the purity of the subunit antigens often results in weaker immunogenicity. Immunologic adjuvants can improve the performance of subunit vaccines by targeting of antigen to APC, eliciting cytokines that direct Th1 or Th2 immune responses, promoting cell-mediated immunity including CTL, and reducing the number of immunizations or the amount of antigen required for protective immunization. Among the recombinant viral vectors, replication-defective forms of vaccinia such as MVA (modified vaccinia virus Ankara) and related poxviruses have been most widely studied and are furthest advanced in human studies (39). Other candidate viral vectors that have been developed and applied to vaccine research include: alpha viruses such as Sindbis-, Venezuelan equine encephalitis- and semliki forest virus (SFV); adenovirus; adeno-associated virus; poliovirus; herpes simplex virus; rabies virus; and vesicular stomatitis virus (39). Direct injection of DNA elicits potent humoral and cellular immune responses in rodents models, but it has been difficult to induce such robust responses in humans and non-human primates (39). However plasmid DNA vectors can be effective if combined with adjuvants, recombinant viruses or proteins in prime-boost regimens (39).

For preclinical evaluation of the efficacy of new combinations of proteins, DNA, adjuvants, viral vectors, administration routes and dosage, a number of animal models have been developed and provided encouraging results (24;80-82). Rhesus and cynomolgus macaques that can be infected with simian immunodeficency virus (SIV), or recombinants thereof containing the envelop of HIV-1 (SHIV) are most widely used. Informative comparison of results from the various vaccine studies is currently difficult due to the many differences in choices of animals, virus strains and vacination-challenge protocols. The most widely used non-primate models for lentivirus infection utilize equine infectious anemia virus and feline immuno-deficiency virus (81). The results of these non-primate vaccine trials demonstrate a remarkable spectrum of vaccine efficacy, ranging from apparently sterilizing protection to severe enhancement of virus replication. Although further studies are required to characterize properties of vaccines that drive immune responses towards protection or enhancement in these models, evaluation strategies for vaccine efficacy in humans and non-human primates should be designed with the potential for vaccine enhancement in mind.

1.5. This thesis - outline

The focus of the work presented in this thesis is on CTL directed against HIV-1. Chapter 2 addresses frequencies of circulating CTL, their specificity at the protein and epitope level, and associations of CTL responses with rapid or slow disease progression in HIV-1 infection. Studies on the capacity of CTL to control reproduction of HIV are presented in chapter 3. Together, the data presented in chapters 2 and 3 support the concept that CTL directed against the early regulatory proteins Rev and Tat are more effective in controlling HIV reproduction than CTL directed against the late structural proteins Gag and RT.

The main hypothesis addressed in this thesis is that CTL control reproduction of HIV more effectively if they are able to recognize infected cells earlier after the entry of virus. This would enable CTL to eliminate more infected cells before release of infectious progeny virus begins, and thus to prevent more subsequent infection cycles.

This hypothesis was also tested *in vivo* by comparing the ability of cynomolgus macaques to control an experimental SIV infection after being vaccinated with Rev and Tat or with Gag and RT. The results of these studies are in described in chapter 4. Chapter 5 discusses the findings presented in this thesis and their significance for AIDS vaccine development. A summary is provided in chapter 6.

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CHAPTER 2

Relationship between cytotoxic T lymphocyte responses and disease progression

Kinetics of Gag-specific Cytotoxic T Lymphocyte Responses during the Clinical Course of HIV-1 Infection: A Longitudinal Analysis of Rapid Progressors and Long-term Asymptomatics

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Summary

To gain more insight into the role of HIV-1–specific cytotoxic T lymphocytes (CTL) in the pathogenesis of AIDS, we investigated temporal relations between HIV-1 Gag-specific precursor CTL (CTLp), HIV-1 viral load, $CD4^+$ T cell counts, and T cell function. Six HIV-1–infected subjects, who were asymptomatic for more than 8 yr with $CD4^+$ counts >500 cells/mm^3, were compared with six subjects who progressed to AIDS within 5 yr after HIV-1 seroconversion. In the long-term asymptomatics, persistent HIV-1 Gag-specific CTL responses and very low numbers of HIV-1–infected $CD4^+$ T cells coincided with normal and stable $CD4^+$ counts and preserved CD3 mAb-induced T cell reactivity for more than 8 yr. In five out of six rapid progressors Gag-specific CTLp were also detected. However, early in infection the number of circulating HIV-1–infected $CD4^+$ T cells increased despite strong and mounting Gag-specific CTL responses. During subsequent clinical progression to AIDS, loss of Gag-specific CTLp coincided with precipitating $CD4^+$ counts and severe deterioration of T cell function. The possible relationships of HIV-1 Gag-specific CTLp to disease progression are discussed.

During progressive HIV-1 infection immune responses deteriorate with subsequent development of AIDS. Although several correlates for progression to AIDS have been identified, the exact mechanisms underlying immune dysfunction remain to be elucidated (1, 2). The clinical course of HIV-1 infection is determined by complex interactions between viral parameters, host properties, and cofactors. Virus-specific CTL that kill virus-infected cells are thought to be a major host defense against viral infections (3). Therefore, HIV-1–specific CTL may be important for controlling viral spread during acute HIV-1 infection (4) and for maintaining viral load at low levels during the asymptomatic phase (5). Observations from cross-sectional studies have shown absent or severely depressed HIV-1–specific CTL responses during advanced stages of HIV-1 infection (6–8). These studies however, have not resolved whether rapid progressors are non-responders to HIV-1 or whether HIV-1–specific CTL responses are elicited which subsequently diminish during progression to AIDS. In contrast, strong HIV-1–specific CTL responses have been proposed to cause immunosuppression in HIV-1 infection rather than being beneficial (9).

To gain more insight in the role of HIV-1–specific CTL in the pathogenesis of AIDS, we analyzed long-term asymptomatics (LTA)[1] and rapid progressors for precursor CTL (CTLp) specific for Gag, the protein of HIV-1 which is most predominantly recognized by CTL during asymptomatic HIV-1 infection (10–16). Longitudinal studies were undertaken to investigate temporal relations between Gag-specific CTLp, HIV-1 viral load, immune status, and clinical course of HIV-1 infection.

[1] *Abbreviations used in this paper:* B-LCL, B-lymphoblastoid cell line; CDC, Centers for Disease Control; CI, confidence interval; CTLp, precursor CTL; LTA, long-term asymptomatics; (N)SI, (non-)syncytium inducing; rVV, recombinant vaccinia virus; TCID, tissue culture infectious dose.

Materials and Methods

Study Population. The Amsterdam Cohort Studies on AIDS were initiated in October 1984 (17). Data about cohort participants were collected in three monthly visits that consisted of a standardized medical history and collection of blood samples for HIV-1 serology and cellular immunology. Date of entry for participants already HIV-1 seropositive at enrollment, or documented HIV-1 seroconversion, taken as the midpoint between the last seronegative and first seropositive visit, were used as reference points for clinical follow-up. Previously it was shown that participants who were HIV-1 seropositive at entry in the study, seroconverted within 1.5 yr before enrollment (18). By December 1991 there were 106 cohort-participants with documented HIV-1 seroconversion. By December 1992, 34 of these seroconvertors were diagnosed with AIDS (19, 20) according to 1987 Centers for Disease Control (CDC) classifications (21). For this study, six cohort participants were studied, who progressed to AIDS within 5 yr after HIV-1 seroconversion: P159, P186, P187, P224, P450, and P748. From 273 cohort-participants who either entered the study as HIV-1 seropositive or seroconverted before January 1986, 61 participants remained asymptomatic (CDC II/III) for more than 7 yr (22). For this study, six LTA were selected, who had at least 8 yr of asymptomatic follow-up and $CD4^+$ T cell counts >500 cells/mm^3: L008, L067, L090, L617, L709, and L206. Subject L206 is a patient who is monitored at The Academic Medical Centre in Amsterdam.

Immunological Markers. T lymphocyte immunophenotyping for CD4 and CD8 membrane markers was carried out at three monthly intervals by flow cytofluorometry. PBMC were stained with CD4 mAb (Leu-3a-PE; Becton Dickinson, Mountain View, CA) or CD8 mAb (Leu-2a-PE; Becton Dickinson) according to the manufacturer's protocols. Polyclonal T cell functions were measured in real time after May 1987 as previously described (23), by measuring the CD3-mAb (CLB-T3/4E; CLB, Amsterdam, The Netherlands) induced proliferative capacity of PBMC in whole-blood cultures. T cell reactivity is expressed as counts per minute.

Recombinant Vaccinia Viruses (rVV). rVV used in these studies were constructed from the Copenhagen strain of Vaccinia virus, and include rVV TG.1144 expressing Gag of HIV-1_{LAI} (24, 25) and control-rVV 186-poly containing no insert; kindly provided by Dr. Y. Rivière (Institut Pasteur, Paris, France) and Dr. M.P. Kieny (Transgène S.A., Strasbourg, France).

Induction of HIV1-specific CTL Responses. HIV-1-specific CTLp were expanded in vitro by Ag-specific stimulation as previously described (15). Frequencies of Gag-specific CTLp were determined using standard methods of limiting dilution analysis (26). Briefly, PBMC isolated and cryopreserved at different time points during the study were thawed and resuspended in IMDM supplemented with antibiotics and 10% pooled human serum. Eight serial dilutions of PBMC ranging from 20,000 to 745 cells/well were seeded in 24-fold in 96-well round-bottom microtiter plates. Stimulator cells were autologous EBV-transformed B-lymphoblastoid cell lines (B-LCL) infected with rVV-TG.1144 and subsequently inactivated with paraformaldehyde. To each well, 10^4 fixed stimulator cells and 10^4 autologous PBMC (30 Gy irradiated) were added, and microcultures were maintained for 15 d at 37°C and 5% CO_2. At day 2 and 9 cultures were fed with medium containing rIL-2 (10 U/ml; Cetus Corp., Emeryville, CA), and at day 7 they were restimulated with 10^4 fixed stimulator cells and rIL-2 (10 U/ml). On day 15, wells were split and effector cells tested for cytotoxicity.

Cytotoxicity Assays. Standard ^{51}Chromium-release assays were performed as previously described (15). Briefly, autologous B-LCL were infected with 5 MOI rVV-TG.1144 or rVV 186-poly and labeled with $Na_2{}^{51}CrO_4$ (Amersham Intl., Amersham, Bucks, UK) for 16 h. After three additional washings, 4×10^3 target cells were added to each well. After 4 h, supernatants were harvested and radioactivity was counted on γ-counter (Cobra II; Packard Instr. Co., Inc., Meriden, CT). Spontaneous ^{51}Cr-release was always <15% of maximum release. Specific lysis was calculated with the formula: 100× ([experimental release − spontaneous release]/[maximum release − spontaneous release]). Wells were considered positive when the ^{51}Cr-release exceeded 10% specific lysis. Statistical analysis was performed using methods as previously has been described by Strijbosch et al. (27). CTLp frequencies are expressed as number of CTLp/10^6 PBMC. Gag-CTLp frequencies were computed as differences between CTLp frequencies determined on Gag-expressing versus control targets. The average CTLp-frequency on control targets was <25/10^6 PBMC.

Virological Markers. Viral load in peripheral blood samples was determined using clonal virus isolation procedures as previously described (28). Briefly, 12,500–25,000 PBMC of HIV-1–infected patients were cocultivated with 10^5 2-d PHA-stimulated PBMC from HIV-1 seronegative blood donors. HIV-1 replication was monitored by screening culture supernatants for p24 production using a p24 capture ELISA. Statistical analysis of positive wells was performed using methods as previously has been described by Strijbosch et al. (27). Viral burden was expressed as tissue culture infectious dose (TCID)/10^6 $CD4^+$ T cells, representing the number of cells productively infected with HIV-1 in the peripheral blood. Biological phenotype of HIV-1 viruses was determined as previously described (29). Briefly, 10^6 PBMC of HIV-1–infected patients were cocultivated with MT2 cells, and cultures were monitored microscopically several times per week to check for syncytium formation to determine the viral phenotype.

Results

Natural History of HIV-1 Infection in Long-term Asymptomatics and Rapid Progressors. Six LTA who were selected for this study remained asymptomatic for >8.0 yr with $CD4^+$ counts >500/mm^3. Total follow-up period until October 1994 was 9.6 ± 0.4 yr. In addition, six cohort-participants who progressed to AIDS within 5 yr after HIV-1 seroconversion were also longitudinally studied. Mean time between HIV-1 seroconversion and AIDS diagnosis was 3.8 ± 1.2 yr. Clinical and laboratory findings of all studied subjects are presented in Table 1. Except for P186, all progressors suffered from severe to mild influenza-like disease in the 3 mo preceding HIV-1 seroconversion indicative for symptomatic acute HIV-1 infection (19), whereas none of the LTA reported history of primary HIV-1 infection in the months preceding seroconversion or enrollment in the cohort study.

$CD4^+$ T cell numbers in LTA were in the range of values found in healthy uninfected controls (90% confidence interval (CI): 560–1,550/mm^3). $CD4^+$ T cell counts of L090, L617, and L709 remained stable, whereas in subjects L008, L067, and L206, $CD4^+$ counts tended to decline towards the end of the study. $CD8^+$ T cell numbers of L617 and L206 were increased, whereas in other LTA $CD8^+$ T cell counts remained within normal range (90% CI: 310–1,000/mm^3). $CD4^+/CD8^+$ ratios were clearly reversed in L617; while in subjects L008, L067, and L206, ratios inverted after ~5–6 yr of follow up (Fig. 1 *A*). Upon HIV-1 seroconversion $CD4^+$ counts rapidly declined in five out of six progressors with

Table 1. *Clinical and Laboratory Data of LTA and Rapid Progressors*

Subjects	HLA Class I*	Seroconversion status‡	Age§	Virus phenotype‖	AIDS diagnosis¶	Follow-up**
LTA						
L008	A2,26;B27,44;Cw1,6	II	38	SI (95)	NA	>119
L067	A26,28;B7,57;Cw7	II	35	NSI	NA	>119
L090	A1,2;B41,57;Cw6	I	41	NSI	NA	116
L617	A2,11;B35,62;Cw3	II	28	NSI	NA	>115
L709	A1,69;B14,57;Cw6	I	29	NSI	NA	108
L206	A3,25;B18,51	II	31	NSI	NA	>111
Progressors						
P159	A1;B8;Cw7	I	46	SI (18)	CAO, PCP	32
P186	A3,24;B60,Cw3,4	I	30	NSI	PCP	42
P187	A1;B8;Cw7	I	34	SI (1)	HSV, TXP	28
P224	A3;B44,51;Cw4,7	I	28	SI (45)	PCP	60
P450	A24,28;B39,44	I	29	SI (52)	KS	65
P748	A1;B8;Cw7	I	29	SI (31)	CD4 <200	47

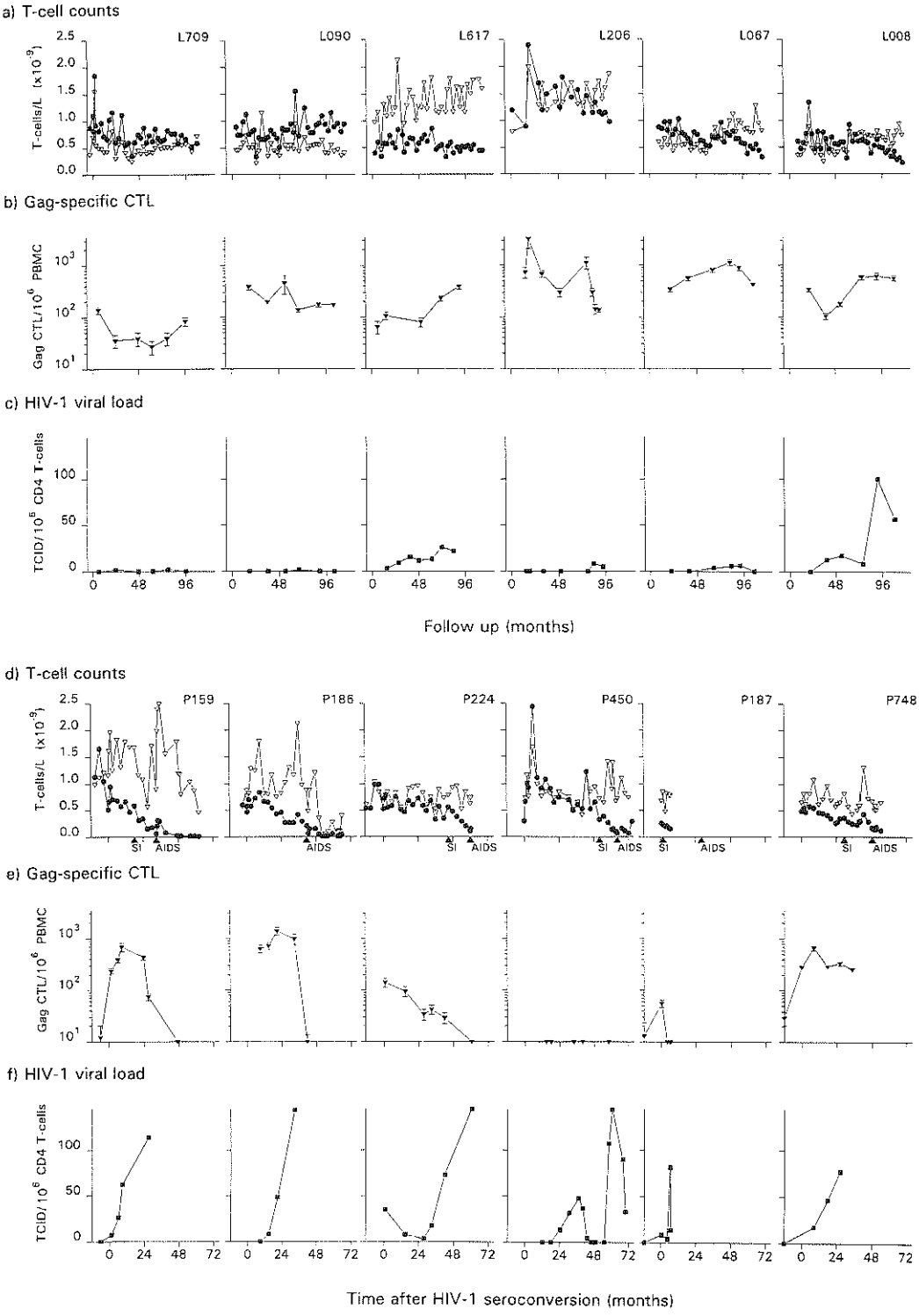
* HLA class-I typings were performed at Department Transplantation Immunology, CLB, Amsterdam, using standard serological typing methods.
‡ Known date of HIV-1 seroconversion (I) or seropositive upon entry in the cohort study (II).
§ Age (yr) at HIV-1 seroconversion or first seropositive visit.
‖ Biological virus phenotype: NSI vs. SI; number of months after seroconversion or seropositive entry at which NSI to SI switch occurred indicated in parentheses.
¶ AIDS diagnosis according to CDC classifications (21): PCP, *pneumocystis carinii* pneumonia; HSV, Herpes Simplex virus infection; TXP, Toxoplasmosis; CAO, *Candida albicans* oesophagitis; KS, Kaposi's sarcoma or $CD4^+$ T cell numbers <200/mm^3; NA, not applicable for LTA.
** Time (mo) between HIV-1 seroconversion or seropositive entry and AIDS diagnosis for progressors or October 1994 for LTA.

>140 cells/mm^3 per year. In subject P224 $CD4^+$ counts initially remained quite stable, but dropped precipitously after 45 mo of infection. In general, $CD4^+/CD8^+$ ratios were inverted after seroconversion, which in patients P159 and P186 was also due to elevated $CD8^+$ T cell numbers (>1,000/mm^3) (Fig. 1 *D*). T cell function of LTA, measured by CD3 mAb-induced proliferation, was stable and within the range of normal values (90% CI: 1,100–10,100 cpm), whereas T cell function in all progressors gradually diminished to below normal values (Fig. 2). We have shown this to be predictive for progression to AIDS (23).

Subject P748 remained asymptomatic during follow-up but $CD4^+$ counts dropped <200 cells/mm^3 within 4 yr after seroconversion. Currently P748 is being treated with AZT and *pneumocystis carinii* pneumonia (PCP) prophylaxis. Subject P187 became infected with HIV-1 after unprotected sexual intercourse with an AIDS patient. Six months after seroconversion he was lost for the cohort study, but kept monitored by a local general practitioner who also conducted AZT antiretroviral therapy and PCP prophylaxis. Subject P450 suffered from multiple allergic complaints during all stages of HIV-1 infection. General skin rash, eczema, erythema, and dermatomycosis were observed, as well as allergic reactions to rubber and allergic skin rash after treatment with erythromycin, cotrimoxazol, and ciprofloxacin. Except for L008 who started AZT treatment at 109 mo after entry, none of the LTA was subjected to anti-retroviral therapy.

Longitudinal Analysis of HIV-1 Gag-specific CTL Responses. During the entire follow-up period of the study Gag-specific CTL responses could be detected in all LTA. The observed CTLp frequencies were between 1/300–1/21,000; in the same range as previously has been reported by other investigators (8, 10, 13). In subjects L067 and L206, strong (average of >300 CTLp/10^6 PBMC) persistent Gag-specific CTL responses were detectable although they tended to decline at later time points (Fig. 1 *B*). Subjects L008, L617, and L090 had stable intermediate (average of 100–300 CTLp/10^6 PBMC) Gag-specific CTL activity during follow-up. Finally, subject L709 showed persistent but lower (average 20–100 CTLp/10^6 PBMC) Gag-specific CTL responses (Fig. 1 *B*). In addition, CTL responses from subjects L206, L008, and L617 were analyzed in greater detail, using series of overlapping peptides spanning the entire Gag sequence of HIV-1_{LAI}. Multiple CTL epitopes were identified mainly localized in Gag-p24 (15). In addition, after 7 yr of follow-up, CTLp frequencies for Nef and Env in subject L206 were only 53 and 32/10^6 PBMC, respectively; 7–10-fold lower than the Gag-specific CTLp frequency. In blood sampled from subject L090 5.7 and 7.7 yr after seroconversion no Env-specific CTL directed against the HIV-1_{LAI} sequence could be detected.

All tested progressors, except for patient P450, showed distinct Gag-specific CTL responses after seroconversion albeit with different kinetics (Fig. 1 *E*). Participants P159, P186, and P748 initially showed strong though transient Gag-specific


a) T-cell counts
L709
L090
L617
L206
L067
L008
T-cells/L (x10⁹)
b) Gag-specific CTL
Gag CTL/10⁶ PBMC
c) HIV-1 viral load
TCID/10⁶ CD4 T-cells
Follow up (months)
d) T-cell counts
P159
P186
P224
P450
P187
P748
SI
AIDS
e) Gag-specific CTL
f) HIV-1 viral load
Time after HIV-1 seroconversion (months)

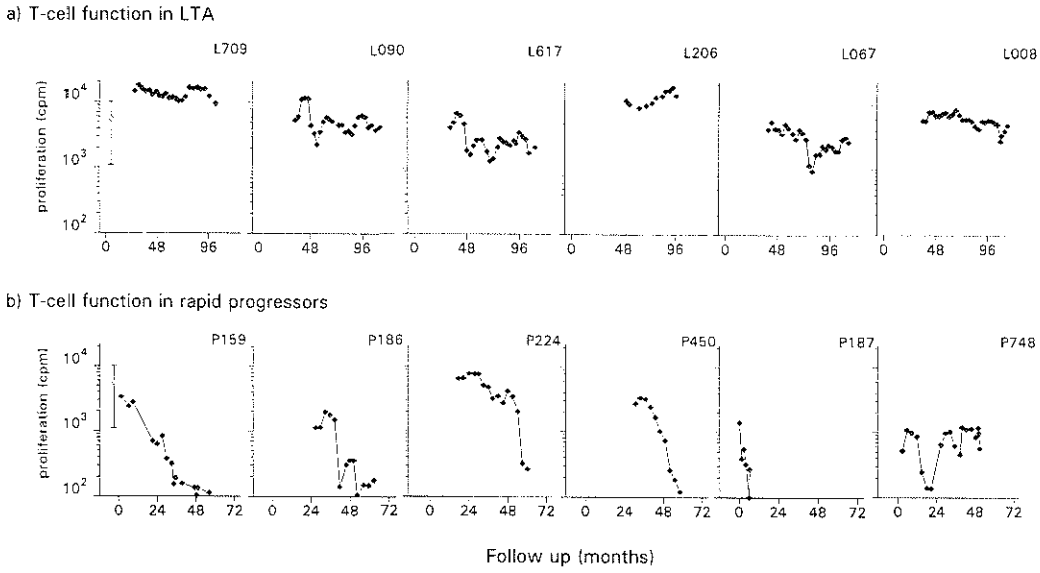


Figure 2. Longitudinal analysis of CD3-mAb induced T cell reactivity. Follow up on x-axis indicates time (mo) after HIV-1 seroconversion or HIV-1-seropositive entry in the study. In vitro T cell function (◆) of LTA (*A*) and progressors (*B*) is measured by whole blood proliferation assays using CD3 mAb, and is expressed as cpm. 90% CI of normal values are indicated by ◇.

CTL responses during the first 2 yr after seroconversion, with frequencies up to 700–1,400/10^6 PBMC. Gag-specific CTL responses in patient P224 gradually subsided over time. In patient P450 repeatedly no Gag-specific CTL responses could be detected in blood samples from 10 different time points (Fig. 1 *E*).

Virological Characteristics during the Clinical Course of HIV-1 Infection. In general, the number of circulating HIV-1–infected CD4$^+$ T cells was low in LTA. From blood samples of subjects L090 and L709 only 2, respectively, 3 virus clones were isolated at 65, respectively, 24 and 78 mo. From subjects L067 and L206, virus could only be isolated at later time points. On average, viral load was <5 TCID/10^6 CD4$^+$ T cells. From L617, virus could be isolated at all time points tested; the frequency of infected cells was low, although it tended to increase towards the end of the study (<25 TCID/10^6 CD4$^+$ T cells). In subject L008, there was a sudden increase of viral load up to 100 TCID/10^6 CD4$^+$ T cells after 91 mo of follow-up, coinciding with a change in viral phenotype (Fig. 1 *C* and Table 1). In all patients studied, viral load increased during progression to AIDS, with the number of infected cells up to 81–415 TCID/10^6 CD4$^+$ T cells. Except for patient P186, all progressors studied here developed AIDS with SI HIV-1 variants. Viral load in patient P450 showed a biphasic course, and the emergence of SI-variants coincided with a high number of HIV-1–infected CD4$^+$ T cells 4 mo before AIDS diagnosis. Increased decline of CD4$^+$ T cells and accelerated progression to AIDS is strongly associated with emergence of SI variants (30), which was most obvious in patient P224 (Fig. 1 *F*).

Discussion

We evaluated HIV-1 Gag-specific CTLp, HIV-1 viral load and general immune status in relation to clinical course of HIV-1 infection, to gain more insight in temporal relations between HIV-1 replication and host immune responses.

Persistent Gag-specific CTL responses and low numbers

Figure 1. Natural history of HIV-1 infection in LTA and rapid progressors. Follow up on x-axis indicates time (mo) after HIV-1 seroconversion or HIV-1-seropositive entry in the study. (*A* and *D*) Longitudinal analysis of CD4 (●) and CD8 (▽) T lymphocyte subsets. Reference values (90% CI) for CD4$^+$ and CD8$^+$ subsets were determined in a group of healthy HIV-1-seronegative volunteers (n = 430), and ranged from 0.56–1.55 and 0.31–1.00 * 10^9 cells/L, respectively. Arrows (▲) indicate time points when NSI to SI-phenotype switch occurred or date of AIDS diagnosis. (*B* and *E*) Longitudinal analysis of HIV-1 Gag-specific CTL responses in cryopreserved blood samples. Ag-specific CTL effectors were tested in split-well ^{51}Chromium-release assay on autologous B-LCL infected with rVV TG.1144 expressing *gag* or control targets infected with rVV 186 poly, containing no insert. Gag-CTLp frequencies (▼) were computed as differences between CTLp frequencies determined on *gag* versus control targets and normalized to the number of CTLp per 10^6 PBMC. Error bars indicate standard error of calculated frequencies. (*C* and *F*) Longitudinal analysis of HIV-1 viral load in peripheral blood samples. HIV-1 viral load was determined with clonal virus isolation procedures. The number of cells productively infected with HIV-1 is expressed as TCID/10^6 CD4$^+$ T cells (■).

of circulating HIV-1–infected CD4+ T cells were observed in LTA, together with stable and normal CD4+ counts and preserved T cell functions for more than 8 yr. This may indicate that HIV-1 Gag-specific CTL contribute to maintenance of the asymptomatic state by effectively controlling HIV-1 replication. However, in four out of six progressors, a rise of Gag-specific CTLp frequencies early in infection was paralleled by increasing numbers of HIV-1–infected CD4+ T cells. During subsequent progression, Gag-CTLp frequencies decreased severely in three out of four progressors. Subject P748 with CD4+ counts dropping below 200 cells/mm^3, impaired T cell function, SI viruses, and increasing viral load, all predictive for rapid progression to AIDS (2, 31), remained asymptomatic during follow-up. In this patient, Gag-specific CTLp remained relatively stable during the study period, which may be related to anti-retroviral treatment (32). In subject P224, Gag-specific CTL responses gradually decreased during progression to AIDS. At the time when CTLp frequencies were very low, an increase in viral load, change in biological viral phenotype and subsequent progressive depletion of CD4+ T cells was observed (1, 2). In progressor P450, no Gag-specific CTLp were detected at all. However, it could be that CTL recognizing strain-specific sequences of autologous HIV-1 variants are present that are not detected using prototype HIV-1 sequences. Furthermore, the presence of efficacious CTL responses directed against other antigens of HIV-1 can also not be excluded at the present time.

This longitudinal analysis revealed that five out of six rapid progressors were able to mount substantial Gag-specific CTL responses early in infection, with magnitudes comparable to those observed in LTA. In contrast to observations in LTA however, Gag-specific CTL responses were only transient and disappeared during progression to AIDS, apparently failing to contain viral replication and spread. Increase of viral load in the face of mounting Gag-specific CTL responses might be due to expanding HIV-1 variants which have escaped from CTL recognition (33), but a clear demonstration that these escape variants have selective advantage in vivo is still lacking (34, 35). Another explanation may be that, although CTL can be detected in vitro, they may not be able to execute effector functions in vivo. For example, IL-10, an immunosuppressive cytokine, which has been reported to induce a state of tolerance by downregulating allogeneic CTL responses in human long-term chimeric patients that received HLA-mismatched bone marrow transplants (36), may have frustrated in vivo CTL function in rapid progressors (37). In addition, as has been shown for mice infected with lymphocytic choriomeningitis virus, persistent viral infections may exhaust virus-specific effector CTL resulting in loss of immune surveillance (38).

Zinkernagel and Hengartner (9), have suggested that strong CTL responses in fact could be instrumental in deteriorating the immune system by depleting HIV-1–infected CD4+ T cells and APC. In LTA however, vigourous Gag-specific responses were not detrimental per se, since little loss of CD4+ T cells and well preserved T cell function were observed for more than 8 yr. In patients P224 and 450, precipitous loss of CD4+ T cells and T cell function were observed, only when changes in viral phenotype and viral load occurred, pointing to a role for HIV-1 next to cellular immunity in determining kinetics of clinical progression (28, 30).

In conclusion, our results show that long-term asymptomatic HIV-1 infection is characterized by sustained HIV-1 Gag-specific CTL responses and low numbers of circulating HIV-1–infected CD4+ T cells. Rapid progressors, however, were not protected from disease progression despite high Gag-specific CTLp frequencies early in HIV-1 infection. Besides quantitative aspects of Gag-specific CTL as analyzed here, repertoire differences and phenotypical and functional differences in CTL may contribute to control of HIV-1 infection (39).

Alternatively, based on these data, one could argue that HIV-1–specific CTL responses do not play a critical role in determining the rate of progression to AIDS. Sustained HIV-1–specific CTL activity may merely be a reflection of preserved cellular immunity as observed during long-term asymptomatic HIV-1 infection (22). Loss of HIV-1–specific CTL may be a reflection of progressive immunodeficiency induced by HIV-1 infection (1, 2). Our observations in the progressors suggest that HIV-1–induced perturbation of the immune system, rather than loss of HIV-1–specific CTL, could be the critical event. Clinical outcome of HIV-1 infection may be determined by host genetics (20), virulence of HIV-1 variants (40, 41), as well as by virus–host interactions already at the time of primary HIV-1 infection (42). Thus, our results warrant more detailed studies into underlying pathogenic mechanisms causing immune dysfunction to better understand differences between long-term asymptomatic HIV-1 infection and rapid progression to AIDS.

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Fine-specificity of cytotoxic T lymphocytes which recognize conserved epitopes of the Gag protein of human immunodeficiency virus type 1

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Human immunodeficiency virus type 1 (HIV-1) Gag-specific cytotoxic T lymphocyte (CTL) responses were studied in seven seropositive long-term asymptomatic individuals (CDC A1) with stable CD4 counts for more than 8 years. Using a set of partially overlapping peptides covering the whole Gag, five 15–20-mer peptides were found to contain CTL epitopes. Further characterization of these epitopes revealed a new HLA-A25-restricted CTL epitope in p24, $p24_{203-212}$ ETINEEAAEW. This region of Gag is highly conserved in clades B and D of HIV-1. Naturally occurring amino acid sequences, containing $p24_{203}$D (consensus HIV-1 clades A, C, F, G and H) or $p24_{204}$I (HIV-2_{ROD}) were not recognized by CTL recognizing the index peptide. No virus variants with mutations in this sequence were found in peripheral blood mononuclear cells from the HIV-1-infected individual concerned during the 8 year observation period, indicating that the virus had not escaped from the observed CTL response.

Introduction

A number of observations suggest a role for cytotoxic T lymphocytes (CTL) in mediating protection against human immunodeficiency virus type 1 (HIV-1) infection. HIV-1-specific CTL have been detected well before neutralizing antibodies (Koup *et al.*, 1994). In seronegative prostitutes who had been exposed to HIV-1 infection, and in seronegative children from HIV-1-infected mothers, the presence of HIV-1-specific CTL may be an indication of their contribution to protective immunity (Rowland-Jones *et al.*, 1993, 1995; Cheynier *et al.*, 1992). In a simian immunodeficiency virus (SIV)–macaque model we showed that vaccine-induced protective immunity may correlate with the presence of a major histocompatibility complex (MHC) class I haplotype and associated CTL responses (Heeney *et al.*, 1994). During the asymptomatic phase following the acute stage of infection, HIV-1-specific CTL can be detected in most individuals, with Gag being the most commonly recognized protein (Riviere *et al.*, 1994; Johnson & Walker, 1994).

In a recent study by Klein *et al.* (1995) we showed that frequencies of CTL detected early after infection, using HIV-1_{LAI} Gag as whole protein antigen, do not have a predictive value for the time that elapses before AIDS is diagnosed. Besides quantitative aspects, qualitative differences in CTL response may be important (Johnson & Walker, 1994; Van der Burg *et al.*, 1995). CTL directed against epitopes located within structurally constrained and thus conserved regions may be most effective in limiting virus replication and spread (Riviere *et al.*, 1994; Johnson & Walker, 1994).

In this paper we present data on the identification of HLA class I-restricted CTL epitopes in conserved parts of Gag, using peripheral blood mononuclear cells (PBMC) from long-term asymptomatic (LTA) individuals and a set of 48 partially overlapping peptides, which together span the entire protein.

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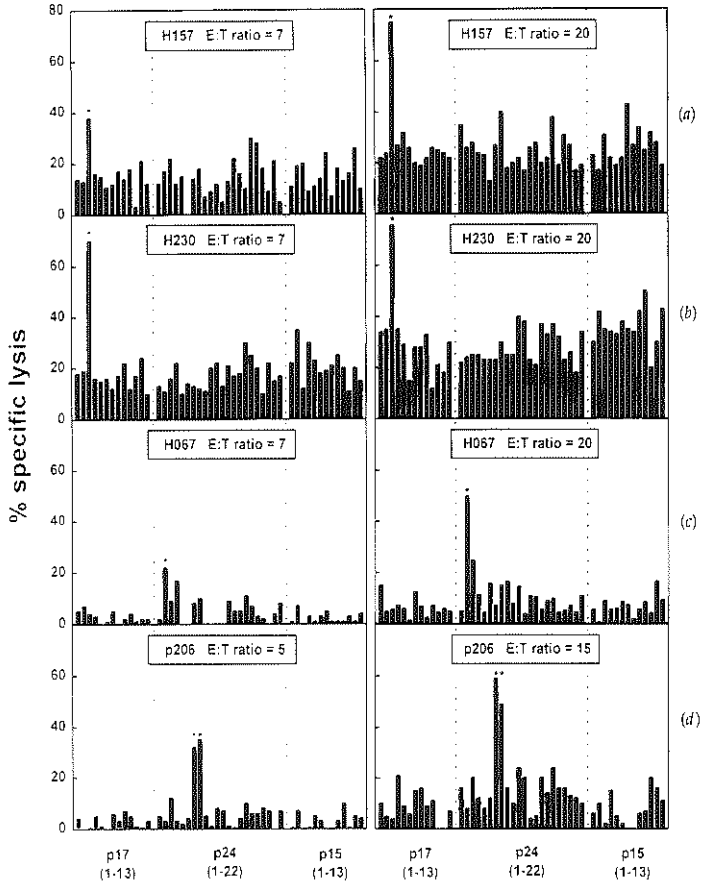

Fig. 1. CTL lines generated from the PBMC of LTA HIV-1 seropositive individuals H157 (*a*), H230 (*b*), H067 (*c*) and p206 (*d*) recognize one or two peptides from a set spanning the entire Gag. CTL lines were tested for lysis of 48 different target cell populations pulsed with one of 13 15-mer peptides (five residue overlap) spanning p17, or one of 22 20-mer peptides (ten residue overlap) spanning p24 or one of 13 15-mer peptides (five residue overlap) spanning p15. Preparation of target cells was as follows: autologous B-LCL were incubated with 100 μCi $Na_2{}^{51}CrO_4$ in 100 μl of medium for 1 h at 37 °C. Cells were washed twice and incubated with 20 μM-peptide in 100 μl of medium for 1 h. Subsequently, 900 μl of medium supplemented with 5% fetal bovine serum was added and cells were incubated overnight. Target cells were washed twice and suspended in 96-well plates at 5×10^3 cells per well. Results are expressed as percentage-specific ^{51}Cr-release (average of duplicates). Effector:target (E:T) ratios are indicated in a box in each graph. * indicates CTL responses directed at peptides that were selected for further characterization.

LTA may represent the best candidates for studies on the identification and characterization of potentially protective CTL and their corresponding epitopes. It may be expected that if indeed CTL are involved in protective immunity, their prevalence will be most pronounced in this group of individuals. Besides the identification of five regions of Gag that have been shown to contain CTL epitopes previously, a novel HLA-A25-restricted and conserved epitope recognized by $CD8^+$ CTL was identified and characterized. Comparison of sequences of the epitope region in samples collected from the LTA individuals concerned over an 8 year observation period did not reveal escape from CTL activity.

Methods

Study population. A total of seven HIV-1-infected individuals who remained asymptomatic with stable CD4 counts [Centers for Disease Control category A1 (Centers for Disease Control, 1992)] for more than 8 years, and who participate in the Amsterdam Cohort Studies on AIDS (de Wolf *et al.*, 1987), were studied. Their clinical characteristics and laboratory markers were recently described (Keet *et al.*, 1994; Klein

et al., 1995). HLA class I typing was performed at the Department of Transplantation Immunology of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. Their types were as follows: H008, HLA-A2, -A26, -B27, -B44, -Cw1; H067, HLA-A26, -A28, -B7, -B57; H157, HLA-A3, -A28, -B13, -B44, -Cw7; p206, HLA-A3, -A25, -B18, -B51; H230, HLA-A3, -B7, -B51, -Cw7; H233, HLA-A3, -A24, -B35, -B60, -Cw3, -Cw4; H358, HLA-A2, -A29, -B18, -Cw5, -Cw7. Individual p206 was monitored at the Academic Medical Centre in Amsterdam.

Peptides. Sets of overlapping peptides spanning the entire Gag of HIV-1 (strain SF2) included 13 15-mer peptides with a five residue overlap spanning p17 (ADP704), 22 20-mer peptides with a ten residue overlap spanning p24 (ADP788) and 13 15-mer peptides with a five residue overlap spanning p15 (ADP703). These peptides were kindly provided by H. Holmes (Medical Research Council AIDS Directed Programme, Potters Bar, South Mimms, UK). The 9-mer and 10-mer peptides spanning $p24_{203-222}$ with a nine amino acid overlap were kindly provided by R. Meloen (Central Veterinary Institute, Lelystad, The Netherlands). The variant $p24_{203-212}$ peptides with sequences that correspond to related lentiviruses were kindly provided by R. van Herwijnen (European Veterinary Laboratory, Woerden, The Netherlands). Amino acid (aa) numbers are according to the consensus sequence of HIV-1 Gag clade B (Myers *et al.*, 1994).

Recombinant vaccinia viruses. Recombinant vaccinia viruses (rVV) TG1144, containing the $pr55^{gag}$ gene from HIV-1_{LAI} (Rautmann *et al.*, 1989; Myers *et al.*, 1994), TG2112, containing the $pr56^{gag}$ gene from HIV-2_{ROD} and rVV 186-poly, containing a polycloning site without insert, were kindly provided by M.-P. Kieny (Transgene SA, Strasbourg, France). The rVV containing the $pr56^{gag}$ gene from SIV_{MM32H} was kindly provided by A. McMichael (Oxford, UK).

Preparation of effector cells. PBMC were isolated by Lymphoprep (Nycomed) gradient centrifugation and cryopreserved. Stimulation of PBMC *in vitro* and maintenance of cell lines was performed as previously described (Van Baalen *et al.*, 1993). Briefly, PBMC were cocultivated with a paraformaldehyde-fixed autologous Epstein–Barr virus-transformed B-lymphocytic cell line (B-LCL) expressing HIV-1 Gag from infection with TG1144 for 14 to 21 days and subsequently used as effector cells in standard ^{51}Cr-release assays. Gag-specific cell lines were subcloned under limiting dilution conditions by stimulation with 1 μg/ml (PHA)-L (Boehringer Mannheim) and irradiated feeder cells as described (Van de Griend *et al.*, 1984). The phenotype of resulting clones was determined by FACScan analysis according to the manufacturer's instructions (Becton Dickinson). Clones were maintained by stimulation every 7 to 14 days alternately with PHA-L (Van de Griend *et al.*, 1984) and by cocultivation with autologous stimulator cells as described (Van Baalen *et al.*, 1993).

^{51}Cr-release assays. Cytotoxicity was determined in standard 4 h ^{51}Cr-release assays as described previously (Van Baalen *et al.*, 1993). Target cells were pulsed with peptides as described in the legends to the figures or infected overnight with rVV TG1144 or 186-poly and subsequently labelled with $Na_2{}^{51}CrO_4$ (Amersham). Effector cells were added at indicated E:T ratios. Supernatants were harvested with the Skatron harvesting system and counted on a gamma counter (LKB Wallac). Percentage specific lysis was determined from the formula: 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Maximum release was determined by lysis of target cells in 3% Triton X-100. Spontaneous release was less than 30% of maximum release.

Virus sequencing. Virus isolation procedures were performed with PBMC samples from p206 collected at seven regular intervals during follow-up. Generation of biological clones and sequence analysis of the $p24_{193-222}$ region and of the gp120 V1, V2 and V3 regions of the biological clones was performed as described (Schuitemaker *et al.*, 1992).

Results

Mapping of the Gag-specific CTL response

PBMC from six LTA individuals (H157, H230, H067, p206, H233 and H385) were screened for HIV-1 Gag-specific CTL activity. Polyclonal T cell lines established by *in vitro* antigen-specific stimulation of the PBMC were tested for cytolytic activity against 48 different target cell populations pulsed with single partially overlapping peptides, spanning the entire Gag. Target cells pulsed with the third peptide of p17 ($p17_{21-35}$), and with the second ($p24_{143-162}$), the seventh ($p24_{193-212}$) and eighth ($p24_{203-222}$) peptides of p24 (Fig. 1*a–d*) were recognized by cells from individuals H157, H230, H067 and p206. Cultures from individuals H233 and H385 exhibited no specific lysis of the peptide-pulsed target cells (data not shown).

Clones from individuals H157 and H067 specific for $p17_{21-35}$ and $p24_{143-162}$ also recognized endogenously expressed HIV-1 Gag as evidenced by lysis of autologous cells expressing Gag upon rVV infection (data not shown). Some clones from H157 recognized HIV-1 Gag but not $p17_{21-35}$ (data not shown), indicating that Gag-specific CTL with fine-specificities other than the dominantly recognized $p17_{21-35}$ were present. Using essentially the same approach it was found that effector cells from a seventh individual, H008, recognized $p24_{263-282}$. The $CD8^+$ fraction of this culture recognized endogenously synthesized Gag and lysed $p24_{263-282}$-pulsed autologous and heterologous HLA-B27-matched target cells but not peptide-pulsed target cell lines matched for the other HLA class I alleles (data not shown).

Identification of $p24_{203-212}$ ETINEEAAEW as an HLA-A25-restricted epitope

As shown in Fig. 1, the CTL response observed with cells from p206 was directed against $p24_{193-212}$ and $p24_{203-222}$. CTL with this specificity were found at all the five time-points tested during the 45 to 93 month follow-up (data not shown). To date, no epitopes located in the region $p24_{203-222}$ and restricted by one of the HLA-A or -B alleles of p206 (HLA-A3, -A25, -B18, -B51) have been described. A T cell line, p206/E2, containing 95% $CD8^+$ T cells (data not shown) recognized both 20-mer peptides $p24_{193-212}$ and $p24_{203-222}$ as well as the 10-mer sequence $p24_{203-212}$ ETINEEAAEW, which is shared by both 20-mers (Fig. 2*a*). Neither the 9-mer peptide ETINEEAAE nor the 10-mer peptide TINEEAAEWD were recognized (Fig. 2*a*), indicating that both the C-terminal residue $p24_{212}$W and the N-terminal residue $p24_{203}$E are essential for T cell recognition. Therefore, it was concluded that $p24_{203-212}$ is the minimal epitope recognized. This peptide was recognized in association with autologous and HLA-A25-

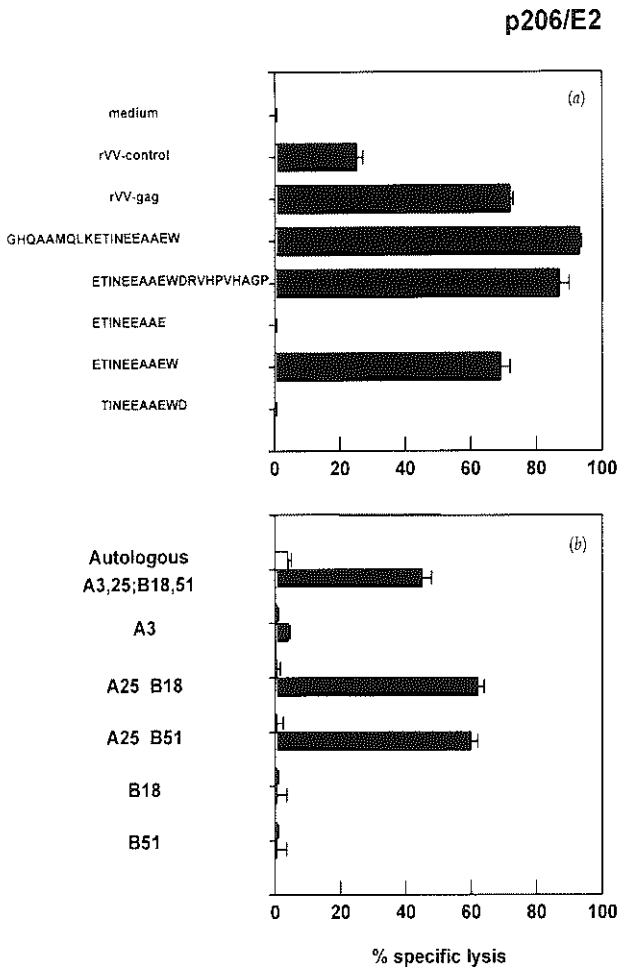

Fig. 2. Characterization of the $p24_{193-222}$-specific CTL from p206. (*a*) The $CD4^-$ $CD8^+$ Gag-specific CTL line p206/E2 was tested for lysis of autologous target cells infected with rVV-*gag* or pulsed with the peptides indicated on the vertical axis. (*b*) A panel of partially HLA class I-matched B-LCL was pulsed with the peptide ETINEEAAEW and tested for lysis by CTL line p206/E2 (black bars). B-LCL without peptide were tested as negative controls (open bars). Results are expressed as percentage specific lysis (average of triplicates ± 1 SD). Incubation with 20-mer peptides was as described in legend to Fig. 1. The 9- and 10-mer peptides were added to ^{51}Cr-labelled target cells at a final concentration of 1 μM 1 h prior to the addition of effector cells.

matched target cells (Fig. 2*b*). In contrast, peptide-pulsed B-LCL matched for HLA-A3, -B18 or -B51 alone were not lysed (Fig. 2*b*). These data indicate that ETINEEAAEW is recognized in the context of HLA-A25.

CTL epitope $p24_{203-212}$ is restricted to HIV-1 clades B and D

Amino acid sequence $p24_{203-212}$ ETINEEAAEW is highly conserved amongst HIV-1 clade B and D sequences (Myers *et al.*, 1994) (Fig. 3). The corresponding regions of the consensus sequences of the other HIV-1 clades differ from those in clade B and D only by an aspartic acid at position $p24_{203}$. Similarly, the corresponding $HIV\text{-}2_{ROD}$ sequence differs only by an isoleucine at position $p24_{204}$. The homologous region of SIV_{MM32H} differs at three positions from the HIV-1 B and D consensus sequences: aspartic acid residues at positions $p24_{203}$ and $p24_{211}$, and isoleucine at position $p24_{204}$. Autologous target cells pulsed with variant 10-mer peptides corresponding to these regions were used in a CTL assay with ETINEEAAEW-specific $CD8^+$ CTL clones from p206 as effector cells. None of these variant peptide sequences were recognized (Fig. 4). Furthermore, rVV-infected target cells expressing Gag of clade B strain $HIV\text{-}1_{LAI}$ were lysed, whereas target cells expressing $HIV\text{-}2_{ROD}$ Gag or SIV_{MM32H} Gag were not (Fig. 4).

No escape variants detectable in PBMC of p206

Since Gag sequences containing either aspartic acid at position $p24_{203}$ or isoleucine at position $p24_{204}$ were not recognized by ETINEEAAEW-specific CTL from p206, but do occur in related lentiviruses, it was speculated that virus escape

Clade	No. of isolates	Amino acid sequence
A-H consensus		DTINEEAAEW
A(31)[1]	22	----------
	1	----G-----
	4	E---------
	1	N---------
	2	--------D-
	1	-A------G-
B(26)	25	E---------
	1	----------
C(6)	6	----------
D(10)	8	E---------
	1	E---D-----
	1	E--I------
F(4)	4	----------
G(3)	3	----------
H(2)	2	----------
HIV-2_{ROD}		-I--------
SIV_{MM32H}		-I------D-

Fig. 3. Sequence variability in the minimum epitope $p24_{203-212}$ in viruses from HIV-1 clades A to H, HIV-2_{ROD} and SIV_{MM32H}. Numbers in parentheses are the number of sequenced isolates within each clade. Figure adapted from Myers *et al.* (1994).

mutations at these positions could occur in p206. Virus isolation procedures carried out with PBMC collected after the baseline visit were successful at 84 and 90 months of follow-up (Klein *et al.*, 1995). Twelve and seven biological clones were generated from these two time-points, respectively. Sequence analyses of a 30 aa region containing the epitope of each of the biologically cloned viruses revealed conservation of the original clade B and D consensus sequence in each of the 19 virus clones tested (Fig. 5). In contrast, the variable regions of gp120, V1, V2 or V3 proved to show aa variability when mutually compared (Fig. 5), showing that the 19 virus clones analysed originated from different viruses.

Discussion

Fine-specificities of HIV-1 Gag-specific CTL were studied in seven seropositive LTA individuals with stable CD4 counts for more than 8 years. Using PBMC from five of them and a panel of overlapping peptides spanning the entire Gag of HIV-1, at least five peptides were identified as areas containing CTL epitopes. These peptides overlap with sequences which have previously been shown to contain CTL epitopes. One of these, $p17_{21-35}$, overlaps with an HLA-A3-restricted epitope, $p17_{18-31}$ (Jassoy *et al.*, 1992). Indeed, the two individuals showing $p17_{21-35}$-specific CTL activity, H157 and H230, expressed HLA-A3. Similarly, peptides $p24_{143-162}$ and $p24_{263-282}$ overlap with regions containing HLA-B57- and HLA-B27-restricted epitopes, respectively (Johnson *et al.*, 1991; Nixon *et al.*, 1988; Buseyne *et al.*, 1993). The individuals recognizing these two peptides, H067 and H008, were positive for HLA-B57 and HLA-B27, respectively. It was indeed confirmed that peptide $p24_{263-282}$ was recognized by HLA-B27-restricted $CD8^+$ CTL (data not shown).

No Gag peptide-specific CTL were detected in PBMC from H233 and H385. This may have been due to the absence of Gag-specific CTL, or to the presence of Gag-specific CTL that recognize epitopes which are not shared with sequences from the laboratory strains of HIV-1 used in this study. Furthermore, not all epitopes may have been generated from the 15- and 20-mer peptides. The latter explanation is in agreement with the observation that a CTL clone from H157 recognized Gag but

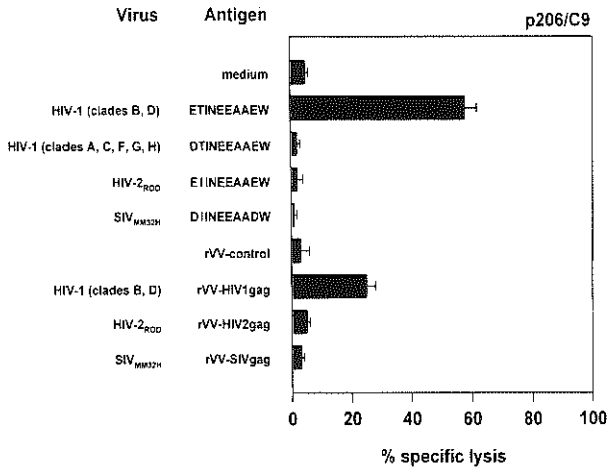

Fig. 4. CTL clones from p206 specific for $p24_{203-212}$ recognize the sequence from HIV-1 clades B and D, but not the corresponding sequences from related lentiviruses. The peptide sequences indicated on the vertical axis were tested for recognition in a ^{51}Cr-release assay using as effector cells four ETINEEAAEW-specific CTL clones from p206. Target cells expressing Gag of HIV-1_{LAI}, HIV-2_{ROD} or SIV_{MM32H} from rVV-infection were also tested. Each clone exhibited a similar recognition pattern and the result of a representative clone, p206/C9, is shown. Peptides were added at a final concentration of 1 μM 1 h prior to the addition of effector cells. Bars represent the percentage specific lysis (average of triplicate measurements ± 1 SD).

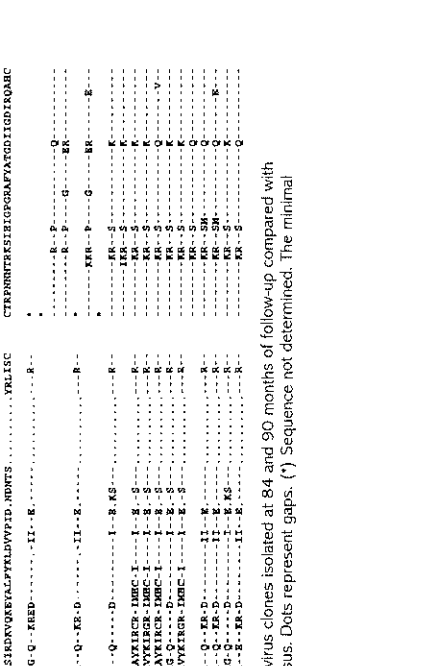

Fig. 5. Sequences of the $p24_{193-222}$ region and the gp120 V1, V2 and V3 regions of biological virus clones isolated at 84 and 90 months of follow-up compared with the consensus sequence of HIV-1 clade B. Dashes represent aa identity with the clade B consensus. Dots represent gaps. (*) Sequence not determined. The minimal HLA-A25-restricted epitope is underlined.

not the peptide $p17_{21-35}$, which was dominantly recognized by the parental T cell line.

A novel CTL epitope was minimally defined to the 10 aa sequence $p24_{203-212}$ ETINEEAAEW, and proved to be recognized by $CD8^+$ CTL in the context of HLA-A25. Target cells expressing Gag from rVV infection were also lysed by $CD8^+$ ETINEEAAEW-specific CTL clones, showing that the epitope can be generated from endogenously synthesized Gag. To our knowledge, this is the first record of an HLA-A25-restricted epitope.

Corresponding sequences from closely related lentiviruses, HIV-1 strains of clades A, C, F, G and H, HIV-2_{ROD} and SIV_{MM32H} were not recognized by $p24_{203-212}$-specific CTL clones from p206. A change from aa $p24_{203}$E to D and from aa $p24_{204}$T to I were each sufficient to abolish recognition, indicating that residues $p24_{203}$E and $p24_{204}$T are essential for functional presentation. Loss of recognition may indicate that the variant peptides have a lower HLA-A25 binding affinity or that complexes of HLA-A25 and the variant peptides can no longer interact with the T cell receptor of the CTL recognizing the index peptide. The observation that *in vitro* recognition of the epitope was abrogated by single mutations at positions $p24_{203}$ or $p24_{204}$ to residues that occur in replicating viruses prompted us to look for virus variants with mutations at these aa positions in PBMC from p206. None of the 19 different biological virus clones tested showed sequence variation in the region $p24_{193-222}$, indicating that the HLA-A25-restricted epitope was conserved within the virus quasispecies of p206. Although it cannot be excluded that potential negative selection conditions for escape variants may have been present during *in vitro* culture, it is most likely that they were not generated *in vivo*. This may be due to structural constraints that limit the ability of the virus to mutate in the epitope region. Consistent with this hypothesis is the observation that 35 out of 36 known clade B and D Gag sequences contain aa $p24_{203}$E and 82 out of 85 known HIV-1 Gag sequences contain aa $p24_{204}$T (Fig. 3) (Myers *et al.*, 1994), indicating that variation at these positions is limited. It may be speculated that CTL, like those specific for ETINEEAAEW, have been instrumental in limiting virus replication in p206 without possibilities for the virus to escape, thereby delaying disease progression. The CTL specific for conserved epitopes in HIV-1 Gag described here originated from long-term asymptomatic individuals. We are presently analysing the CTL responses from individuals who progress to AIDS more rapidly, to determine whether CTL responses against epitopes like those found in this study may play a role in the prolonged survival after HIV infection.

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Human immunodeficiency virus type 1 Rev- and Tat-specific cytotoxic T lymphocyte frequencies inversely correlate with rapid progression to AIDS

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Immunological correlates of AIDS-free survival after human immunodeficiency virus type 1 (HIV-1) infection are largely unknown. Cytotoxic T lymphocyte (CTL) responses are generally believed to be a major component of protective immunity against viral infections. However, the relationship between HIV-1-specific CTL responses and disease progression rate is presently unclear. Here we show in twelve HIV-1-infected individuals that detection of Rev-specific CTL precursors (CTLp) early in the asymptomatic stage, as well as detection of Rev- and Tat-specific CTLp later during follow-up, inversely correlate with rapid disease progression. No such correlation was found for detection of CTLp against Gag, RT or Nef. Further studies are required to determine whether a protective mechanism is indeed the basis of the observed correlation. The data presented are in agreement with the hypothesis that CTL against proteins that are important for early viral transcription and translation are of particular importance in protection from rapid disease progression.

The duration of the asymptomatic period after human immunodeficiency virus type 1 (HIV-1) infection varies considerably (Klein & Miedema, 1995; Haynes *et al.*, 1996), and is inversely related to plasma RNA levels following initial viraemia (Jurriaans *et al.*, 1994; Mellors *et al.*, 1996). These parameters may be determined by viral characteristics, host genetics and immunological factors (Klein & Miedema, 1995; Bollinger *et al.*, 1996; Haynes *et al.*, 1996). Cytotoxic T lymphocyte (CTL) responses are associated with initial control of viraemia, persist in asymptomatic individuals, and eventually decline with disease progression (Carmichael *et al.*, 1993; Borrow *et al.*, 1994; Koup *et al.*, 1994; Klein *et al.*, 1995; Rinaldo *et al.*, 1995; Bollinger *et al.*, 1996; Geretti *et al.*, 1996). This decline coincides with HIV-1-induced $CD4^+$ T cell loss and perturbation of T cell function (Klein *et al.*, 1995; Geretti *et al.*, 1996).

Recent reports on virus escape from HIV-1-specific CTL responses clearly indicate that CTL exert pressure on virus replication *in vivo*, but that the overall CTL efficacy may be dependent on the (lack of) conservation in the epitope sequences (Borrow *et al.*, 1997; Goulder *et al.*, 1997). In addition, it has been suggested that CTL responses against early expressed epitopes may be more efficacious than those against late expressed epitopes (Ranki *et al.*, 1994; Riviere *et al.*, 1994). Qualitative and quantitative analyses of specific CTL responses, before the immune status has deteriorated, may identify the requirements for a CTL response to be protective. In asymptomatic HIV-1-infected individuals the early expressed proteins Rev and Tat are generally less frequently recognized than the late expressed structural proteins Gag and RT (Johnson & Walker, 1994; Riviere *et al.*, 1994; Lamhamedi-Cherradi *et al.*, 1995).

Here, we investigated whether differences between CTL responses against HIV-1 Gag, RT, Nef, Rev and Tat are related to differences in disease progression rates. CTL precursor (CTLp) frequencies were determined in twelve participants of the Amsterdam Cohort Studies on AIDS, who were selected on the basis of their disease progression rates and HLA phenotypes (Table 1). Seven of these individuals remained AIDS-free for more than a decade (median 129 months, range 110–140) after seroconversion (L090, L658, L709 and L434) or

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Table 1. Characteristics of the seven LTA and five progressors selected for this study

Individual*	HLA phenotype A	HLA phenotype B	Serostatus at entry†	Seroconversion interval (months)	Clinical status (months after seroconversion or entry) AIDS	CD4 < 200‡	Asymptomatic Follow-up	CD4 slope (cells/μl per month)	Virus load§ (RNA copies/ml)	Sampling for CTL assays (months after seroconversion or entry)	Corresponding CD4 cell count (cells/μl)
L090	1,2	41,57	I	2·6	NA	NA	> 129	+1·1	$< 10^3$	24	860
										103	1160
L658	1,2	8,61	I	3·3	NA	NA	> 110	−1·3	$8·3 \times 10^4$	4	950
										69	690
L211	1,2	8,57	II	NA	NA	NA	> 139	−3·1	NT	24	730
										74	710
L709	1,69	14,57	I	3·4	NA	NA	> 122	−3·5	$2·5 \times 10^3$	8	850
										84	770
L434	2,28	7,27	I	3·0	NA	NA	> 129	−3·7	$7·4 \times 10^3$	8	630
L008	2,26	27,44	II	NA	NA	132	> 140	−4·5	NT	97	490
L157	3,28	13,14	II	NA	NA	130	> 139	−5·6	NT	88	710
P493	1,2	8,35	I	3·0	40	28		−3·1	$4·7 \times 10^5$	5	420
										9	450
P1215	1,2	7,8	I	3·0	72	50		−4·4	$3·2 \times 10^5$	21	310
										62	280
P356	2,28	27,38	I	3·0	41	38		−7	$1·9 \times 10^4$	13	420
P424	1,2	8,61	I	10·6	43	46		−14	$4·0 \times 10^4$	15	510
P039	1,2	8,44	I	3·4	39	39		−19	$7·4 \times 10^4$	4	870
										15	730

* L, LTA; P, progressor. The number following L or P indicates the number of the participant in the Amsterdam Cohort studies on AIDS.
† I, seronegative; II, seropositive. Seroconversion interval is time between last seronegative and first seropositive visit.
‡ First time-point at which $CD4^+$ T cell count was below 200 cells/μl.
§ Mean serum viral RNA load in first year after seroconversion.
NA, Not applicable.
NT, Not tested.

study entry (L211, L008 and L157). The other five individuals developed AIDS within 3–6 years (median 47 months, range 39–72) after seroconversion (P493, P1215, P356, P424 and P039). In accordance with our previous studies (Van Baalen *et al.*, 1993, 1996; Klein & Miedema, 1995; Klein *et al.*, 1995), and to highlight the profound difference in progression rates, these individuals are referred to as LTA and progressors, respectively. AIDS-defining symptoms of the progressors were: Kaposi's sarcoma (P493); *Candida albicans* oesophagitis (P1215, P424 and P039); *Pneumocystis carinii* pneumonia (P356). Rates of $CD4^+$ T cell decline (slopes) were calculated from $CD4^+$ T cell counts measured at regular 3 monthly intervals during the entire follow-up period (Table 1). Only two of the twelve individuals received antiviral therapy during follow-up. For L008 and P1215 AZT therapy was started at 109 and 51 months after entry, respectively, and DDC therapy was started at 126 and 69 months, respectively. No CTLp frequencies were determined after the start of antiviral therapy. HLA-A and -B phenotypes of the individuals were serologically determined at the Department of Transplantation Immunology, CLB, Amsterdam (Table 1).

Because CTL responses may be impaired as a result of disease progression, we decided to test samples collected before deterioration of the immune status had become evident. Retrospective CTLp frequency analyses were performed on the earliest available PBMC samples. Most of the individuals could also be tested during follow-up. The PBMC were cultured *in vitro* in RPMI 1640 containing 10% human pooled serum and recombinant IL-2 under limiting dilution conditions for 14–20 days, as described previously (Van Baalen *et al.*, 1993; Klein *et al.*, 1995). On day 0 and day 7, cultures were stimulated by addition of paraformaldehyde-fixed autologous B lymphoblastoid cell lines infected with recombinant vaccinia viruses VVTG1144 (Gag), VVTG4163 (RT), VVTG1147 (Nef), VVTG4113 (Rev) or VVTG3196 (Tat), kindly provided by M. P. Kieny (Transgène, Strasbourg, France). Cytotoxic activity was measured by standard 4 h ^{51}Cr-release assays (Van Baalen *et al.*, 1993; Klein *et al.*, 1995). CTLp frequency calculations were performed as described previously (Geretti *et al.*, 1996).

The most striking finding of these studies was that Rev- and Tat-specific CTLp were predominantly detected in the LTA (Fig. 1): their frequencies in LTA and progressors proved to be

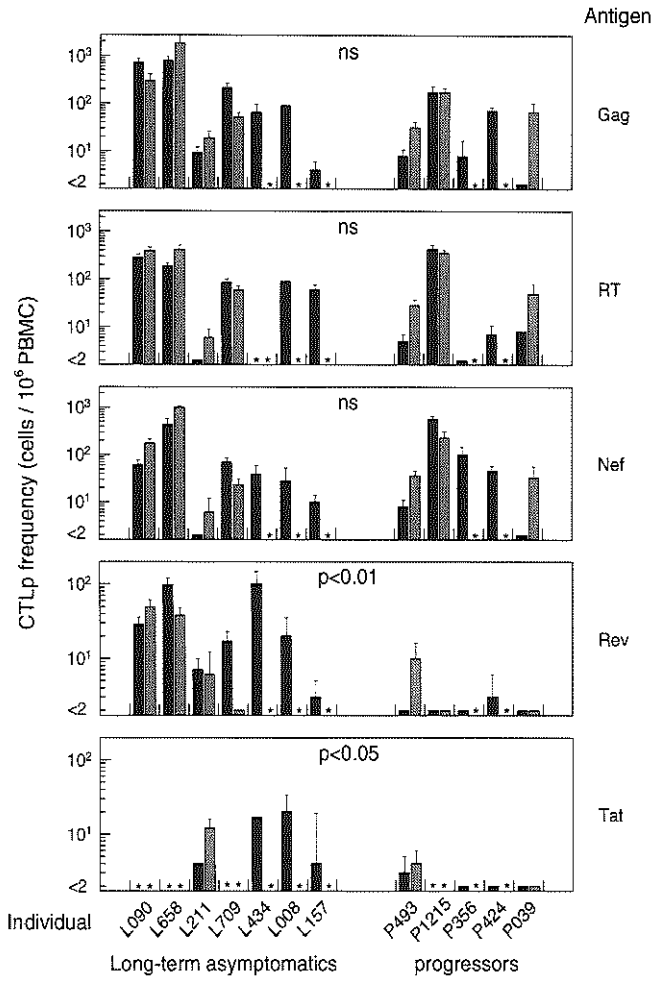

Fig. 1. Frequencies of CTLp against HIV-1 Gag, RT, Nef, Rev and Tat detected in the asymptomatic stage of seven LTA and five progressors. Cultures were established using cryopreserved PBMC sampled from LTA and progressors at time-points indicated in Table 1. Progressor samples were tested in parallel with those of LTA sharing at least three HLA-A and -B alleles. Solid bars, first available sampling time-points; hatched bars, second sampling time points. Error bars indicate standard error of calculated frequencies. *P* values result from the Mann–Whitney analyses in which the CTLp frequencies of the LTA were compared to those of the progressors. ns, not significant; * not tested.

significantly different (Mann–Whitney $P < 0{\cdot}01$ and $P < 0{\cdot}05$, respectively). In contrast, CTLp directed against Gag, RT or Nef were found at similar frequencies in individuals of both groups.

Since all the progressors and four of the seven LTA were seronegative at study entry, the data could be analysed in relation to the time elapsed after infection. During the first 24 months after seroconversion, Rev-specific CTLp, but not Gag-, RT- or Nef-specific CTLp, proved to be significantly more prevalent in these LTA (Mann–Whitney $P < 0{\cdot}02$). Due to limited numbers of PBMC, this analysis could not be made for Tat-specific CTLp. Collectively, these data show that the detection of Rev-specific CTLp early in the asymptomatic stage, as well as the detection of Rev- and Tat-specific CTLp during follow-up, are inversely correlated with rapid disease progression.

The failure to detect responses against Rev and Tat in the progressors could have resulted from an overall decline in CTLp frequencies or from the early impairment of $CD4^+$ T cell function. However, as for the LTA, frequencies of CTLp against Gag, RT and Nef remained stable or even increased early after infection. This indicates that the absence of detectable Rev- and Tat-specific CTL activity in progressors was not due to a general failure of CTL responses *in vivo*.

To explain the specific absence of detectable Rev- and Tat-specific CTL responses in the progressors, a number of hypotheses may be considered. These include the absence of functional HLA–epitope complexes due to either host genetic (Klein *et al.*, 1994; Kaslow *et al.*, 1996) or viral characteristics (Phillips *et al.*, 1991; Couillin *et al.*, 1994; Koenig *et al.*, 1995), and the mobilization of a restricted T-cell receptor repertoire (Pantaleo *et al.*, 1994; Kalams *et al.*, 1996). It may be expected

Table 2. HLA class I peptide-binding motifs of Rev sequences obtained from non-cultured PBMC of L658 and P424

The individuals L658 and P424 share HLA-A1, 2; -B8, 40, 61; -C2, 7; -DR3, 6, 13; -DR52; -DQ1, 2. Sequences were determined for 20 and 19 individual recombinant PCR clones generated from PCR amplification products of the individuals, respectively. Sequences were analysed for the presence of HLA-A1, 2 and -B8, 61 peptide-binding motifs (Rammensee *et al.*, 1995). Anchor residues are underlined. Motifs of HIV-1_{Lai} Rev, which was used for CTL detection, are indicated for reference purposes.

	HLA-A1 (X[ST]XXXXXXY)		HLA-A2 (X[LM]XXXXXX[VL])				HLA-B8(XX[KR]X[KR]XXX[IL])	
	Sequence	Frequency	Sequence	Frequency	Sequence	Frequency	Sequence	Frequency
Lai	ISERILSTY		YLGRSAEPV		ILVESPTVL		RWRERQRQI	
L658	L.GWL...T	16/20	.T.....T	20/20	.T.....T	19/20	..T.T...T	20/20
	L.GWLI..T	4/20			.T.....A.	1/20		
P424	.TGW...TS	15/19	S.....K.T	19/19	.T.....E.T	18/19	..TQ...T	19/19
	.TGW...NS	4/19			.T.G..E.T	1/19		

on the basis of protein size that the number of CTL epitopes on Rev and Tat is smaller than that on Gag and RT. This may also explain the overall lower levels of CTLp against Rev and Tat in the LTA as compared to those against Gag, RT and Nef.

Given the number of matching HLA class I alleles between progressors and LTA, it may be considered that variation in viral sequences has had an impact on the generation of HLA–epitope complexes in these individuals. In support of this hypothesis, we found differences in the Rev sequences of viruses obtained from LTA L658 and progressor P424, who differed markedly in their CTL response to Rev but were identical for the HLA class I and class II alleles tested (Table 2). High-molecular-mass DNA was isolated from PBMC using cell lite beads (Boom *et al.*, 1991). A nested PCR was used to amplify the second exon of *rev*. Outer primers were AAATGTCAGCACAGTACAATGT and CATTGGTCTTAAAGGTACCTG, inner primers were GTACTTTCTATAGTGAATAGAGTTAGGC and CCTATCTGTCCCCTCAGCTACT. PCR conditions were: 5 min 95 °C, 1 min 55 °C, 1 min 30 s 72 °C for one cycle then 50 s 95 °C, 50 s 55 °C, 1 min 30 s 72 °C for 30 cycles and 7 min 72 °C extension for both the outer and inner amplification. Amplified fragments were cloned with a pCR2 kit according to the manufacturer's protocol (Invitrogen). Clones were sequenced with a Taq Dye Deoxy Terminator sequencing kit on an Applied Biosystems 373A sequencing system. All clones were sequenced on both strands with the inner primers. Sequences were analysed with Geneworks (IntelliGenetics). In viral sequences from L658, who developed Rev-specific CTL, the anchor residues of a previously described HLA-A1 peptide-binding motif were present. One of these anchor residues was not present in all viral sequences obtained from P424, who did not show Rev-specific CTL responses. Considerable variation was found in HLA-A2 and HLA-B8 peptide-binding motifs of Rev. The influence of this variation on the antigenicity of these sequences remains to be elucidated. In addition, the presence of HLA class I alleles unique to the LTA may also, at least in part, explain the observed differences in CTL responses between LTA and progressors. Three of the seven LTA, but none of the progressors, were positive for HLA-B57. This allele has been suggested to be associated with prolonged survival (Klein *et al.*, 1994; Kaslow *et al.*, 1996). To address this point, a larger number of HLA-matched individuals would have to be studied. Whether Rev and Tat epitopes may be presented in the context of HLA-B57 is presently being studied.

To investigate the relationship between viral loads and disease progression rates in these individuals, serum viral RNA loads were determined in the individuals with a known seroconversion time-point (Table 1). Mean HIV-1 RNA loads in the first year after infection were quantified using a nucleic acid sequence-based amplification assay (NASBA HIV-1 RNA QT; Organon Teknika), according to the manufacturer's instructions. As expected, these mean viral loads were relatively low in most LTA and high in the progressors. These differences could be due to viral characteristics. However, the *in vitro* replication of viruses isolated early after infection from these groups showed similar kinetics (H. Schuitemaker and others, unpublished), which is in line with the observation that predominantly slowly replicating NSI variants are transmitted (Connor & Ho, 1994).

Rev- and Tat-specific CTL may influence viral load more efficiently than CTL against structural proteins in different ways. Firstly, in the asymptomatic stage, many infected cells, both in circulation and in lymph nodes, do not actively produce virus, but do express multiple spliced mRNAs that encode the regulatory proteins (Seshamma *et al.*, 1992; Embretson *et al.*, 1993). This renders these cells targets for CTL against regulatory, but not against structural proteins. Secondly, in the replicative cycle, Rev and Tat are expressed earlier than the structural proteins. This may allow specific CTL to kill infected cells well before release of progeny virus (Ranki *et al.*, 1994; Riviere *et al.*, 1994). Although both considerations would also hold true for Nef, specific CTL responses to this protein were observed in most progressors. This may be

related to a lack of major structural constraints on Nef (Couillin *et al.*, 1994; Koenig *et al.*, 1995).

Our data which indicate that Rev- and Tat-specific CTL are involved in protection from rapid disease progression are in line with earlier suggestions that CTL responses against conserved and early proteins of HIV-1 would be protective (Johnson & Walker, 1994; Riviere *et al.*, 1994, 1995; Harrer *et al.*, 1996; Van Baalen *et al.*, 1996). Recent data obtained from studies in SIV_{mac}-infected macaques (Hulskotte *et al.*, 1995) indicate that in these animals also, Rev-specific CTL responses inversely correlate with disease progression (A. M. Geretti, unpublished). Analysis of the efficacy of CTL specific for the early and regulatory proteins Rev and Tat in containing HIV-1 infection in *in vitro* and *in vivo* models, which are prompted by the results of the present study, will contribute to our understanding of the pathogenesis of human and animal lentivirus infections. This will facilitate the development of rational strategies for vaccination and specific immunotherapy.

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CHAPTER 3

Antiviral activity of HIV-1-specific cytotoxic T lymphocytes

Kinetics of Antiviral Activity by Human Immunodeficiency Virus Type 1-Specific Cytotoxic T Lymphocytes (CTL) and Rapid Selection of CTL Escape Virus In Vitro

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The antiviral activity of a $CD8^+$ cytotoxic T-lymphocyte (CTL) clone (TCC108) directed against a newly identified HLA-B14-restricted epitope, human immunodeficiency virus type 1 (HIV-1) Rev(67-75) SAEPVP LQL, was analyzed with respect to its kinetics of target cell lysis and inhibition of HIV-1 production. Addition of TCC108 cells or $CD8^+$ reverse transcriptase-specific CTLs to HLA-matched $CD4^+$ T cells at different times after infection with HIV-1 IIIB showed that infected cells became susceptible to CTL-mediated lysis before peak virus production but after the onset of progeny virus release. When either of these CTLs were added to part of the infected cells immediately after infection, p55 expression and virus production were significantly suppressed. These data support a model in which CTLs, apart from exerting cytolytic activity which may prevent continued virus release, can interfere with viral protein expression during the eclipse phase via noncytolytic mechanisms. TCC108-mediated inhibition of virus replication in peripheral blood mononuclear cells caused rapid selection of a virus with a mutation (69E→K) in the Rev(67-75) CTL epitope which abolished recognition by TCC108 cells. Taken together, these data suggest that both cytolytic and noncytolytic antiviral mechanisms of CTLs can be specifically targeted to HIV-1-infected cells.

Identification of immune responses that may limit progression toward AIDS and may eliminate infected cells that persist despite effective antiviral therapy (6, 20, 30) is a major goal for current research aimed at the development of vaccines and immunotherapies against AIDS (1). It is generally assumed that an effective vaccine against human immunodeficiency virus type 1 (HIV-1) should elicit an antiviral immune response which includes virus-specific major histocompatibility complex class I-restricted $CD8^+$ cytotoxic T lymphocytes (CTL), because their presence is associated with the control of primate lentivirus replication and they have been detected in individuals exposed to but apparently uninfected with HIV (reviewed in reference 8). Furthermore, CTL have been shown to exert pressure on virus replication in vivo (2, 9) and in vitro (3, 27, 32). CTL clones directed against the late viral proteins Gag, reverse transcriptase (RT), and Env, have been shown to lyse HIV-1-infected cells before peak virus production (31) and to suppress HIV-1 replication in immortalized $CD4^+$ T-cell lines such as H9 and T1 (32). Env-specific CTL have been shown to eliminate HIV-1-infected $CD4^+$ peripheral blood mononuclear cells (PBMC) and H9 cells (32), indicating that inhibition of HIV-1 replication involved cytolytic mechanisms. In addition to exerting cytolytic activity, HIV-specific CTL have been shown to suppress virus replication by the excretion of soluble factors (3, 32). Although CTL against late viral proteins do exhibit antiviral activity, elimination of nonproductively infected cells with still incomplete protein expression (18) and suppression of low-level virus replication (20) may require CTL directed against the regulatory viral proteins Tat and Rev. These proteins are translated early in the replication cycle of HIV-1 and are necessary for transcription (Tat) or expression of the intermediate and late proteins (Rev) (14, 15). Consistent with such a protective role of CTL against Rev and Tat, we have shown previously that CTL with these specificities were preferentially found in individuals who experienced a long-term asymptomatic course of disease progression (28). In contrast, CTL responses against the late proteins Gag and RT did not correlate with the rate of disease progression (28).

Here we present a detailed analysis of the Rev-specific CTL response in one individual who has been infected for more than 12 years without developing symptoms. Rev-specific CTL clones were generated, and a minimal epitope as well as the HLA class I restriction of its recognition were identified. It is shown that both Rev- and RT-specific CTL can suppress HIV-1 production before they exert cytolytic activity and that Rev-specific CTL-mediated inhibition of virus replication in PBMC leads to the rapid selection of virus mutated in the CTL epitope.

MATERIALS AND METHODS

Cells. All cells were maintained in RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (10 μg/ml), and 10% pooled human serum (R10H) for PBMC and T cells or 10% fetal bovine serum (BioWhittaker) (R10F) for B-LCL cells. CTL clones and the $CD4^+$ TCL2H7 cells were stimulated at 2-week intervals with phytohemagglutinin-L (PHA-L) (1 μg/ml; Boehringer Mannheim, Germany) and gamma-irradiated (3,000 rads) allogenic feeder cells and maintained in R10H containing recombinant interleukin-2 (rIL2) (50 U/ml; Eurocetus, Amsterdam, The Netherlands). International Histocompatibility Workshop B-LCL cells were obtained from the European Collection of Cell Cultures (Salisbury, United Kingdom).

Generation of CTL clones. HIV-1 Rev-specific T-cell lines obtained from individual L709, HLA-A1,28;−B14,57, were seeded at 0.3, 1, and 3 cells per well in 60-well Terasaki plates (Greiner, Alphen a/d Rijn, The Netherlands) in a final volume of 20 μl per well containing PHA-L (1 μg/ml), rIL2 (50 U/ml), and irradiated allogenic feeder cells. The plates were incubated at 37°C in a humidified chamber with 5% CO_2. After 10 to 14 days, growing cell cultures from plates showing growth in 10 to 15% of wells were restimulated. Cell samples from these cultures were analyzed for Rev-specific CTL activity on autologous B-LCL cells

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TABLE 1. Specificities of $CD8^+$ CTL clones from individual L709

CTL clone	% Specific lysis[a]	
	rVV-control	rVV-rev
TCC102	1 ± 3	61 ± 5
TCC104	1 ± 2	59 ± 3
TCC106	0 ± 0	86 ± 9
TCC108	−1 ± 0	78 ± 5
TCC110	0 ± 1	79 ± 5
TCC112	0 ± 1	3 ± 1

[a] Average (± standard error; triplicates) percentages of CTL-mediated chromium release by autologous rVV-infected B-LCL cells.

infected with recombinant vaccinia virus (rVV) containing the HIV-1_{LAI} *rev* gene (TG4113; rVV-rev) in chromium release assays as described below. The Rev-specific TCC108 clone expressed T-cell receptor VB14S1 uniformly (data not shown), confirming its clonality. Since the five Rev-specific CTL clones were restricted by the same HLA class I allele, recognized the same 20-mer peptide, and were established from the same Rev-specific cell line, these clones most likely originated from the same cell. For practical reasons, further analyses were performed with one of these CTL clones, TCC108.

Chromium release assays. Target cells were labelled for 1 h with 100 μCi of $Na_2\ ^{51}CrO_4$ (Amersham, Buckinghamshire, United Kingdom), washed three times, and adjusted to a concentration of 2×10^5 cells per ml in R5F. A volume of 50 μl (10^4 cells) was plated in 96-well V-bottom plates. Effector cells were added at ratios of between 3:1 and 10:1 in a final volume of 150 μl. The spontaneous and maximum chromium releases were determined by the incubation of the target cells with R5F only or with 5% Triton X-100, respectively. Triplicate incubations were performed in all assays. After incubation at 37°C for 4 h, supernatants were harvested with a harvesting device (Skatron, Oslo, Norway), and radioactivity was counted in a gamma counter (LKBWallac, Turku, Finland). The percent specific lysis was calculated as (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. To ensure sufficient HIV-1-infected cells at each time point, TCL2H7 cells were infected at a multiplicity of infection (MOI) of approximately 1.0.

Flow cytometry. The expression of CD4, CD8, and HLA-A2 was analyzed by incubation of viable cells with CD4-fluorescein isothiocyanate (CD4-FITC) or CD8-phycoerythrin (Becton Dickinson, Leiden, The Netherlands) or HLA-A2 specific monoclonal antibody BB7.2 (kindly provided by W. Biddison). FITC-conjugated goat anti-mouse immunoglobulin (Becton Dickinson) was used as a second antibody for detection of expression of HLA-A2. For detection of HIV-1 p55 expression, cells were incubated subsequentially with paraformaldehyde–lyso-lecithin, cold absolute methanol, Nonidet P-40 (Sigma), and FITC- or phycoerythrin-labelled anti-HIV-1 p55 monoclonal antibody (clone KC57) according to the instructions of the manufacturer (Coulter, Mijdrecht, The Netherlands).

Peptides. The 20-mer peptides with 10 residues of overlap, together spanning the entire HIV-1 Rev sequence were kindly provided by H. Holmes (Medical Research Council, South Mimms, Potters Bar, United Kingdom). For the preparation of target cells, 0.5×10^6 to 1×10^6 B-LCL cells were incubated with these peptides at 20 μM in 100 μl of R0. After 1 h, 900 μl of R5F was added and cells were incubated overnight. The peptides used for fine mapping of the CTL epitope recognized by TCC108 cells were manufactured at the European Veterinary Laboratory (Woerden, The Netherlands). Chromium-labelled B-LCL cells were incubated with these shorter peptides for 1 h at concentrations ranging from 1×10^{-9} to 3×10^{-4} M, washed twice, and used as target cells.

Virus stocks. rVV-rev and rVV containing the polylinker without insert (186-poly; rVV-control) were kindly provided by M. P. Kieny (Transgène, Strasbourg, France). Stocks were prepared on RK13 cells and stored at concentrations of 1×10^8 to 3×10^8 PFU/ml at −70°C. For the preparation of rVV-infected target cells, B-LCL cells were incubated with 10 PFU per cell at 10^7 cells per ml for 1 h. Subsequently, the cells were diluted to 10^6/ml with R10F and incubated overnight.

An HIV-1 IIIB stock was prepared on freshly infected PHA-activated $CD4^+$ TCC cells and stored at −70°C. The RT activity of this stock was 10^5 RT cpm/ml. The infectious viral titer was determined by infection of PHA-activated PBMC or TCL2H7 cells with serial fivefold dilutions of this stock in quadruplicate. With both cell types an estimated titer of 10^5 infectious particles per ml was found.

RT assay. RT activity was assayed in a microassay as previously described by Gregersen et al. (10) and adapted by Siebelink et al. (25). Culture supernatants were precipitated with 32% polyethylene glycol 6000–1.5 M NaCl. The pellets were resuspended in 15 μl of lysis buffer (50 mM Tris [pH 8.3], 20 mM dithiothreitol, 0.25% Triton X-100) and mixed with 35 μl of H_2O and 50 μl of RT cocktail [100 mM Tris (pH 7.9), 150 mM KCl, 10 mM $MgCl_2$, 4 mM dithiothreitol, 0.6 U of poly(rA)-oligo(dT), 60 μCi of [^{3}H]TTP per ml]. After incubation at 37°C for 1 h, the DNA was precipitated with 20 μl of 120 mM $Na_4P_2O_7 \cdot 10H_2O$ in 60% trichloroacetic acid for 15 min at 4°C. The DNA was harvested on glass fiber filters with a Skatron cell harvester and washed with 12 mM $Na_4P_2O_7 \cdot 10H_2O$ in 5% trichloroacetic acid. The filters were dried at 80°C, and [^{3}H]TTP incorporation was measured in a beta scintillation counter (LKBWallac).

Kinetics of target cell recognition. Three days after their most recent stimulation with PHA-L, 1.2×10^6 $CD4^+$ TCL2H7 cells were incubated with IIIB at 1.2×10^6 RT cpm (MOI of approximately 1) for 3 h at 37°C. The cells were washed three times and cultured at 3×10^5 cells per ml in R10H supplemented with rIL2 (50 U/ml). At various time points, a volume of 0.6 ml, including cells, was harvested. After centrifugation, the supernatants were stored at −70°C for RT assays, part of the cells were fixed for flow cytometric analysis of p55, CD4, and CD8 expression, and part of the cells were labelled with chromium for analysis in chromium release assays.

Coculture of CTL and acutely infected PBMC. PBMC (2×10^7) isolated from a buffy coat were incubated with HIV-1 IIIB at 10^5 RT cpm for 3 h at 37°C. The cells were washed three times and cultured at 5×10^6 cells per 10 ml of R10H supplemented with PHA-L (1 μg/ml) and rIL2 (50 U/ml) in 25-cm^2 flasks. Effector cells were added at a ratio of 0.1:1. At various time points, 2 ml of the culture was harvested and centrifuged (250 × *g*). The supernatants were stored at −70°C for analyses of RT activity and viral RNA sequences, and the cells were fixed with paraformaldehyde–lyso-lecithin for flow cytometric analysis of HIV-1 p55, CD4, CD8, and HLA-A2 expression. At days 6 and 11 postinfection, part of the cells were not fixed but were separated into a $CD8^+$ fraction and a $CD8^-$ fraction with an anti-CD8 monoclonal antibody covalently conjugated to magnetic beads (Becton Dickinson) according to the manufacturer's instructions. After treatment of the $CD8^+$ fraction with DetachaBead (Becton Dickinson) and overnight incubation at 37°C, these cells were analyzed for cytolytic activity against rVV-rev- and rVV-control-infected autologous B-LCL cells. $CD8^+$ PBMC could be discriminated from TCC108 and TCC112 cells by flow cytometric analysis of the expression of HLA-A2, which was present on the PBMC only.

Sequencing. Viral RNA was isolated from supernatants harvested from cultures of HIV-1-infected PBMC with and without the Rev-specific TCC108 cells. The second exon of *rev* was amplified by RT-PCR. After reverse transcription with random primers, cDNA was amplified with primers GTACTTTCTATAG TGAATAGAGTTAGGC and CCTATCTGTCCCCTCAGCTACT. PCR conditions were as follows: 30 s at 95°C, 30 s at 50°C, and 30 s at 72°C for 30 cycles and then 7 min of extension at 72°C. Amplified fragments were sequenced directly on both strands by using the PCR primers with the *Taq* Dye Deoxy Terminator sequencing kit on a 373A sequencing system from Applied Biosystems (Foster City, Calif.). Sequences were analyzed with Geneworks (Intelligenetics, Mountain View, Calif.).

RESULTS

HLA restriction and fine specificity of Rev-specific CTL clones. To analyze the Rev-specific CTL response of a long-term asymptomatic individual in more detail, we cloned a Rev-specific T-cell line generated in a previous study from PBMC of individual L709 (28). Five $CD4^-$ $CD8^+$ Rev-specific CTL clones, TCC102, TCC104, TCC106, TCC108, and TCC110, and one $CD4^-$ $CD8^+$ non-HIV-specific CTL clone, TCC112, were obtained (Tables 1 and 2).

The HLA restriction of the Rev-specific CTL clones was determined with a panel of partially HLA class I-matched B-LCL cells infected with rVV-rev or rVV-control in standard chromium release assays (Fig. 1A). All Rev-specific CTL clones lysed the four rVV-rev-infected heterologous target cells

TABLE 2. Lysis of HIV-1 IIIB-infected cells by $CD8^+$ T cells used in this study[a]

$CD8^+$ T-cell line	Specificity		Effector/target ratio	% Specific lysis (mean ± SD)	
	Protein	Epitope		Mock infection	HIV-1 IIIB infection
TCC108	Rev	SAEPVPLQL	10	5 ± 4	48 ± 6
			3	0 ± 3	37 ± 1
TCC112	ND[b]	ND	10	−1 ± 1	4 ± 2
			3	−4 ± 1	−8 ± 1
TCL1C11	RT	IVLPEKDSW	10	−1 ± 1	63 ± 8
			3	−1 ± 1	62 ± 9

[a] Target cells were $CD4^+$ TCL2H7 cells 10 days after mock or HIV-1 IIIB infection (MOI of approximately 0.05).

[b] ND, not determined.

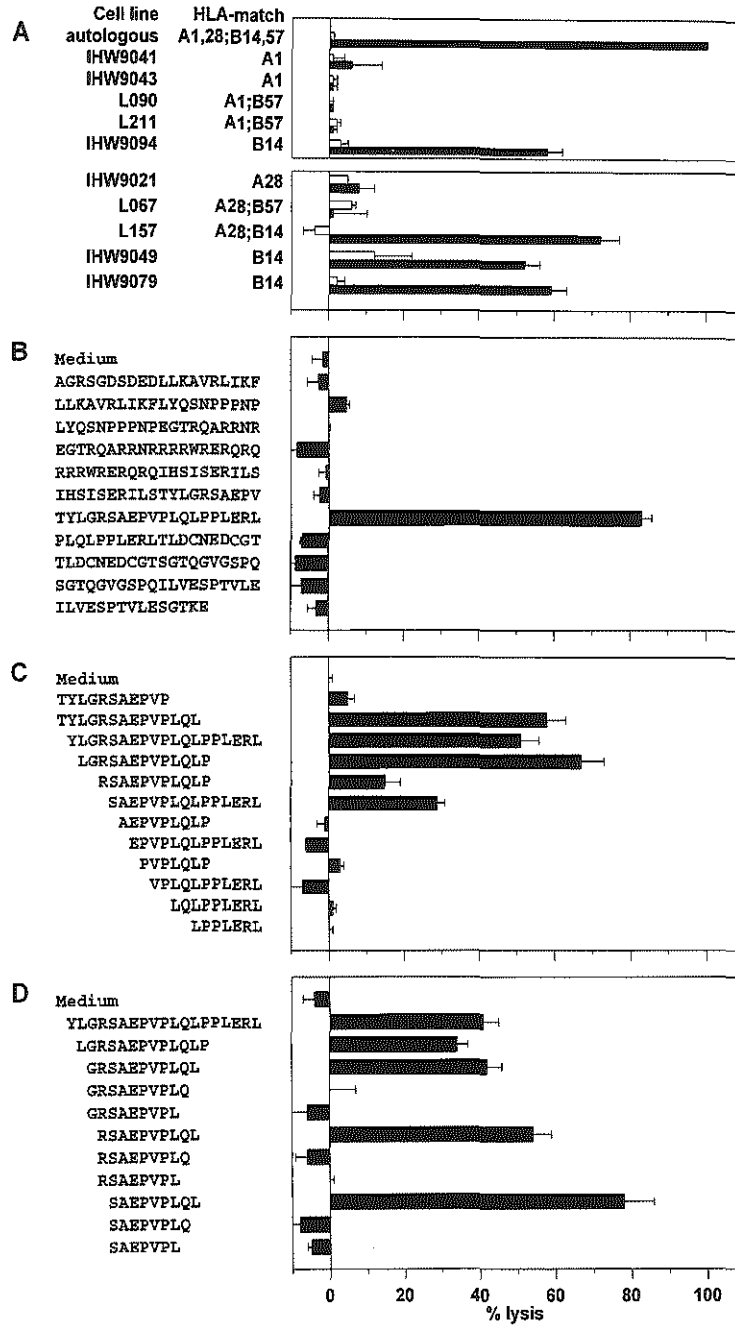

FIG. 1. HLA restriction and fine specificity of HIV-1 Rev-specific CTL clone TCC108. (A) Autologous and partially HLA class I-matched B-LCL cells were infected with rVV-rev (filled bars) or rVV-control (open bars) and analyzed for recognition by TCC108 cells in a standard chromium release assay (upper panel). Additional B-LCL cells were analyzed in a separate assay to confirm HLA-B14 restriction (lower panel). (B to D) Peptide-pulsed autologous B-LCL cells were analyzed for recognition by TCC108 cells in standard chromium release assays. Chromium-labelled target cells were incubated overnight with one of the 11 20-mer peptides together spanning the entire Rev sequence (B) or for 1 h with the N- and C-terminally truncated peptides before the addition of effector cells (C and D, respectively). Effector-to-target ratios were between 3:1 and 10:1 in all assays. The average percent specific lysis (with standard error) for triplicates is shown. Results similar to those presented in panels A and B were obtained with the Rev-specific CTL clones TCC102, TCC104, TCC106, and TCC110 (data not shown). The non-Rev-specific clone TCC112 did not lyse any of the rVV-infected or peptide-pulsed target cells (data not shown).

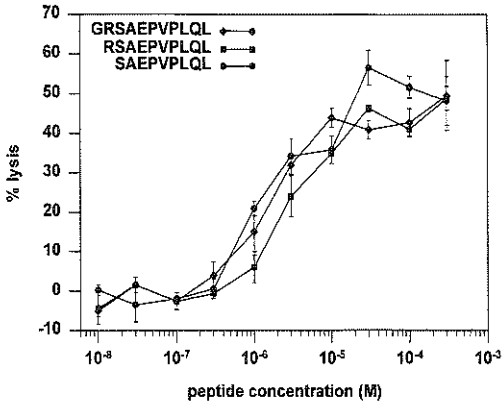

FIG. 2. Titration of peptides recognized by the Rev-specific clone TCC108. Chromium-labelled autologous B-LCL cells were incubated with the peptide Rev(65-75) GRSAEPVPLQL, Rev(66-75) RSAEPVPLQL, or Rev(67-75) SA EPVPLQL for 1 h at the concentrations indicated. Subsequently, the target cells were washed and cocultivated with TCC108 cells for 4 h. The effector-to-target cell ratios were 10:1. The average percent specific lysis (with standard error) for triplicates is shown. No lysis of B-LCL cells without peptide was observed (data not shown).

that shared HLA-B14 (Fig. 1A shows the results obtained with TCC108 cells). Heterologous target cell lines that were matched for HLA-A1 (four cell lines), HLA-A28 (three cell lines), or HLA-B57 (three cell lines) were not lysed by the Rev-specific CTL clones (Fig. 1A). These data indicate that recognition of Rev by the CTL clones was restricted by HLA-B14.

The location of the CTL epitope within the Rev protein was estimated by using 11 20-mer peptides with 10 amino acid residues of overlap, together spanning the entire Rev sequence. All five clones specifically lysed autologous B-LCL cells pulsed with the peptide Rev(62-81) TYLGRSAEPVPLQ LPPLERL but not those pulsed with the other peptides (Fig. 1B). Truncated peptides lacking the N-terminal residues 62T, 63Y, 64L, 65G, and 66R were recognized by TCC108 cells, but those without 67S were not (Fig. 1C), indicating that 67S defines the N terminus of the minimal epitope. TCC108 cells recognized peptides truncated C terminally at 75L but not peptides truncated at 74Q or 73L (Fig. 1D). These data show that Rev(67-75) SAEPVPLQL is the minimal epitope recognized by TCC108 cells. The amino acid arginine (R) has been described to serve as an anchor at position 2 in HLA-B14 binding peptides (5), and it flanks the minimal epitope at position 66 of Rev. Titration of the peptides SAEPVPLQL, RSA EPVPLQL, and GRSAEPVPLQL on autologous B-LCL cells revealed that all three peptides required a concentration of at least 1 μM to be recognized by TCC108 cells and showed a similar increase in specific lysis with increasing concentrations (Fig. 2). Thus, the additional residues 66R and 65G did not contribute to the optimal recognition of the CTL epitope.

Kinetics of target cell lysis and suppression of HIV-1 production by Rev- and RT-specific CTL. To evaluate the temporal relationship between protein expression in infected cells and the antiviral activity of CTL, we compared the kinetics of cytolysis of infected cells by CTL against the early protein Rev (TCC108 cells) and by CTL against the late protein RT [TCL1C11 cells; specific for RT(244-252) IVLPEKDSW in the context of HLA-B57 (29)]. Both TCC108 and TCL1C11 cells were shown to lyse an HIV-1 IIIB-infected $CD4^+$ T-cell line,

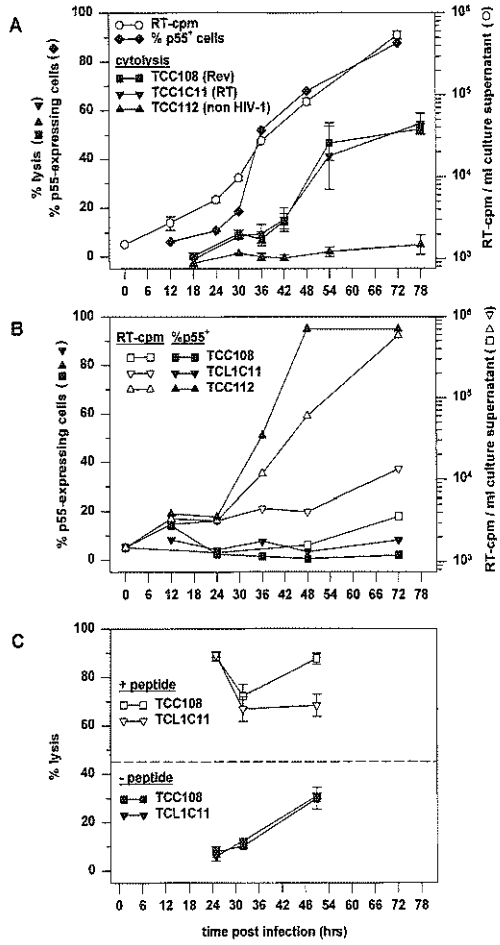

FIG. 3. Kinetics of HIV-1 production and lysis of infected cells by Rev- and RT-specific CTL. (A) $CD4^+$ TCL2H7 cells were infected as described in Materials and Methods, and culture supernatants were harvested at the indicated times for analysis of virus production. The TCL2H7 cells were analyzed for p55 expression and for susceptibility to CTL-mediated lysis by Rev-specific clone TCC108, RT-specific clone TCL1C11, and non-HIV-specific clone TCC112. The effector-to-target ratios were 10:1. Lysis of uninfected $CD4^+$ TCL2H7 cells was below 5% in all assays (data not shown). The chromium release data are plotted as the average (with standard error) for triplicates at the time point at which the chromium release assay was terminated, i.e., 6 hours after the addition of chromium. This time was required for the chromium labelling (1 h), washing of the target cells and preparing the cocultures of the effector and target cells (1 h), and incubation (4 h). (B) p55 expression (closed symbols) and virus production (open symbols) by TCL2H7 cells in the presence of TCC108 cells, TCL1C11 cells, or TCC112 cells. Effector and target cells were discriminated by flow cytometric analyses of CD8 and CD4 expression, respectively. The population of p55-expressing TCL2H7 cells is expressed as a percentage of the $CD8^-$ cells and not of the $CD4^+$ cells, since CD4 was down-regulated in a major fraction of the infected cells. (C) Infected TCL2H7 cells were analyzed in chromium release assays after incubation without peptide or with the relevant peptides at 10 μM: SAEPVPLQL for TCC108 cells and IVLPEKDSW for TCL1C11 cells. The average specific lysis (with standard error) for triplicates is shown. The dashed line shows the percentage of p55-expressing cells at 48 h after infection. Lysis of uninfected TCL2H7 cells without peptides was always below 5% (data not shown), and peptide-pulsed uninfected TCL2H7 cells were lysed as efficiently as peptide-pulsed infected cells (data not shown).

TCL2H7, obtained from individual L709 (Table 2). At 12 h after infection of TCL2H7 cells with HIV-1 IIIB (MOI of approximately 1.0), no significant population of p55-expressing cells was detected (Fig. 3A). The percentage of p55-expressing cells increased slightly between 12 and 24 h and increased rapidly thereafter: from 18% at 30 h to 50% at 36 h, 68% at 48 h, and 89% at 72 h. Significant virus production was found at 30 h after infection, in agreement with previous reports on the replication cycle of HIV-1 (14, 21), and production was increased at 36, 48, and 72 h (Fig. 3A). These data indicate that all cells expressing detectable levels of p55 at 48 h (here 68% of the cells) had been infected by the initial inoculum. Since Rev-encoding mRNA and Rev protein have been detected at between 16 and 18 h after infection in IIIB-infected $CD4^+$ T cells (14, 22), we expected to find significant specific lysis by TCC108 cells added between 26 and 30 h or between 32 and 36 h after infection. Although the percentage of specific lysis was higher at these time points than at 14 to 18 h, it did not exceed 10% (Fig. 3A). At 38 to 42 h it was still only 15%, and at 50 to 54 h it was 42%, reaching a maximum of 55% after 74 to 78 h (Fig. 3A). The RT-specific CTL lysed the infected cells at similar levels and with similar kinetics as the Rev-specific CTL (Fig. 3A). As expected, the infected cells were not lysed by the non-HIV-specific TCC112 cells at any time (Fig. 3A). In a parallel experiment, part of the infected TCL2H7 cells were cocultured with the CTL immediately after infection. In cultures containing TCC108 or TCL1C11 cells, p55 expression and virus production were significantly suppressed at all times (Fig. 3B). The presence of the TCC112 cells did not affect p55 expression or virus production (Fig. 3B).

That TCC108 and TCL1C11 cells were cytolytic at all time points was verified in a separate assay: early after infection, target cells loaded with the relevant synthetic peptides were indeed lysed efficiently (Fig. 3C). Later, at 47 to 51 h, specific lysis of infected cells not loaded with the peptides also was observed (Fig. 3C), confirming the results shown in Fig. 3A.

Together these data indicate that infected cells cultured in the absence of HIV-specific CTL are not susceptible to cytolysis before they produce virus. Furthermore, they suggest that mechanisms other than cytolysis caused the observed CTL-mediated inhibition of virus production in the cocultures of effector and target cells.

In vitro inhibition of HIV-1 replication by TCC108 cells. Subsequently, we investigated the longevity of the TCC108-mediated antiviral activity with freshly isolated PBMC. HLA-B14-matched PBMC were infected with HIV-1 IIIB (MOI of approximately 0.05) and cocultured with TCC108 cells or the non-HIV-1-specific TCC112 cells for 11 days. The TCC-to-CD4 cell ratio was 0.2 at the start of the experiment. Virus production was detected in cultures without TCC108 cells on days 6, 9, and 11 (Fig. 4A). In the coculture with TCC108 cells, only a low level of RT activity was detected on day 9. On day 11 this level was similar to that in the control cultures. Flow cytometric analyses showed that the number of TCC108 and TCC112 cells increased 100-fold during the culture period (Fig. 4B). Furthermore, $CD8^+$ cells, recovered from the culture containing TCC108 cells on days 6 and 11 by magnetic bead selection, showed significant Rev-specific CTL activity (Fig. 4C). These data indicate that the lack of control of virus replication could not have been due to the disappearance of the clone from the culture or to impairment of CTL function.

To test whether the virus had escaped CTL recognition by mutation in the epitope, we sequenced the second exon of Rev directly on the amplicons from the total virus pool produced in the presence or absence of TCC108 cells. Sequencing was performed on samples from day 11 only, since amplifications

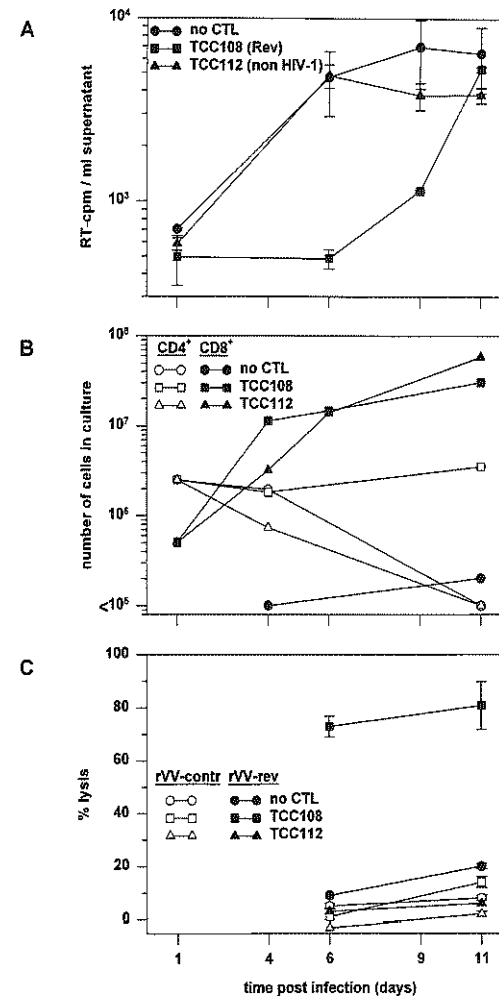

FIG. 4. Inhibition of HIV-1 replication by Rev-specific CTL. HLA-B14-expressing PBMC from an HIV-seronegative individual were infected with HIV-1 IIIB as indicated in Materials and Methods. PBMC (5×10^6) were cocultivated without CTL, with 5×10^5 Rev-specific TCC108 cells, or with 5×10^5 non-HIV-specific TCC112 cells. Both types of TCC cells had been stimulated 7 days before addition to the PBMC. (A) Virus production was analyzed by quantification of the RT activity in culture supernatants. The average RT activity (with standard error) for triplicates is shown. (B) The fates of the $CD4^+$ PBMC and the $CD8^+$ TCC108 and TCC112 cells were determined by counting of the cells and flow cytometric analysis of membrane-expressed CD4, CD8, and HLA-A2. HLA-A2 was included to discriminate between $CD8^+$ PBMC (expressing HLA-A2) and the added TCC108 and TCC112 cells (both expressing HLA-A1 and -A28). (C) $CD8^+$ cells were recovered from the cultures on days 6 and 11 by magnetic bead selection and analyzed for Rev-specific CTL activity on autologous B-LCL cells infected with rVV-rev or rVV-control. The average percent specific lysis (with standard error) for triplicates is shown.

carried out with earlier samples did not yield PCR products. Only the virus from the coculture with TCC108 cells was found to have a mutation, 69E→K, located in the third residue of the minimal epitope recognized by TCC108 cells (Fig. 5). The sequence signal was uniform, indicating that >90% of the virus population contained the mutation. The mutant peptide SAK PVPLQL was not recognized by TCC108 cells at concentra-

HIV-1 Rev

2nd exon	27	31	41	51	61	71	81	91	101	111
$IIIB_{BH}$	PPPN	PEGTRQARRN	RRRRWRERQR	QIHSISERIL	STYLGR	SAEP VPLQL	PPLER	LTLDCNEDCG	TSGTQGVGSP	QILVESPTVL	ESGTKE
$IIIB_{HXB2R}$	----	----------	----------	----------	G-----	---- -----	-----	----------	----------	----------	------
$IIIB_{NL43}$	----	----------	----------	----------	------	---- -----	-----	----------	----------	----------	------
IIIB	----	----------	----------	----------	------	---- -----	-----	----------	----------	----------	------
$IIIB_{66K}$	----	----------	----------	----------	------	--K- -----	-----	----------	----------	----------	------

FIG. 5. Sequence analysis of the second-exon Rev from virus cultured in the absence or presence of Rev-specific TCC108 cells. Viral RNA was isolated from culture supernatants from the experiment shown in Fig. 4 on day 11 postinfection. The second-exon Rev sequences of virus cultured in the absence or presence of TCC108 cells are shown below the sequences of three known IIIB clones (19) for reference purposes. The CTL epitope region is in boldface.

tions ranging from 10 nM to 300 μM (data not shown), indicating that the new virus was indeed an escape variant.

To assess whether escape from a Rev-specific CTL response had occurred in vivo, we attempted to amplify the plasma virus of individual L709 at different times after seroconversion. Primers and PCR conditions were the same as for the amplification of the in vitro-cultured virus. However, no PCR products were obtained, most likely due to the low viral load in this individual.

DISCUSSION

The fine specificity, kinetics of target cell lysis, and capacity to inhibit HIV-1 replication of Rev-specific CTL from an individual infected with HIV-1 for more than 12 years without developing symptoms were analyzed. CTL clones generated from this individual's PBMC recognized the 9-mer peptide Rev(67-75) SAEPVPLQL as their minimal epitope in the context of HLA-B14. The presence of residue L75 at position 9 is consistent with the reported motif for HLA-B14 binding peptides (5). Other predicted anchor residues were not present. Two longer peptides containing a potential anchor, 66R, were recognized at similar levels of efficiency as the minimal epitope, indicating that this residue did not enhance presentation to the CTL. Analysis of the interactions between HIV-1-specific CTL and HIV-1-infected target cells, i.e., immortalized polyclonal $CD4^+$ T cells (TCL2H7 cells) infected with HIV-1 IIIB, showed target cell lysis by both Rev- and RT-specific CTL. This indicates that their respective epitopes, as defined by rVV and synthetic peptide analyses, were indeed generated in these HIV-1-infected cells. The kinetics of Rev- and RT-specific CTL-mediated cytolysis, first observed well before peak virus production, indicate that CTL may prevent a significant quantity of virus from being produced. This is in agreement with the kinetics of Gag-, RT-, and Env-specific CTL-mediated lysis of HIV-1-infected immortalized H9 and T1 cells, as reported by Yang et al., who sampled at 24, 48, 72, and 96 h after infection (31). As a result of frequent sampling between 24 and 48 h after infection, we were able to extend their findings by showing that infected cells, in the absence of CTL, produce virus before they become susceptible to CTL-mediated lysis. A peptide-pulsing experiment revealed that limited target cell lysis during the first 42 h after infection could not be explained by insufficient effector cell numbers or impaired effector cell function. Also, the possibility that limiting antigen levels had affected the efficiency of cytolysis (26) is probably not relevant for our system, considering the extent of viral protein expression and virus production observed at 30 to 36 h after infection. The similarity in the kinetics of cytolysis targeted at the early protein Rev and at the late protein RT suggests that HIV-1 infection had interfered, transiently, with a general aspect of the antigen processing and presentation pathway. A 20 to 50% reduction of HLA class I surface expression after HIV-1 infection has been reported (13, 23, 31). This down-regulation was shown to decrease cytolysis by HLA-specific CTL (13, 23) but had no appreciable effect on the capacity of infected cells to present synthetic peptides (31). The latter finding is consistent with our observation that TCL2H7 cells were susceptible to CTL-mediated lysis early after infection when pulsed with the relevant peptides. The presentation of endogenous epitopes, however, may be affected when the intracellular expression of new HLA class I molecules is impaired. Indeed, recently it has been shown that HIV-1 Nef is involved in protecting infected cells from specific CTL-mediated lysis by affecting the HLA class I surface expression (4). Also, other mechanisms, such as Tat-mediated interference with the proteasome function (24), may have impeded the generation of HLA class I presentable peptides early after infection.

If lysis of infected cells were the only inhibitory mechanism of the CTL, one would expect a steady increase of the levels of p55 expression and virus production by infected cells, even in the presence of CTL, until the time at which they became susceptible to CTL-mediated lysis. However, when the Rev- or RT-specific CTL were added to the infected cells immediately after infection, viral protein expression and virus production were suppressed without delay during the entire coculture period. These results suggest that early antiviral activity involved noncytolytic mechanisms. Indeed, $CD8^+$ T cells have been shown to inhibit HIV-1 replication by the production of soluble factors (3, 16, 32), to inhibit hepatitis B virus gene expression by a noncytolytic mechanism (11), and to exert antiviral effects against murine rotavirus and VV by perforin- and Fas-independent mechanisms (7, 12). Because the Rev- and RT-specific CTL were added to the target cells after infection, factors that prevent binding or entry of HIV-1 could not have been involved in the observed suppression of HIV-1 production. It is possible that the suppression was mediated by $CD8^+$-T-cell-derived factors that interfere with viral transcription, like IL-16 (17, 33) or $CD8^+$ T-cell antiviral factor (16). If noncytolytic antiviral mechanisms of TCC108 cells also contributed to the observed suppression of viral replication in HLA-B14-matched PBMC, they must have been specifically targeted toward the infected cells expressing the appropriate HLA epitope complex, since the replication of virus that had escaped CTL recognition by a mutation in the HLA-B14-restricted epitope was not significantly affected. Noncytolytic interference with transcription and translation may, like cytoly-

sis, require that CTL are targeted to infected cells via major histocompatibility complex-epitope complexes, for this would be a safeguard against harming bystander cells. To determine the relative contributions of CTL against different proteins to the control of viral replication, further characterization of (i) CTL against late proteins with respect to their capacity to inhibit viral replication, (ii) the affinities of the different epitopes for their HLA restriction elements, and (iii) the affinity of each clone for its HLA peptide complex is required.

In summary, the present data show that CTL against early and late viral proteins can lyse acutely HIV-1-infected cells efficiently, but only after the production of progeny virus has started. Yet, virus replication in freshly isolated PBMC was significantly suppressed by CTL against the Rev protein, which resulted in the rapid selection of CTL escape virus in vitro. It is important to realize that the antiviral effect of CTL responses in vivo is determined by multiple factors, including the breadth of the CTL response. Such factors will be difficult to address in vitro with a limited set of CTL clones. However, studies on the kinetics of viral protein-mediated interference with target cell killing and on the relative contributions of CTL against early and late viral proteins to the inhibition of viral replication will shed new light on complications which the immune system encounters in clearing virus and accordingly may contribute to the development of vaccines and immunotherapies against AIDS.

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Macrophage Tropism of Human Immunodeficiency Virus Type 1 Facilitates In Vivo Escape from Cytotoxic T-Lymphocyte Pressure

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Early after seroconversion, macrophage-tropic human immunodeficiency virus type 1 (HIV-1) variants are predominantly found, even when a mixture of macrophage-tropic and non-macrophage-tropic variants was transmitted. For virus contracted by sexual transmission, this is presently explained by selection at the port of entry, where macrophages are infected and T cells are relatively rare. Here we explore an additional mechanism to explain the selection of macrophage-tropic variants in cases where the mucosa is bypassed during transmission, such as blood transfusion, needle-stick accidents, or intravenous drug abuse. With molecularly cloned primary isolates of HIV-1 in irradiated mice that had been reconstituted with a high dose of human peripheral blood mononuclear cells, we found that a macrophage-tropic HIV-1 clone escaped more efficiently from specific cytotoxic T-lymphocyte (CTL) pressure than its non-macrophage-tropic counterpart. We propose that CTLs favor the selective outgrowth of macrophage-tropic HIV-1 variants because infected macrophages are less susceptible to CTL activity than infected T cells.

The predominant biological phenotype of human immunodeficiency virus type 1 (HIV-1) isolates changes during the course of infection. Early after seroconversion, usually only macrophage-tropic, non-syncytium-inducing (NSI) variants are found. With progression to AIDS, HIV-1 isolates tend to lose their capacity to infect macrophages and may gain the ability to induce syncytia (SI) (10, 14). It has been well documented that only macrophage-tropic viruses persist directly after seroconversion (21), even when a mixture of variants was transmitted (2, 8). Data obtained in the simian immunodeficiency virus macaque model have suggested that Langerhans cells or macrophages are the primary target cell after sexual transmission (12). It has been proposed that these primary target cells act as a selective barrier against variants that are not capable of infecting them, i.e., SI variants (12, 20). This physical barrier is not effective if HIV enters the body via other routes, e.g., through blood transfusion, needle-stick accidents, or intravenous drug abuse. Also, in those cases in which macrophages are less likely to be the sole primary target cell type, the selective outgrowth of macrophage-tropic/NSI viruses is observed (2, 12).

Therefore, it must be assumed that additional mechanisms select against non-macrophage-tropic variants after the virus has entered the body. HIV-1-specific cytotoxic T lymphocytes (CTLs) have been shown to exert strong selective pressure on HIV-1 quasispecies during seroconversion (1, 7) and are thus a likely candidate. CTL pressure on replication of non-macrophage-tropic and macrophage-tropic variants was analyzed in a previously described xenograft versus host disease (GvHD) mouse model, because it supports high-level replication of both virus types in their characteristic target cells (6).

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MATERIALS AND METHODS

Animals. XID mice (CBA/HNOlaHsd; Harlan Nederland BV, Zeist, The Netherlands) received total body irradiation with syngeneic bone marrow support. Human peripheral blood mononuclear cells (PBMC) were isolated from the whole blood of HLA B14-matched seronegative individuals by Ficoll gradient. After one wash step, cells were administered intraperitoneally (i.p.) at 3×10^6 to 5×10^6 cells per gram of mouse body weight, which results in an acute GvHD situation within 6 to 14 days (6). After PBMC were administered, CTLs (10^7 per mouse) were injected i.p. together with 10^4 IU of recombinant human interleukin-2. This was repeated every other day in accordance with the optimal dose determined in previous studies of passively transferred CTLs in the HuPBL-SCID mouse model (19).

One hour after reconstitution, mice were challenged with 30 50% mouse infectious doses of the respective HIV-1 or HIV-2 isolates i.p. Six days after grafting and infection of the human PBMC were done, the first signs of the acute GvHD reaction were observed, after which the mice were sacrificed. Cells from the peritoneal lavages were analyzed for viral load using an infectious center test. To this end, the cells were titrated in duplicate starting at 2×10^6 cells per well using fivefold dilution steps and cultured in the presence of HIV-permissive feeder cells. The lowest number of cells required to detect virus by reverse transcriptase assay after 7 days of culture was taken as a measure of the viral load.

Viruses. The HIV strains selected for the present studies were HIV-1 ACH 320.2A.1.2 (molecularly cloned, primary, SI, non-macrophage tropic; in short, HIV-1 #1.2) and HIV-1 ACH 320.2A.2.1 (molecularly cloned, primary, NSI, macrophage tropic; in short, HIV-1 #2.1). These closely related viruses were isolated from participant ACH320 from the Amsterdam cohort studies (ACH) of HIV infection and AIDS in homosexual men, as previously described (3). As a control, HIV-2 RH2 to 5 A10 (biologically cloned, primary, NSI, macrophage-tropic; in short, HIV-2 #RH2–5) from the Rotterdam cohort of HIV-2-infected persons (5) was used. Replication of the viruses in $CD4^+$ T cells has been described previously (17). Primary sequences of the second exon of Rev, which includes the TCC108 epitope, were determined as previously described (16).

CTL clones. Two CTL clones, TCC108 and TCC112, obtained via limiting dilution from participant ACH709 from the ACH, have been described in detail

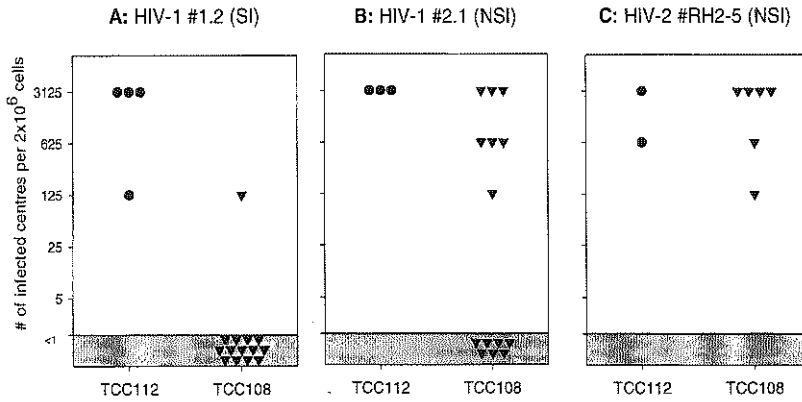

FIG. 1. Viral load determined by an infectious center test in HIV-1-infected (#1.2 [SI]; #2.1 [NSI]) and HIV-2-infected (#RH2–5 [NSI]) GvHD mice that had received either the non-HIV-specific (TCC112) or the HIV-1 Rev-specific (TCC108) $CD8^+$ CTL clone. The lowest amount of cells required to detect virus was taken as a measure of viral load. Each symbol represents the viral load of an individual mouse.

(16, 17). Both clones are $CD4^-$ $CD8^+$ as determined by flow cytometry. TCC112 did not lyse autologous $CD4^+$ T cells (TCL2H7) infected with HIV-1. TCC108 was shown to recognize HIV-1 amino acids 67 to 75 of the Rev protein (SAEP VPLQL) in the context of HLA B14. CTL clones were administered to the mice 7 to 10 days after in vitro stimulation. The presence of TCC108 cells in the lavages and their functionality were determined by flow cytometry and a chromium release assay (15, 17). In vitro CTL assays on autologous B and T cells have been described previously (17). In vitro CTL assays were performed for 4 h at an effector-to-target ratio of 10 to 1 and a peptide concentration of 10 μM.

RESULTS

HIV variants replicate readily in human PBMC in GvHD mice, without suppression by a non-HIV-specific $CD8^+$ clone and with the same tropism as those used in vitro. All viruses in this study established infection in GvHD mice in the presence of non-HIV-specific TCC112 cells: high numbers of HIV-1- or HIV-2-infected cells were reisolated despite the presence of TCC112 cells (Fig. 1), similar to the results from previous studies where no $CD8^+$ cells were added (11). No differences in the viral load were observed for HIV-1 variants #1.2 and #2.1 in this respect (Fig. 1A and B). Combined CD68 immunohistochemistry and HIV RNA in situ hybridization on tissues from GvHD mice showed that $CD68^+$ cells did not contain RNA from SI variant #1.2. By contrast, RNAs from NSI variants #2.1 and #RH2–5 were easily detected in $CD68^+$ cells (18), indicating that these viruses did replicate in macrophages in vivo in accordance with their in vitro tropism (5, 9).

Macrophage-tropic HIV-1 escapes more easily from a Rev-specific CTL clone in vivo. In the presence of HIV-1 Rev-specific CTL (TCC108), clear differences in the numbers of infected cells were observed, depending on the virus used. Replication of HIV-2 #RH2–5, which does not contain the CTL epitope, was not suppressed by the HIV-1 Rev-specific CTLs (Fig. 1C). The non-macrophage-tropic primary isolate HIV-1 #1.2 (SI) was efficiently suppressed in 13 of 14 animals (Fig. 1A). By contrast, high numbers of infected cells were found in 7 of 14 animals infected with the macrophage-tropic primary virus HIV-1 #2.1 (NSI) (Fig. 1B). Regardless of whether virus could be detected in these mice, the numbers of functional TCC108 cells in the peritoneal lavages were comparable in all animals, as determined by flow cytometry and chromium release assays (data not shown).

HIV variants escape from specific CTL pressure by mutations in the minimal epitope. The HIV-1 strains that had been passaged through these GvHD mice (Table 1) were subsequently screened for mutations in Rev. For this purpose, the second exon of Rev, including the minimal epitope for TCC108, was amplified by PCR and sequenced (16). No mutations were observed in viruses passaged through mice which had received the non-HIV-specific TCC112 cells (Table 1). By contrast, viruses that could be recovered from mice despite the

TABLE 1. In vitro characterization of HIV-1 variants after passage in GvHD mice

HIV-1 strain[a]	Origin[b]	Minimal epitope[c]	% Lysis in chromium release assay[d]	% $p55^+$ cells[e]
#1.2nm1	#1.2 + TCC112	SAEPVPLQL	44 ± 2	8
#2.1nm1	#2.1 + TCC112	SAEPVPLQL	44 ± 2	40
#1.2rm1	#1.2 + TCC108	SEEPVPLQL	1 ± 4	136
#2.1rm1	#2.1 + TCC108	SAEHVPLQL	4 ± 3	113
#2.1rm2	#2.1 + TCC108	SAESVPLQL	70 ± 0	28
#2.1rm3	#2.1 + TCC108	SVEPVPLQL	74 ± 4	2
#2.1rm4	#2.1 + TCC108	SAEPVPFQL	NT[f]	
#2.1rm5	#2.1 + TCC108	SLEPVPLQL	NT	
#2.1rm6	#2.1 + TCC108	SAEPVPFQL	NT	
#2.1rm7	#2.1 + TCC108	SAEPVPFQL	NT	

[a] nm, not mutated; rm, Rev mutated.

[b] The virus strains were obtained by short-term coculture of mitogen-stimulated PBMC depleted of $CD8^+$ cells with cells isolated from the peritoneal cavity of GvHD mice.

[c] Predicted amino acid sequence of the minimal epitope is given, and mutations relative to the sequence of the parental strains are underlined. Nine-amino-acid-long peptides were generated on the basis of the predicted amino acid sequences of the minimal epitope of their respective viruses.

[d] Lysis of a B14-matched Epstein-Barr virus-transformed B-cell line pulsed with 10 μM indicated peptide was determined in a chromium release assay as previously described (16).

[e] TCL 2H7 cells were infected with the HIV-1 reisolates and subsequently cultured in the absence (no clone) or presence of TCC108. After 11 days, the percentage of HIV-1 p55-expressing $CD4^+$ cells was determined by flow cytometry. The number of $p55^+$ cells is expressed as a percentage of positive cells compared with $p55^+$ cells in the absence of TCC108 cells (100%).

[f] NT, not tested.

presence of Rev-specific TCC108 cells, all proved to have a mutation in the minimal epitope SAEPVPLQL (Table 1), but not outside the epitope region (not shown). These data indicate that TCC108 cells exerted selective pressure on HIV-1 replication in an antigen-specific and MHC class I-restricted manner.

Some of the escape mutants are no longer recognized in in vitro assays. Nine-mer peptides mimicking the various wild-type or mutant Rev epitopes were tested for in vitro recognition by TCC108, when presented on an autologous B-cell line. Peptides corresponding to the index epitope, as found in HIV-1 #1.2nm1 and HIV-1 #2.1nm1 (wild type for the Rev epitope), were efficiently recognized. Accordingly, virus replication was suppressed when autologous T cells were infected with HIV-1 #1.2nm1 or HIV-1 #2.1nm1 and cultured in the presence of TCC108 (Table 1). HIV-1 #1.2rm1 and HIV-1 #2.1rm1, which could be isolated despite the presence of TCC108 in vivo, were no longer suppressed in these in vitro cultures. As expected, the two peptides corresponding to the TCC108 epitope from these viruses were not recognized in vitro.

Other escape mutants are still recognized in vitro, but not in GvHD mice. Unexpectedly, two other viruses that had escaped in vivo CTL pressure (HIV-1 #2.1rm2 and HIV-1 #2.1rm3) were still suppressed by TCC108 in in vitro cultures (Table 1), despite a mutation in their Rev epitope. Accordingly, the synthetic peptides representing these mutant epitopes were still recognized when presented on autologous B cells (Table 1). To confirm that HIV-1 #2.1rm2 and HIV-1 #2.1rm3 were indeed CTL escape variants in vivo, GvHD mice grafted with HLA B14-matched human PBMC were challenged with HIV-1 #2.1rm2 and HIV-1 #2.1rm3 in the presence of TCC108. Virus could be isolated from all the mice, and no additional mutations were observed, indicating that these viruses had indeed escaped from CTL pressure in vivo (data not shown).

DISCUSSION

Here we have used the GvHD mouse model to study interactions between CTLs and different HIV variants. In contrast to data obtained in the HuPBL-SCID model (19), we found no evidence for non-HLA-restricted suppression of HIV replication (11). As anticipated, replication of viruses containing the wild-type Rev epitope was suppressed by specific CTLs in an HLA-restricted manner and virus could escape from this pressure by mutation of the minimal epitope.

Thus, we defined a model system to study the interactions between CTL and HIV-1 variants, mimicking interactions in early HIV infection. Macrophage-tropic HIV-1 #2.1 was more efficient in escaping CTL pressure than its closely related non-macrophage-tropic counterpart HIV-1 #1.2. Which factors, other than tropism, could have contributed to this more successful escape from CTL pressure? Differences in the fidelity of the reverse transcriptase enzymes of these clones are not a likely explanation, given their overall close relatedness and similarity (4). Furthermore, the primary sequences of the CTL epitope itself and of the flanking regions are identical for these clones (4). This excludes differences in processing and presentation of the epitope for these HIV-1 variants.

How may the macrophage tropism of transmitted viruses contribute to escape from the immune pressure exerted by CTLs? HIV-1 #2.1 could have escaped from CTL pressure more easily if it had more replication cycles to acquire mutations than HIV-1 #1.2, i.e., if infected macrophages were less susceptible to CTL activity than infected T cells. Macrophages migrate easily into peripheral tissues, which may protect them from CTL activity, since CTLs can only affect target cells in their immediate proximity. In addition, T cells and macrophages differ in the expression levels of adhesion molecules, which may also influence the CTL-target cell interaction. Finally, the processing of antigens may differ among cells of different lineages or depend on the activation state of cells (13). This may also help explain the somewhat enigmatic observation that some variants were recognized in T cells in vitro but not in PBMC in vivo.

Irrespective of the mechanism involved, CTLs appear to control wild-type macrophage-tropic virus replication less efficiently than non-macrophage-tropic virus replication in vivo. Reduced pressure on macrophage-tropic variants allows for extra replication cycles, enabling the virus to acquire mutations that help it escape from CTL recognition (1) and establish chronic infection. We therefore propose that macrophages, in addition to acting as a barrier for non-macrophage-tropic HIV-1 variants at the port of entry (12), serve as a sanctuary from CTL activity for macrophage-tropic variants.

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Construction and characterisation of infectious recombinant HIV-1 clones containing CTL epitopes from structural proteins in Nef

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Abstract

In this study the construction is described of HIV-1 molecular clones in which CTL epitopes from RT or Env late proteins were inserted into the Nef early protein. The ectopic epitopes were efficiently processed from the recombinant Nef proteins, were recognized by their cognate CTL in cytolytic assays, and did not perturb virus replication or viral protein expression in vitro. These recombinant viruses will therefore be an important tool in studying the effect of distinct epitope expression kinetics on the efficiency of CTL-mediated suppression of HIV-1 replication.

Keywords: HIV-1; Recombination; CTL; Epitopes

HIV is a lentivirus for which protein expression is strictly regulated during the replication cycle (for a review, see Cullen, 1992). After entry into the target cell, the viral proteins Tat, Rev and Nef are the first to be expressed. The regulatory proteins Tat and Rev are crucial for viral replication, via enhancement of transcription and regulation of HIV-1 mRNA export to the cytoplasm (Cullen, 1992; Jeang et al., 1999; Pollard and Malim, 1998). The accessory genes encoding Vpu, Vif and Vpr are expressed next, together with Env Gag and Pol are the last proteins to become detectable, after which assembly of viral particles begins (Feinberg et al., 1986; Kim et al., 1989).

Infection with HIV leads to a chronic infection and although the virus cannot be cleared, immunity plays a role in suppressing the virus spread. Recent reports have shown that an efficient immune response against the regulatory proteins correlates with the control of primary viremia (Allen et al., 2000) and chronic infection (Geretti et al., 1999) in SIV-infected monkeys, and with a

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better prognosis for HIV-1 infected individuals (van Baalen et al., 1997; Zagury et al., 1998). This is substantiated further by a recent study, which showed no significant differences between the control of viremia in infected individuals and the presence of CTL responses against Tat and Rev, but demonstrated that the breadth of the CTL response against Tat correlates with an efficient control of disease progression (Addo et al., 2001). To test whether the early expression of Tat and Rev contributes to the more effective control of HIV/SIV viremia it was decided to manipulate the expression kinetics of CTL epitopes. Deletion, insertion or mutation in the *tat* and *rev* genes is problematic, because the open reading frames (ORFs) partially overlap with each other and with *env* (Myers et al., 1998). In contrast, the early expressed *nef* gene does not overlap with other ORFs (Myers et al., 1998). Moreover, although Nef-deleted HIV-1 variants display a lower pathogenicity in vivo, they are replication-competent in vitro (Harris, 1996), which makes the *nef* gene a more suitable candidate for genetic modifications.

The construction of full length, infectious HIV-1 molecular clones is described, in which CTL epitopes from the late HIV-1 proteins RT and Env have been inserted in frame into the *nef* ORF. The molecular clone pACH320.2A.2.1 (HIV-$1_{2.1WT}$), which has been described previously (Groenink et al., 1991; Guillon et al., 1995), was subcloned in two halves named pXE2.1 and pEX2.1, respectively (Guillon et al., 1997). DNA was amplified using *Escherichia coli* STBL2 (Gibco-BRL), grown at 30 °C to avoid recombination (Joshi and Jeang, 1993), and purified on cesium chloride gradients using standard methods (Sambrook et al., 1989). We inserted ectopic epitopes in the unique Bpu1102I site (position 67–73 in the *nef* gene), which was located close to a natural CTL epitope of Nef, providing a favourable context for antigen processing (Korber et al., 1999). pEX2.1 subclones were digested with Bpu1102I (MBI Fermentas), dephosphorylated using Calf Intestine Phosphatase (Boehringer–Mannheim), and purified on agarose gels (DNA extraction kit, Boehringer–Mannheim). The nucleotide sequences encoding the $Env_{589-597}$ (ERYLKDQQL) and $RT_{244-252}$ (IVLPEKDSW) CTL epitopes, which are recognised in the context of HLA-B14 and -B57, respectively (Johnson et al., 1992; Klein et al., 1998), were generated by annealing complementary oligonucleotides (Fig. 1A). These sequences were ligated into pEX2.1 to create pEX2.1EN and pEX2.1RN, respectively. Clones were sequenced to confirm the correct presence of the epitopes. To generate the pEX2.1NM constructs, subclones were digested with Xho I (position 101–106 in the *nef* gene) and incubated with Kleenow DNA polymerase (New England Biolabs) in the presence of 30 mM dNTPs. This resulted in a frame shift after amino acid 35 and a truncated Nef protein of 56 amino acids (Fig. 1C). pEX2.1EN, pEX2.1RN and pEX2.1-NM were cloned back into full-length pACH320.2A.2.1 (Fig. 1B) and transfected into 293-T cells for the production of infectious HIV-$1_{2.1EN}$, HIV-$1_{2.1RN}$, HIV-$1_{2.1NM}$ and HIV-$1_{2.1WT}$ stocks, as described previously (Pear et al., 1993).

The replication characteristics of these recombinant viruses were evaluated using TCL2H7 cells, a non-transformed CD4+ T cell line supporting replication of primary HIV isolates and presenting HLA B14- and B57-restricted epitopes (van Baalen et al., 1998). The cells require re-stimulation every 14 days using PHA-L (1 μg/ml) and gamma-irradiated feeder cells, and susceptibility to HIV infection was optimal at day 3 after stimulation (van Baalen et al., 1998). Therefore, virus replication could be monitored for 10 days, without the need for re-stimulation or adding freshly stimulated cells. Cells were incubated with 2-, 20- and 200-fold dilutions of the transfection supernatants for 90 min, and washed subsequently twice to remove unbound virions. Cultures were initiated with 3×10^5 cells, and virus production in the supernatant was measured using a double sandwich p24 ELISA (Cheynet et al., 1993; Guillon et al., 1997). In parallel, 5-fold dilutions of the cells inoculated with the highest virus concentration were cultured in quadruplicate with uninfected TCL2H7 cells for 2 weeks, and the number of positive wells for each of the cell dilutions was used to estimate the initial fraction of infected cells. At the start, cultures infected with HIV-$1_{2.1WT}$, HIV-$1_{2.1EN}$, HIV-$1_{2.1RN}$ or HIV-$1_{2.1NM}$ contained a number of infected cells

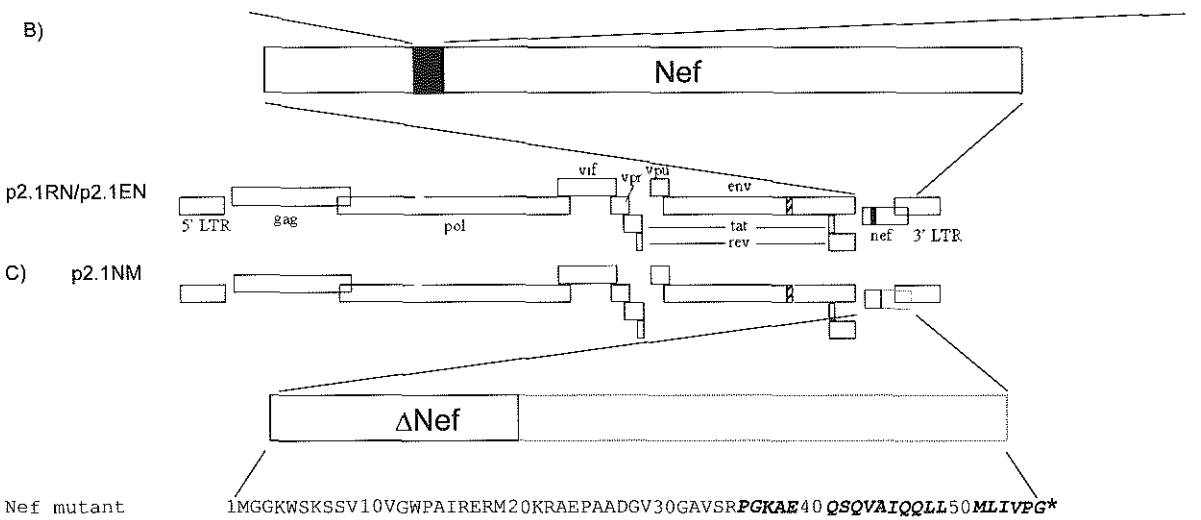
A)

```
                    K21  R   A                                                  E   P   A26
pACH320.2A.2.1   5'- AAA CGA GC                                              T GAG CCA GCA -3'
                 3'- TTT GCT CGA CT                                             C GGT CGT -5'

                          E   R   Y   L   K   D   Q   Q   L   L
EnvEpi-sense     5'-    T GAG AGA TAC CTA AAG GAT CAA CAG CTG CT                          -3'
EnvEpi-antisense 3'-       C TCT ATG GAT TTC CTA GTT GTC GAC GAA CT                       -5'

                          E   P   I   V   L   P   E   K   D   S   W   T
RTEpi-sense      5'-    T GAA CCC ATA GTG CTA CCA GAA AAA GAC AGC TGG AC                   -3'
RTEpi-antisense  3'-       T GGG TAT CAC GAC GGT CTT TTT CTG TCG ACC TGA CT               -5'
```

B)



Nef mutant 1MGGKWSKSSV10VGWPAIRERM20KRAEPAADGV30GAVSR***PGKAE***40 ***QSQVAIQQLL***50***MLIVPG****

Fig. 1. Generation of the artificial epitopes. (A) Description of the oligonucleotides depicted as annealing product. The DNA strands coding for the epitopes are shown in bold. (B) Recombinant full-length HIV-1 molecular clones generated in this study. The ectopic epitopes are depicted as grey and striped areas at their respective natural position and in the recombinant *nef* gene for the p2.1EN and p2.1RN constructs. (C) Aminoacid sequence of the p2.1NM Nef protein. Residues in italic are the result of the frameshift in the *nef* ORF.

proportional to 418, 187, 17 and 125 ID_{50} per 3×10^5 cells, respectively. As shown in Fig. 2, the insertions into or truncation of Nef did not affect in vitro replication kinetics in TCL2H7 cells. At the peak of virus production, p24 concentrations in the supernatant were similar for all infections, between 500 and 1000 ng/ml. Similar results were observed when viruses were used to infect PHA-stimulated PBMCs (data not shown). The inserts and the truncation were stable, since the *nef* genes of virus isolated at the end of the observation period were identical to the *nef* genes of the input virus (data not shown). The differences in growth kinetics correlated with the differences in the estimated initial fraction of infected cells. Tenfold dilutions of the inoculum resulted in a 1–2-day delay in peak virus production, as was shown previously by Dimitrov et al. (1993).

Infected cells were lysed at the peak of p24 replication to assess Nef and Gag expression from the recombinant viruses by Western blot using Nef-specific monoclonal antibodies EVA3068.1 and EVA3067.5 clone 3A2 (Ovod et al., 1992) or with p24-specific antibody 14D4E11 (Janvier et al., 1990). As shown in Fig. 3, upper panel, Nef expression was detected in HIV-$1_{2.1EN}$ and HIV-$1_{2.1RN}$ infected cells. Nef proteins containing the ectopic epitopes (Fig. 3, lanes 2 and 3) showed a slightly higher apparent molecular weight than the wild-type Nef (Fig. 3, lane 1), but all proteins had a molecular weight around the expected size of 32 kD. Thus, the insertion of Env- or RT-specific epitopes in the *nef* ORF of the full-length ACH320.2A.2.1 molecular clone did not perturb viral replication or Nef expression. Nef could not be detected in cells infected with the Nef-truncated HIV-$1_{2.1NM}$ virus (Fig. 3, lane 4), but Gag proteins were detected by Western blots on the same lysate (Fig. 3, lower panel), indicating that viral proteins were expressed normally, despite the lack of Nef expression of HIV-$1_{2.1NM}$.

To assess whether the ectopic epitopes could be processed and presented from the Nef protein, we constructed recombinant vaccinia viruses (rVV) for the expression of the recombinant Nef proteins. The *nef* genes were amplified from the pEX2.1EN or pEX2.1RN plasmid, with primers 5'Nef-SalI (5'-GTCGACGGGGATGGGTG-GCAAGTGGTCAAA-3') and 3'Nef-EcoRI (5'-GAATTCTTAGCAGTCCTTGTAGTACTCCG-3'), where restriction sites are underlined. The full-length *nef* ORFs were then cloned into the pTG186.poly plasmid (Kieny et al., 1986) using the SalI and EcoRI restriction sites. Recombinant vaccinia viruses were generated by homologous recombination and TK-selection as described (Kieny et al., 1986). Plaque purified rVV clones containing the correct *nef* gene and showing Nef expression, $rVV_{2.1EN}$ and $rVV_{2.1RN}$, were selected by sequence analyses and Western blots (data not

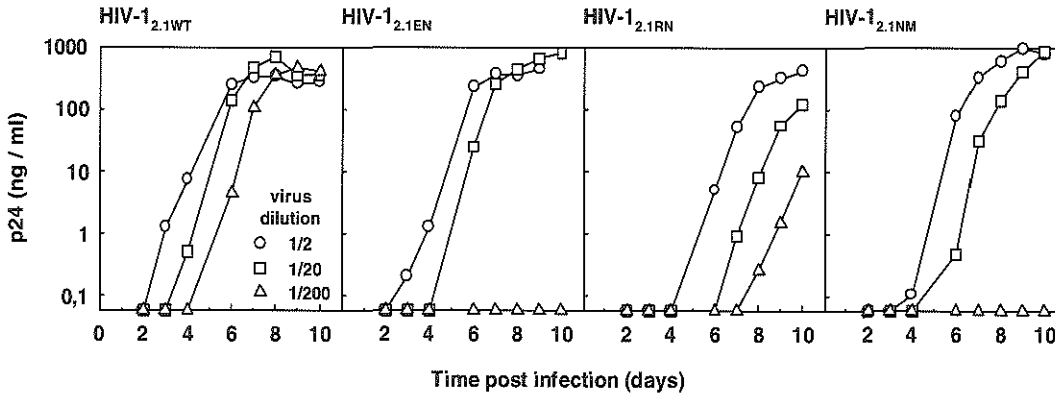


Fig. 2. Kinetics of viral replication in TCL2H7 cells. Levels of p24 were measured in supernatants of cell cultures infected with 2-, 20-, or 200-fold dilutions of HIV-$1_{2.1WT}$, HIV-$1_{2.1EN}$, HIV-$1_{2.1RN}$ or HIV-$1_{2.1NM}$ virus stocks.

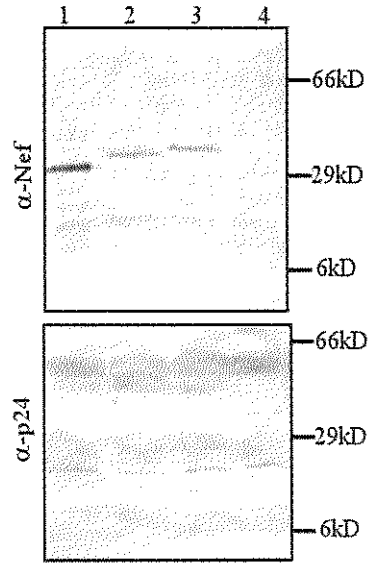


Fig. 3. Nef expression from recombinant HIV-1 viruses in PBMC. Crude cell lysates of HIV-$1_{2.1WT}$, HIV-$1_{2.1EN}$, HIV-$1_{2.1RN}$, HIV-$1_{2.1NM}$-infected PBMC were analysed in Western blot with (upper panel) Nef-specific monoclonal antibodies EVA3068.1 and EVA3067.5 clone 3A2 (Ovod et al., 1992) or (lower panel) with p24-specific antibody 14D4E11 (Janvier et al., 1990). Lane 1, HIV-$1_{2.1WT}$; lane 2, HIV-$1_{2.1EN}$; lane 3, HIV-$1_{2.1RN}$; and lane 4, HIV-$1_{2.1NM}$.

shown). The B-lymphoblastoid cell line BLCL709 (HLA B14, B57) was infected with $rVV_{2.1EN}$ or $rVV_{2.1RN}$ and after o/n incubation cocultured at a 1:10 ratio in a standard 4 h chromium-release assay (van Baalen et al., 1998), using Env-specific CTL clone KMTCC4-1280 ($CD8^+$, HLA-B14-restricted, ERYLDQQL-specific, which is similar to a previously described clone (Johnson et al., 1992)) or RT-specific CTL clone 090TCL1C11 ($CD8^+$, HLA-B57-restricted, IVLPEKDSW-specific (Klein et al., 1998)). As a control, the BLCL709 cells were pulsed with 10 μM of the minimal Env or RT epitope peptide. As shown in Fig. 4, target cells infected by $rVV_{2.1EN}$ or $rVV_{2.1RN}$ were only lysed efficiently by the CTL specific for the inserted epitope. The level of lysis was comparable or higher for rVV-infected cells than for cells pulsed with the minimal epitope peptides (Fig. 4). This showed that the Env and RT-ectopic epitopes were efficiently processed from the recombinant Nef gene and recognised by their specific CTLs.

RT and Env epitopes were inserted in a region of Nef located 4 residues downstream of the C-terminal end of a naturally processed Nef CTL epitope. In line with previous reports which addressed the insertion of CTL epitopes in heterologous proteins (Beekman et al., 2000; Weidt et al., 1995), the antigenic inserts from our constructs were presented efficiently to CTL in a MHC class I restricted manner. One cannot exclude that the heterologous context of the ectopic epitopes might have influenced epitope processing from the EpiNef constructs, either in size of the generated peptides or in the protease pathway involved (Del Val et al., 1991; Niedermann et al., 1999). However, the influence of the site of insertion on epitope processing in our system has to be limited since rVV infected target cells were very efficiently lysed. The insertion of epitopes in Nef did not impair viral replication kinetics, even when the infection was performed at a low MOI, suggesting the recombination did not perturb the natural function of Nef in the replication cycle (Chowers et al., 1994). However, the insertion site

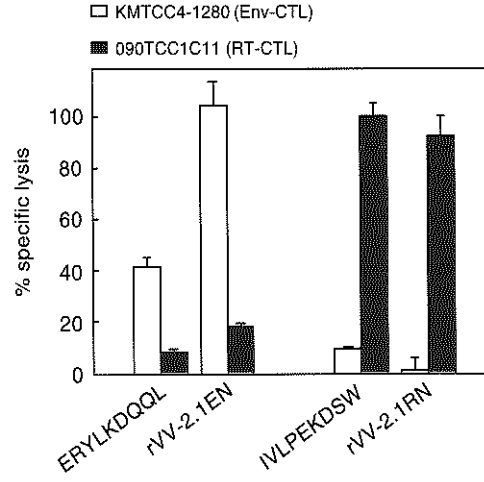


Fig. 4. Recognition of ectopic epitopes by their specific CTL. Percent specific lysis of BLCL709 cells infected with $rVV_{2.1EN}$ or $rVV_{2.1RN}$, or pulsed with peptides corresponding to the minimal epitopes, by Env- or RT-specific CTL. Means of triplicates with standard error are shown.

is located close to a domain involved in the ability of Nef to modulate expression of class I MHC molecules (Collins et al., 1998; Piguet and Trono, 1999), and additional experiments are therefore needed to assess the effect of the insertions in Nef on this mechanism.

In this study, CTL epitopes from the structural HIV-1 proteins Env or RT were inserted in the early expressed Nef ORF of HIV-1. These insertions were not deleterious for HIV-1 replication in vitro, and did not perturb Nef expression. In addition, the inserted CTL epitopes were processed and efficiently recognised by specific CTL despite their ectopic position. Moreover, this system uses a primary HIV-1 isolate and a primary, non-immortalized, CD4-positive T-cell clone to be as close as possible from the in vivo situation. These recombinant viruses have since been used successfully to demonstrate the effect of distinct epitope expression kinetics on the ability of CTL to inhibit HIV replication (van Baalen et al., submitted for publication), and could provide new insights in the mechanisms underlying the control of HIV-1 replication by the immune system in vivo.

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Impact of antigen expression kinetics on the effectiveness of HIV-specific cytotoxic T lymphocytes

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(Submitted)

Abstract

Previous studies indicate that the time required for virus infected cells to become vulnerable for the activity of cytotoxic T lymphocytes (CTL) can be of significance for the capacity of CTL to control ongoing viral reproduction. To investigate whether this applies to the effectiveness of HIV-1-specific CTL, we measured virus production in co-cultures of infected $CD4^+$ T cells with CTL clones directed against an early protein, Rev, or a late protein, RT. The Rev-specific CTL prevented at least 2 $\log_{10}$ more HIV-1 production in 10 days, than similar numbers of RT-specific CTL. To study the contribution of parameters that cannot be independently assessed *in vitro*, we developed a mathematical model that describes CTL-target cell interactions and viral reproduction dynamics. The results show that the rate at which CTL have to eliminate infected cells to preclude increasing numbers of new infections, was substantially lower when they can start early after HIV enters the cell. Furthermore, *in vitro* experiments with HIV recombinant viruses showed that expression of a RT epitope as part of the early protein Nef, significantly enhanced the effectiveness of RT-specific CTL. Together these results indicate that CTL can control reproduction more effectively if they are able to recognize infected cells earlier after viral entry. This provides rationale for immunization strategies that aim at inducing, boosting or skewing CTL responses to early regulatory proteins in AIDS vaccine development.

1 Introduction

HIV-specific CTL can inhibit virus replication by killing infected cells and by secreting non-lytic antiviral factors [1]. The *in vivo* dynamics of CTL responses, viral loads and emergence of viral escape variants indicate that CTL exert considerable pressure on HIV replication during the primary and chronic stages of infection [2-6]. Furthermore, depletion of $CD8^+$ cells, before or after infection, results in increased viremia, prolonged depletion of $CD4^+$ T cells and accelerated disease progression in macaque models for AIDS [7-9]. In most individuals, however, CTL are not capable of controlling ongoing viral reproduction after primary viremia and the effectiveness of CTL responses seems variable among infected individuals [5;10].

Previously we observed that prognosis for HIV-1 infected individuals [11] and resolution of primary viremia in SIV infected macaques [12] were better if CTL responses were not only directed against the structural proteins of the virus, but also against the regulatory proteins Rev and Tat. These findings support the hypothesis that the latter are better able to control HIV by virtue of their specificity for early viral proteins [11;13]. This neither implies that they are expected to prevent AIDS entirely in all individuals, nor that CTL against other proteins do not affect virus production. Indeed their presence will add to the breadth of the CTL response, which has been reported to be beneficial for immune control of virus production [14-18].

Previous studies indicated that kinetic aspects of viral protein production can significantly influence the ability of CTL to control actively replicating virus [19], and implicate presentation of epitopes derived from proteins that are available early after viral entry, in more effective control of viral reproduction by CTL [19;20]. The HIV proteins Tat, Rev and Nef have been detected as early as 6 hours following acute infection in T cells [21]. These proteins are translated from multiple spliced transcripts of about 2 kb that are the first to be induced [22;23], and can be detected between 6 to 8 hours after infection of T cells with HIV-1 [24]. Levels of unspliced transcripts that are translated to produce the stuctural proteins Gag and Pol remain

low until 24 hours after acute infection in T cells, followed by a sharp increase which coincides with release of virions during the next 12 hours [22]. Most HIV-specific CTL clones and lines that have been analysed for their ability to inhibit HIV production were directed against epitopes derived from intermediate and late structural proteins [25-28].

Here we further explore the hypothesis that early recognition of target cells contributes to the effectiveness of CTL in controlling reproduction of HIV. $CD4^+$ T cells are infected at low multiplicity of infection (MOI) and co-cultured with CTL directed against early (Rev) or late (RT) HIV proteins. The results from these experiments are used to calibrate a mathemathical model that simulates CTL-target cell interactions during multiple rounds of infection. This allows us to vary parameters individually and to determine their contribution to control virus propagation. To verify the validity of the model, HIV recombinants are used in which the production kinetics of CTL epitopes has been manipulated.

2 Results

2.1 Experimental approach

To analyse the ability of CTL of different protein specificity to control HIV reproduction, we co-cultured HIV infected cells with CTL against early (Rev-CTL) and late (RT-CTL) proteins. Non-immortalized $CD4^+$ TCL2H7 cells, expressing HLA-B14 and -B57, were stimulated and, after 3 days, inoculated with a low MOI of primary HIV-1. This allowed for the monitoring of p24 production during 10 days, without the need for re-stimulation or addition of fresh cells. Under the experimental conditions, the virus used, HIV-$1_{ACH320.2A.2.1}$ (HIV-$1_{2.1WT}$) was detectable from day 4 onwards and increased exponentially over a 3 $\log_{10}$ range, untill a plateau was reached by day 9 (Fig 1a, open symbols) [29]cf.[30].

2.2 Inhibition of virus reproduction by CTL.

Target cells were co-cultured with CTL at different ratios, and cell populations were followed by FACS analysis to monitor the actual CD8-to-CD4 cell ratio during the experiment (Fig 1: triangles and squares for RT- and Rev-CTL respectively). In

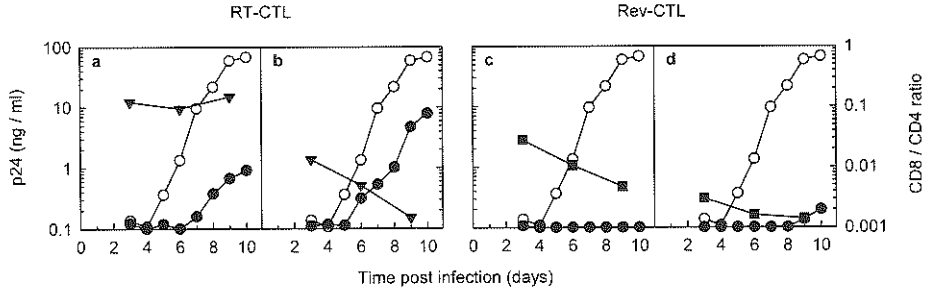


Fig. 1. Inhibition of HIV reproduction by RT- and Rev-CTL. a-d, Kinetics of virus production by TCL2H7 cells ($CD4^+$ expressing both HLA-B14 and HLA-B57 [34]) infected with HIV-$1_{2.1WT}$ (a molecular clone of the primary, non-syncytium-inducing virus isolate HIV-$1_{ACH320.2A.2.1}$ [52;53]) and cultured without (open circles) or with (closed circles) CTL at different ratios. Virus levels were quantified with a p24 ELISA [54] (left axis). Co-cultures were initiated with $2x10^5$ $CD4^+$ T cells and two different quantities of RT-CTL (TCL1C11: $RT_{244-252}$IVLPEKDSW-specific, HLA-B57 restricted [37]) (**a** and **b**; triangles), or Rev-CTL (TCC108: Rev_{67-75}SAEPVPLQL-specific, HLA-B14 restricted [34]) (**c** and **d**; squares). The CD8-to-CD4 cell ratio was assessed by flowcytometry (right axis).

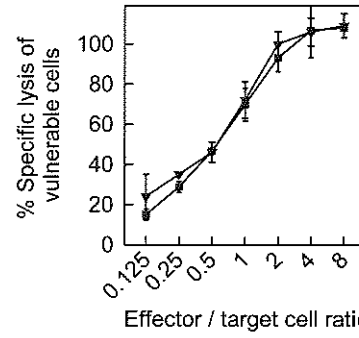
cultures containing RT-CTL and CD4 cells at a ratio of 1:10 on day 2, HIV production was delayed, resulting in a 2 $\log_{10}$ reduction of p24 levels by day 10 (Fig.1a, closed circles). A ten-fold lower initial CD8-to-CD4 cell ratio resulted in exponentially increasing p24 levels between day 6 and 10, reaching ten-fold higher p24 levels (Fig.1b). This indicates that the antiviral effect is dose dependent, as previously reported in a similar assay [27]. Sequence analyses of virus released in culture supernatant revealed no mutations in the epitope or its flanking regions (data not shown), despite the continued presence of RT-CTL.

In cultures with Rev-CTL at CD8-to-CD4 cell ratios that were similar to the co-cultures with RT-CTL in Fig. 1b, no virus was detected during the course of the experiment (Fig.1c, closed circles). Only in cultures initiated with ten-fold less Rev-CTL, low levels (< 0.2 ng/ml) of p24 were detected at the end of the experiment, when $CD8^+$ cell numbers had decreased to the detection limit (Fig. 1d, closed circles). Virus isolated from these cultures on day 10 had no mutations in the epitope or flanking regions (data not shown).

2.3 Cytolytic capacity of the Rev- and RT-CTL.

We next investigated whether the Rev-CTL and the RT-CTL differed in their capacity to lyse vulnerable target cells. Peptide labeled TCL2H7 cells were incubated with different numbers of effector cells in a chromium release assay. The lowest effector-to-target cell (E/T) ratio required to lyse all vulnerable cells was 4:1 for both CTL populations (Fig. 2). Half-maximal lysis was achieved for both the RT-CTL (Fig 2, triangles) and Rev-CTL (squares) at an E/T ratio of 0.7:1. Infection at low MOI was mimicked by mixing peptide labeled target cells with a ten-fold excess of unlabeled TCL2H7 cells. This did not significantly influence the percentage of cells lysed by the CTL at various E/T ratios (data not shown). Thus, the difference of these CTL in containing HIV reproduction could not be explained by a difference in their cytolytic capacity.

Fig. 2. Cytolytic capacity of the RT- and Rev-CTL. Peptide labeled TCL2H7 cells were incubated with RT-CTL (triangles) or Rev-CTL (squares) at indicated effector-to-target cell ratios in a chromium release assay as described previously [34]. Synthetic peptides corresponding to HIV-1 $RT_{244\text{-}252}$IVLPEKDSW or $Rev_{67\text{-}75}$ SAEPVPLQL were used. Mean values of triplicate incubations are shown. Maximal lysis was reached by 5 hours at effector-to-target ratios > 4; RT- and Rev-peptide labeled target cells contained 100% and 80% vulnerable cells, respectively. Results are expressed as percentage of maximal lysis of vulnerable cells, allowing estimation of the number of CTL required for half maximal lysis.



2.4 An in silico model for CTL effectiveness.

CTL can reduce virus production by infected cells via mechanisms other than cytolysis [1;25]. To exclude variability in the potency of effector mechanims, we explored individual parameters in a mathematical model based on a previous model by Klenerman et al. [31]. Figure 3 depicts a model of the stages susceptible cells go through after infection and when they encounter specific CTL. In both models elimination of infected cells is a exponential decay process that occurs at a rate α, and

elimination can begin when infected cells become vulnerable at time a_V after infection. The most significant difference with the model of Klenerman is that our model takes into account that progeny virus can start new infection cycles. Infected cells produce virus that generates newly infected cells at a maximum rate of m per day. To obtain estimates for m we fitted values in the model to p24 levels observed in the *in vitro* experiments shown in Fig. 1 and Fig. 5. The estimates ranged from 12-14 infections per day. Considering essentially the same parameter values for the onset of virus production after infection (a_P = 1day), and the lifetime of productively infected cells (a_D - a_P = 2.5 days) as Klenerman et al., this implies that, in the absence of CTL, one infected cell will cause 30-35 new infections. This number, called the virus' reproduction ratio and denoted R_0, is in the same range as estimates for *in vivo* HIV-1 production during primary viremia [32]. It should be reduced to less than 1 to quench ongoing viral reproduction. By how much a specific CTL population will reduce R_0 depends on its action, which has two components, a_V and α. From the model framework, depicted in Fig. 3, the virus' reproduction ratio R_0 can be derived and is equal to:

$$R_0 = m\, e^{\alpha a_V} \frac{e^{-\alpha a_P} - e^{-\alpha a_D}}{\alpha} \qquad (1)$$

which is the product of the infection rate m and survival of infected cells during the producing stage.

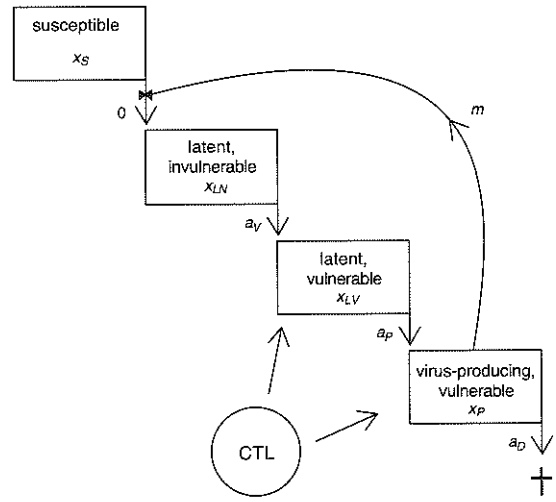


Fig 3. Schematic representation of the model combining virus kinetics and infection cycles. The densities of susceptible cells, non-vulnerable latent cells, latent cells vulnerable to CTL attack and virus-producing cells (vulnerable) are given by x_S, x_{LN}, x_{LV} and x_P respectively. The 'age' of a cell measures the time since infection: cells become vulnerable to CTL attack at age a_V, start to produce virus at age a_P and die at age a_D. The rate at which infected cells are eliminated from the replication cycle by CTL is α. The reproduction ratio R_0 of the virus in absence of CTL is $\bar{R}_0 = m(a_D - a_P)$; in presence of CTL it is given by equation (1) from the text; the relative amount of virus production prevented by CTL is therefore $F = R_0 / \bar{R}_0$.

2.5 Requirements for CTL to control virus spread.

Using the model we varied individual parameters to determine their contribution to the effectiveness of the CTL. Input values for the moment that cells become vulnerable for CTL (early: a_V = 0.3d; late: a_V = 0.9d) were the same as those analysed by Klenerman et al. [31]. In figure 4a the whole shaded area represents the amount virus produced in the absence of CTL. If RT-CTL can eliminate target cells from 0.9

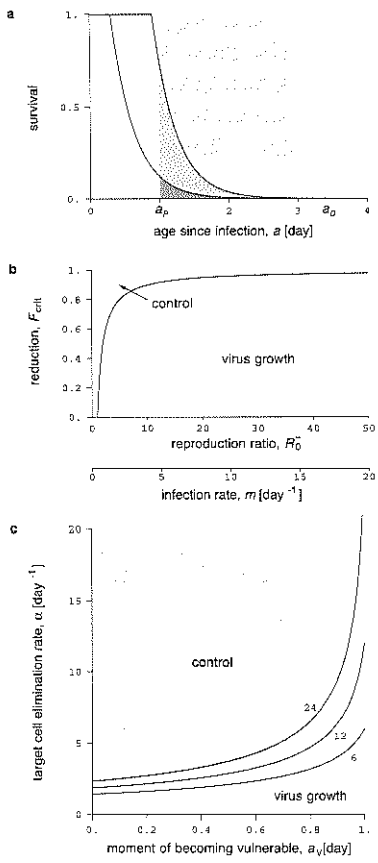


Fig 4. Reduction of virus production by CTL. a, Expected survival of infected cells as a function of age since infection, in the presence of CTL that either attack from age a_V = 0.3 onward (left curve), or from age a_V = 0.9 onward (right curve); both eliminate infected cells at rate α = 3.0 d^{-1}. The virus' reproduction ratio R_0 is proportional to amount of virus produced (shaded areas: light in absence of CTL, intermediate for late-acting CTL, dark for early-acting CTL). **b**, Critical reduction (difference between the shaded areas in panel a) of the virus' reproduction ratio in absence of CTL, necessary for control of virus growth. Also indicated how the virus' R_0 depends on the rate of infection m. **c**, Combinations of the timing of becoming vulnerable (a_V) and target cell elimination rate (α) that result in control of virus replication. In the shaded area the virus replication ratio R_0 is less than unity for the case where the infection rate m equals twelve; the drawn curves indicate how the area changes with infection rate (m = 6, 12 or 24).

days on, the fraction of cells that survive decays rapidly and virus production is reduced by 90% (medium gray). Rev-CTL, that can start recognising cells at 0.3 days, have a larger impact on virus production (98% reduction, dark gray), if they eliminate cells at the same rate as RT-CTL (α = 3.0 in this example). Thus, Rev-CTL have a slightly larger impact on virus production per replication cycle, but these calculations do not resolve whether the required $R_0 < 1$ can be achieved. In Fig. 4b the fraction of virus production that has to be prevented to meet this criterium (shaded area) has been calculated as function of virus reproduction (R_0 or m). At low virus reproduction ratio's, a small reduction of the amount of virus produced will suffice. However, virus production during each individual round of replication should be reduced by more than 97% at moderate reproduction rates, like for HIV-1 in T cells (m=12-14).

The impact of early recognition on CTL effectiveness is depicted in figure 4c. For CTL directed against early produced epitopes, an α in the order of 2 to 3 day^{-1} suffices. If target cells can be recognised only shortly before virus production starts, the CTL mediated elimination rate must be considerably higher, α of 6 to 7 day^{-1}. The beneficial effect of early recognition is more pronounced when the infection rate m *is* larger (cf. m=6, 12, 24 in Fig 4c). Together, our calculations indicate that, under our *in vitro* experimental conditions, the CTL that can eliminate target cells longer before the onset of virion release, have a higher per cell capacity to control reproduction of HIV.

2.6 Impact of epitope expression kinetics on CTL effectiveness in vitro.

To test these predictions in vitro, we constructed recombinant viruses with sequences encoding epitopes derived from late proteins inserted into the early expressed *nef* gene [29]. HIV-$1_{2.1RN}$ contains the RT epitope recognised by the RT-CTL

used in this study and HIV-$1_{2.1EN}$ contains a previously described Env epitope [33]. Insertion of the epitopes did not perturb production of full length Nef [29] or Nef-mediated down modulation HLA class I expression on the cell surface (data not shown). Cells expressing the recombinant Nef proteins were recognised by their cognate CTL, indicating that the epitopes were correctly processed and presented, and HIV-$1_{2.1RN}$ and HIV-$1_{2.1EN}$ replicated with similar kinetics as HIV-$1_{2.1WT}$ (Fig. 5a-d, open symbols, cf. [29]).

HIV-$1_{2.1EN}$ and HIV-$1_{2.1RN}$ infected cells were co-cultured with different numbers of RT-CTL. The CD8-to-CD4 cell ratios were ~ 1:3 and 1:50 at day 2 and declined during the observation period, due to different growth kinetics of CTL and target cells (Fig. 5a-d, triangles). The effect of the RT-CTL on HIV-$1_{2.1EN}$ was dose dependent with minimal reduction of virus replication at low density and a 3 to 4 day delay in virus growth kinetics at higher CD8-to-CD4 cell ratios (Fig. 5a-b), comparable with cultures infected with HIV-$1_{2.1WT}$ (data not shown). This indicates that target cell recognition by RT-CTL was not affected by the insertion of the Env epitope in Nef. By contrast, HIV-$1_{2.1RN}$ was completely suppressed by the same CTL, even at low density (Fig. 5c-d). No changes were observed in the primary sequence

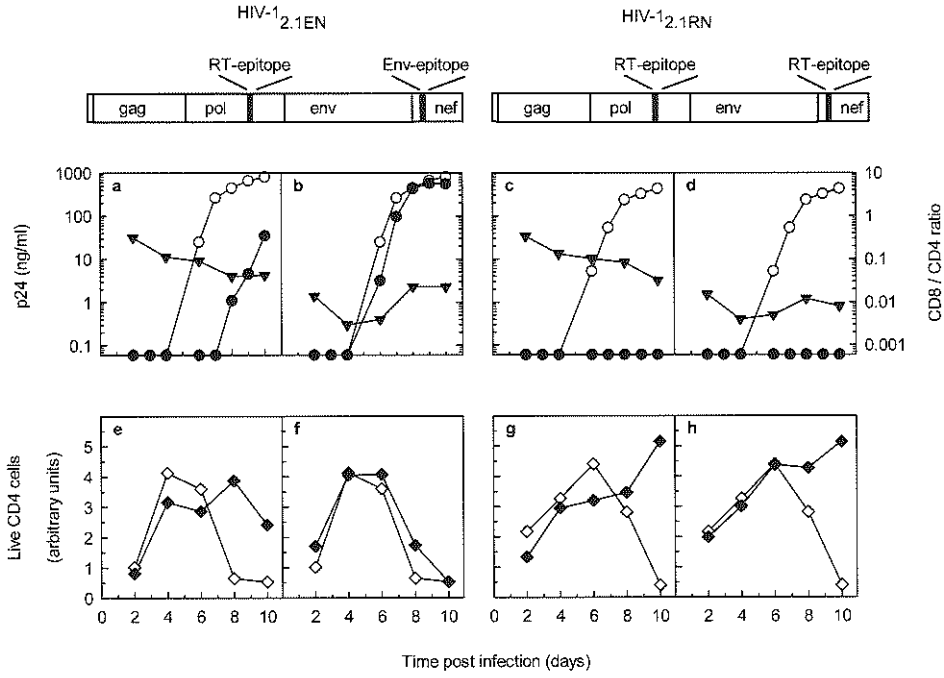


Fig 5. Kinetics of epitope expression influence the ability of RT-CTL to control virus reproduction. Kinetics of virus production by CD4+ TCL2H7 cells infected with recombinant HIV-$1_{2.1EN}$ (**a-b**), or HIV-$1_{2.1RN}$ (**c-d**). These viruses contain an Env- or RT-epitope in Nef, respectively, created by replacing the *nef* gene of HIV-$1_{2.1WT}$ with the recombinant *nef* genes containing the sequences encoding IVLPEKDSW (2.1rn) or ERYLKDQQL (2.1en). Levels of p24 (left axis) were quantified in samples from cultures initiated with $3x10^5$ CD4+ T cells, containing a number of infected cells proportional to approximately 17 ID_{50}, together with no (open circles), $3x10^5$ (**a** and **c**; closed circles) or $3x10^4$ (**b** and **d**; closed circles) RT-CTL. The CD8-to-CD4 cell ratio (right axis) in cultures containing RT-CTL (triangles) was assessed by flowcytometry at the indicated times. **e-h**, Dynamics of life, 7AAD-, CD4+ T cells during the culture period in the absence (open diamonds) or presence (closed diamonds) of RT-CTL (**e-h** correspond to the same cultures as **a-d**). Data are presented as event count ($x10^{-3}$) acquired in 90 seconds from 200 µl samples.

of the recombinant *nef* genes of the viruses isolated at the end of the observation period (data not shown).

2.7 Effect of CTL on CD4⁺ T cells in the presence of HIV.

In the same experiment we followed the number of viable CD4⁺ cells, to see whether CTL were protective or destructive to the CD4⁺ T cell population in HIV infection. Without the presence of CTL, the number of CD4⁺ cells declined concurrently with the increasing virus levels, indicating that cell death was related to virus production (Fig. 5e-h, open symbols). In presence of RT-CTL, CD4⁺ cell death occurred later and paralleled the delay in HIV-$1_{2.1EN}$ production (Fig. 5e-f, closed symbols). The control of HIV-$1_{2.1RN}$ reproduction by the RT-CTL was associated with continued proliferation of the CD4⁺ cells during the follow-up period (Fig. 5g-h, closed symbols), similar to uninfected CD4⁺ cell cultures (data not shown). Thus, expression of the RT epitope from the recombinant *nef* gene not only rendered the RT-CTL more effective in inhibiting virus reproduction, but also enabeled them to protect the CD4⁺ cell population from virus-related cell death.

3. Discussion

3.1 Differences in effectiveness of CTL directed against early or late HIV proteins

In this study we provide evidence for the contribution of early target cell recognition to the capacity of HIV-1-specific CTL to control reproduction of virus in a susceptible CD4⁺ T cell population. Previously reported data indicated that CTL directed against an early HIV protein (Rev) prevented slightly more virus production during a single infection cycle than CTL directed against a late protein (RT), >97% and ~92% reduction by 48 hours after infection, respectively [34]. In the present study this small difference was shown to increase significantly in experiments designed to allow the residual progeny virus to start new rounds of infection. In cultures that were initiated by low multiplicity of infection, i.e. containing mostly uninfected susceptible cells, Rev-specific CTL had prevented at least 2 $\log_{10}$ more virus production than a similar number of RT-specific CTL by day 10. This difference was not compensated for by ten-fold more effector cells. These data are in line with the notion that if CTL are to be antivirally active, they have to lyse infected cells within a given time window [35], and that small differences in their capacity to reduce virus production can have dramatic effects on overall virus control because of the capacity of virus populations to expand exponentially [36]

3.2 Differences in potency of CTL effector mechanisms and in target cell vulnerability

Several mechanisms that could explain these results were addressed. The specific epitopes and their flanking regions of virus recovered at the end of the observation period were unchanged, indicating that escape by mutation had not occurred. In standard four hour chromium release assays, the RT-specific CTL required less peptide for half-maximal lysis than the Rev-specific CTL [34;37]. If saturating amounts of peptide were used, both effector cell populations lysed half of the target cells at an effector-to-target cell ratio of about 0.7:1 (this study). Thus neither escape by mutation of virus, nor differences in direct lytic capacity of CTL are likely to explain the observed differences in CTL effectiveness.

Previous studies have shown that RT-specific CTL lysed lower fractions of infected cells than Gag-specific CTL, which was associated with lower amounts of HLA-epitope complexes on the surface of target cells [38]. These data were obtained with immortalized T cells chronically infected with a T cell line-adapted virus strain, and culture conditions precluded new rounds of infection. It is therefore not clear whether the implications of these results apply to our system, which utilizes non-transformed, PHA-activated, $CD4^+$ T cells, primary virus isolates and allows for successive rounds of new infection. In fact, the RT-specific CTL used in the present study consistently lysed similar or higher fractions of infected cells if compared to the Rev-specific CTL [34]. This indicates that differences in fractions of virus producing cells that become vulnerable to lysis are not likely to explain the lower capacity of the RT-specific CTL to control viral reproduction. Furthermore, also Gag-specific CTL that were able to lyse all infected cells at some point after infection, could not control ongoing viral reproduction, even in cultures containing 50% specific CTL and 50% $CD4^+$ cells that had been infected with 10^{-2} $TCID_{50}$ per cell prior to cocultivation [27].

We did not yet directly address possible differences in the number of vulnerable cells that one effector cell can eliminate during its lifetime, the rate of serial killing, or the efficiency of non-cytolytic effector mechanisms. However, reproduction of a recombinant virus that encoded the minimal RT-epitope in the early expressed *nef* gene remained undetectable in presence of the RT-specific CTL for 10 days. That they could not efficiently control reproduction of the parental virus can therefore not be explained by insufficient potency of effector mechanisms of the CTL, but is most probably due to their inability to attack enough infected cells before progeny virions initiated new infection cycles. Further studies are required to directly assess the average and range of the time period during which cells infected with the parental or recombinant virus are vulnerable before production of progeny virions.

The precise mechanism that underlies the higher susceptibility of cells infected with the recombinant virus for CTL activity directed against the RT-epitope is presently unclear. The epitope is expected to be generated earlier and at higher levels, which both would result in earlier vulnerability of the target cells. But the epitope insert in the *nef* gene could also have affected the ability of Nef to reduce MHC class-I expression [39], and thereby have caused a larger fraction of infected cells to become vulnerable before the onset of virion release. This possibility is unlikely, however, because MHC class-I molecules were expressed at similar levels on the surface of cells infected with the recombinant or parental virus. Finally, if Nef-mediated effects on MHC-peptide production delay onset of target cell vulnerabilty, early epitopes are more likely than late epitopes to be presented before Nef can interfere with their presentation. Although the relative contribution of different mechanisms to the timing of target cell vulnerability requires further analyses, our results indicate that it is critical for CTL-mediated control of HIV reproduction and propagation.

3.3 Mathematical model for CTL-target cell interaction and viral dynamics

Direct measurement of the interval between the onset of target cell vulnerability and virion release is problematic because it is influenced by multiple factors in infected cells, but also by characteristics of effector cells, including the density of TCR molecules on the surface, differentiation state, and the potency of effector

mechanisms. Moreover, whether a certain interval is sufficient for CTL to control virus spreading will also depend on kinetics of virus reproduction and rates of serial target cell elimination by CTL. As more information on these parameters becomes available, integration of experimentally determined values in mathematical models can help to assess the relative contribution of each parameter to the final outcome.

The activity of CTL is effective if they reduce the virus' reproduction ratio (R_0) i.e. the number of newly infected cells that arise from one infected cell, to less than 1 [40]. We analysed 2 components of CTL pressure on R_0: the time at which target cells become vulnerable to CTL (a_V) and α, the CTL-mediated target cell elimination rate. In the present model, target cell elimination refers to loss of the infected cell in terms of their ability to contribute to new infection cycles. Biologically, this could be achieved both by lysis of infected cells and by suppression of viral protein production via non-cytolytic mechanisms.

The results show that if CTL eliminate infected cells from the reproduction cycle with an exponential decay rate of approximately 2 to 3 per day, they can control the infection, provided that infected cells become vulnerable within approximately 16 hours after infection. After that time, the target cell elimination rate, e.g. the effector cell number, has to increase considerably as there is less time before release of progeny virus begins. The results of our model reveal how differences in epitope production kinetics in infected cels can influence the per cell capacity of CTL to control ongoing viral reproduction. The model provides a plausible explanation for the observed differences in the *in vitro* effectiveness between Rev- and RT-specific CTL.

3.4 Implications for pathogenesis of HIV infection and vaccine design

The present data indicate that the association of CTL responses directed against Rev and Tat with slower rate of disease progression [11] can be explained by the ability of CTL directed against early proteins to recognize infected cell earlier during the eclips phase, which increases their capacity to control HIV reproduction. But if this is so, why have Nef-CTL not been associated with better control of HIV infection? Nef-specific CTL have been frequently detected in the early stages of infection, also in individuals who rapidly progressed to AIDS [11;41]. Because the *nef* gene, unlike *tat* and *rev*, does not overlap with other open reading frames, variation resulting in escape may be tolerated more frequently. This notion is supported by the dynamical balance between Nef-escape variants and variant-specific CTL responses observed in infected individuals [42], and by the rapid escape by mutation from recognition by adoptively transfered Nef-specific CTL [43]. In our *in vitro* assay, the number of replication cycles is most likely too limited for escape mutants to emerge.

Evasion of HIV from immune pressure during the different stages of infection has been attributed to escape by mutation [44;45], persistence of virus in latently infected cells, immuneprivelidged sites [46] or FDC-networks [47], and impairment of immune responses [5;10]. Our data suggest that the failure of CTL to control ongoing viral reproduction after primary viremia *in vivo* may also result, in part, from the fact that the dominant CTL response is generally directed against late proteins, which limits the pressure exerted by these CTL on the reproduction of HIV [48].

Several studies have shown that vaccination with regulatory proteins induces effective immune responses. We and others showed that vaccination with Tat, alone or in combination with Rev, gave mild transient viremia and a beneficial follow up

after challenge in macaques [49-51]. More recently we observed that pre-existing CTL responses in Rev and Tat vaccinated cynomolgus macaques, correlated with better control of primary SIV viremia than in Gag and Pol vaccinated macaques (K.J. Stittelaar et al submitted). Considering the number of proteins, their size and levels of expression, it is unlikely that the higher protection level induced by the Rev/Tat vaccine was due to more broadly directed immune responses. Collectively, our data provide rationale for further evaluation of immunization strategies aimed at induction, boosting or skewing CTL responses to early regulatory proteins in AIDS vaccine development.

4 Materials and Methods

4.1 Cells.

The $CD8^+$ Rev_{67-75}SAEPVPLQL-specific, HLA-B14 restricted, TCC108 CTL clone was generated from PBMC of participant L709 from the Amsterdam Cohort Studies on AIDS (ACH) [34]. The $CD8^+$ $RT_{244-252}$IVLPEKDSW-specific, HLA-B57 restricted, TCL1C11 CTL line was generated from PBMC of ACH participant L090[37]. The $CD4^+$ TCL2H7 cells express both HLA-B14 and HLA-B57, and were obtained from the same participant L709 as the Rev-specific CTL[34]. Cells were stimulated with phytohemagglutinin-L (PHA) (1μg/ml; Boehringer Mannheim, Germany) and gamma-irradiated feeder cells every 10-14 days as previously described[34]. Cell concentrations were kept between $0.3x10^6$/ml and $1.0x10^6$/ml.

4.2 Viruses.

HIV-$1_{320.2A.2.1}$, referred to as HIV-$1_{2.1WT}$, is a molecular clone of a primary, non syncytium inducing virus isolate from patient 320 of the ACH[52;53]. Recombinant HIV-$1_{2.1RN}$ and HIV-$1_{2.1EN}$ were generated by replacing the nef gene of HIV-$1_{2.1WT}$ with the recombinant nef genes containing the sequences encoding IVLPEKDSW (2.1rn) or ERYLKDQQL (2.1en) [29]. The Bpu1102I site, position 67-73 in the nef gene, was used for the insertions because it is close to a natural Nef epitope. Virus stocks, generated by transfection of 293T cells, were used to infect TCL2H7 cells. Virus production was monitored with a p24 ELISA as described[54]. For estimation of the fraction of infected cells at the start of the experiment, six five-fold dilutions of the inoculated cells were cultured in quadruplicate with uninfected TCL2H7 cells for 14 days. The fraction p24 positive wells for each of the dilutions was used to calculate the ID_{50} (Kärber estimate).

4.3 Flowcytometry.

At indicated times, 110 μl samples of the cultures were centrifuged and cells were incubated with CD4-fluorescein isothiocyanate, CD8-phycoerytrine (Dako), and 7 amino-actinomycine-D (7AAD) (Sigma) for 20 minutes, washed, resuspended in 200μl PBS containing 2% paraformaldehyde (Merck), and analysed on a FACScan (Becton-Dickinson). This allows the discrimination between CTL ($CD4^-$ $CD8^+$) and target cells ($CD4^+$ $CD8^-$) and live ($7AAD^-$) and dead ($7AAD^+$) cells [55]. In the experiment shown in Fig. 5, acquisition-time was held constant allowing semi-quantative estimation of cell numbers in the cultures.

4.4 Chromium-release assays.

Lysis of peptide labelled target cells was assessed as described previously[34]. Mean values of triplicate incubations were calculated as follows: % specific lysis = 100 x [(experimental release-spontaneous release)/(maximal release-spontaneous release)]. Synthetic peptides corresponding to HIV-1 $RT_{244\text{-}252}$ IVLPEKDSW and $Rev_{67\text{-}75}$SAEPVPLQL were manufactured by EVL (Woerden, The Netherlands).

4.5 Mathematical model.

Our dynamic model is based on the static model analysed by Klenerman et al.[31], and describes changes in densities of virus susceptible cells, infected but invulnerable cells, cells vulnerable to CTL attack, and virus-producing cells, see Fig. 3. The dynamics are given by a set of four delay-differential equations:

Susceptible cells (x_S) become infected at rate r (see equation A5)

$$dx_S(t)/dt = -r(t) \qquad \text{(A1)}$$

Infected cells that are latent, i.e. do not yet produce virus, and not vulnerable to CTL attack (x_{LN}), become vulnerable at $t = a_V$

$$dx_{LN}(t)/dt = r(t) - r(t-a_V) \qquad \text{(A2)}$$

Latent but vulnerable cells (x_{LV}) are eliminated by CTL at rate α and start to produce virus at $t = a_P$

$$dx_{LV}(t)/dt = r(t-a_V) - \alpha - r(t-a_P)e^{-a(a_P-a_V)} \qquad \text{(A3)}$$

Virus producing cells (x_P) are eliminated by CTL at rate α or die at $t = a_D$ due to the virus

$$dx_P(t)/dt = r(t-a_P)e^{-a(a_P-a_V)} - \alpha - r(t-a_D)e^{-a(a_D-a_V)} \qquad \text{(A4)}$$

Recruitment of latent cells (infection) at time t is given by

$$r(t) = mx_S(t)\, x_P\,(t) \qquad \text{(A5)}$$

Initial conditions are $x_S\,(0) = 1$, $x_{LN}\,(0) = x_{LV}\,(0) = x_P\,(0) = 0$; the model is started with a short pulse of infection (for details of how to analyse delay-differential equations, see Gurney et al [56]). As in the static model, we assume that infected cells start to produce virus at $t = a_P = 1$ day after infection. An insignificant difference is that Klenerman et al. assume distributed cell deaths after the onset of production of virus (cells die with a rate of $c = 0.4$ per day) whereas we assume that all virus-producing cells die at a fixed moment (at $t = a_D = 1/c = 2.5$ days after the onset of virus production). We did the analysis assuming distributed cell deaths, but we obtained virtually the same results. The most significant change to the model is that multiple infection cycles occur. That is, a virus-producing cell generates newly infected cells with a maximum rate of m per day (m is the rate of infection in the beginning of the experiment, the effective rate will of course decline in the course of the experiment due to depletion of susceptible cells).

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CHAPTER 4

Vaccination with regulatory versus structural viral proteins

Vaccination with Rev and Tat against AIDS

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More than 15 years after the discovery of HIV-1 as the causative agent of AIDS, and numerous attempts to develop a vaccine, it has become clear that the efficacy of the currently considered HIV-1 vaccine candidates will generally be limited [1]. This is at least in part due to the relative resistance of so-called primary HIV strains to neutralization by HIV-1 envelope specific antibodies [2,3]: even the most potent HIV-1 neutralizing antibodies failed to provide protection in in vivo models, at concentrations that can be maintained for longer periods in human vaccinees [4,5]. Therefore we strongly advocate that vaccination studies in primate lentivirus models should also focus on the induction of cell mediated immunity. So far few candidate vaccines have been shown to induce protective immunity in these models. Virtually all these vaccines have been based on the use of structural proteins of the virus. Our recent data suggest that soon after infection disease progression in humans and macaques infected with HIV-1 and SIV respectively, correlates inversely with the presence of cytotoxic T lymphocytes specific for the early proteins Rev and Tat [6,7]. To investigate the putative protective role of early protein specific CTL in the containment of primate lentivirus infection, we performed a pilot study in adult cynomolgus monkeys (*Macaca fascicularis*). Two animals were vaccinated at four week intervals twice intramuscularly with 10^8 and 10^9 $TCID_{50}$ recombinant semliki forest virus (SFV) and twice with 10^8 and 10^9 $TCID_{50}$ recombinant vaccinia virus (modified virus Ankara: MVA) both producing SIVmac 32H Rev and Tat. Two control animals were vaccinated in parallel with the same amounts of recombinant SFV and MVA, both expressing LacZ instead of SIV genes. Two weeks after the last vaccination all four animals were challenged intravenously with 50 MID_{50} SIVmac 32H (pJ5), as previously described [8]. As expected, the two control animals became persistently infected, showing SIV infected cell loads ranging from $10^{1.2}$ to $10^{4.2}$ infected cells per 10^6 peripheral blood mononuclear cells, during the 10 weeks follow up period. In contrast, no cell associated viraemia was found in the two Rev and Tat vaccinated animals during this period (Fig. 1). Using a quantitative competitive SIV *gag* based RT-PCR, the two control monkeys showed a plasma virus load above 3×10^7 RNA copies per ml, which persisted for the entire follow-up period of 36 weeks. In the vaccinated animals plasma viral RNA could only be demonstrated transiently, during the first four weeks after infection, whereafter no plasma viral RNA could be detected for the entire observation period of 36 weeks (not shown).

These data show that vaccination with live vectors expressing the early proteins Rev and/or Tat, may induce protective –albeit no sterile– immunity against primate lentivirus infection. We therefore deduce that the induction of early viral protein specific CTL may be a promising alternative to the currently little successful approaches of lentivirus vaccine development, which are virtually all based on the use of late structural proteins of the virus. Further studies in more animals should confirm that, as speculated from our previous data [7], the underlying mechanism of the

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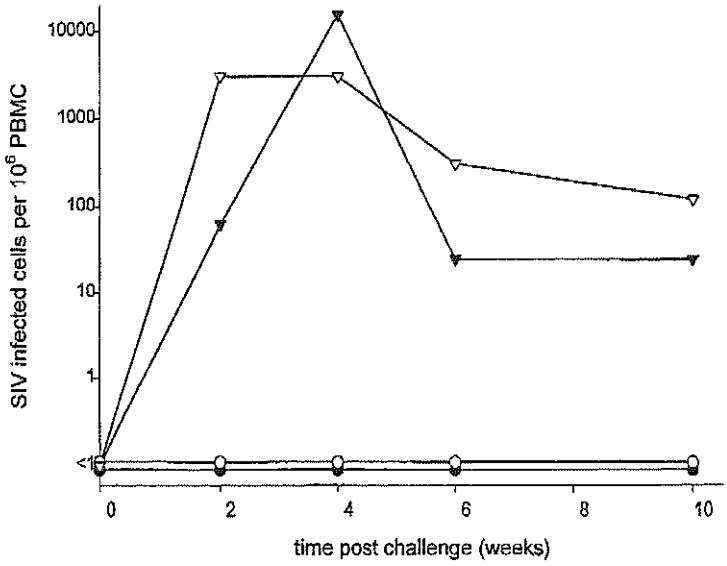

Fig. 1. SIV cell associated viral load. As previously described, serial 5 fold dilutions of PBMC were incubated with C8166 cells for 6 weeks. The number of infected cells per 10^6 cells was calculated from the highest dilution that was positive in a $p27^{gag}$ antigen capture ELISA. ●/○ SIV Rev and SIV Tat vaccinated macaques, ▼/▽ control macaques vaccinated with nonSIV proteins.

observed protection is indeed the induction of Rev and/or Tat specific CTL.

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Comparison of the efficacy of early versus late viral proteins in vaccination against SIV

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Vaccine, in press

Abstract

The immune response against early regulatory proteins of simian- and human immunodeficiency virus (SIV, HIV) has been associated with a milder course of infection. Here, we directly compared vaccination with Tat/Rev versus Pol/Gag. Challenge infection with SIVmac32H(pJ5) suggested that vaccination with Tat/Rev induced cellular immune responses that enabled cynomolgus macaques to more efficiently control SIV replication than the vaccine-induced immune response against Pol/Gag. Vaccination with Tat/Rev resulted in reduced plasma SIV loads compared with control ($p=0.058$) or Pol/Gag vaccinated ($p=0.089$) animals, with undectectable plasma viral loads in two of the four Tat/Rev-vaccinated animals. Therefore, the results warrant further investigation of the early regulatory proteins and their potential for vaccination against HIV.

Introduction

Candidate lentivirus vaccines have been tested with variable degrees of success but correlates of protective immunity remain largely elusive. Most of the efforts have focussed on the induction of both humoral and cellular responses against structural proteins [1]. However, lentivirus infection generally results in chronic infection and progression to disease despite the presence of these immune responses [2,3].

We have previously found that longterm asymptomatic HIV seropositive individuals have cytotoxic T lymphocyte (CTL) responses against all the HIV proteins tested, whereas rapid progressors initially have comparable CTL responses against structural proteins, but not or very limitedly against Tat and Rev [4]. The possibility that CTL responses against early regulatory proteins are more effective in controlling lentivirus infection was recently also supported by studies on SIV infection in macaques [5-7].

Preliminary vaccination experiments in macaques with Tat alone or Tat in combination with Rev yielded encouraging results showing reduced primary SIV viraemia after challenge [5,8,9]. In other studies, however, vaccination with structural proteins in some cases also led to reduction of primary viraemia [10]. Comparing efficacy of the different vaccine formulations and schedules described is difficult, since different primate models for lentivirus infection vary markedly in their read-out parameters [11]. Therefore, we directly compared the ability of vaccine-induced immunity against the early regulatory proteins Tat and Rev with that against the structural proteins Pol and Gag to control SIV replication after challenge.

Materials and Methods

Immunization and challenge of macaques.

Twelve macaques (*Macaca fascicularis*) were vaccinated four times intramuscularly in a prime-boost regimen in which two vaccinations with recombinant Semliki Forest virus (rSFV; at weeks 0 and 6) were followed by two vaccinations with recombinant modified vaccinia virus Ankara (rMVA; at weeks 12 and 16) constructs expressing Tat and Rev (n=4), Pol and Gag (n=4) or β-gal (n=4) as described previously [8].

EDTA-blood samples were collected every two weeks for plasma and isolation of peripheral blood mononuclear cells (PBMC). Five weeks after the last immunization all macaques were challenged intravenously with 50 MID_{50} SIVmac 32H (pJ5).

Biochemical analysis of vaccines.

The genes of the respective SIV proteins are all expressed under specific control of the same promotor and similar levels of Rev and Gag expression have been observed *in vitro* (Fig. 1). CEF cells grown in 6-well-tissue culture plates were infected at an MOI of 10 with MVA or rMVA. After 24 h infected monolayers were harvested by cell lysis. Lysates corresponding to about $5x10^5$ infected CEF were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were electroblotted onto nitrocellulose membranes for 2 h in a buffer containing 25 mM Tris, 192 mM glycine and 20% methanol (pH 8.6). After blocking overnight in PBS-2% BSA, blots were probed with 1000-fold dilutions of mouse monoclonal antibodies directed against SIV Rev (MAB 6.2, kindly provided by Kai Krohn, University of Tampere, Finland) or Gag-p27 proteins (KK60, kindly provided by Karen Kent, NIBSC, Potters Bar, UK) in PBS-2% BSA for 1 h. After being washed with PBS-0.1% Nonidet P-40, the blot was incubated for 1 h with alkaline phosphatase-conjugated polyclonal goat anti-mouse antibody (Promega, Madison WI) diluted 2000-fold in PBS-2% BSA, washed again, and developed using Western blue substrate (Promega, Madison WI).

Serologic assays.

MVA-specific plasma IgG was determined using a FACS-measured immunofluorescence assay as described previously with an additional pre-incubation of plasma's on uninfected RK-13 cells [12]. SFV-specific plasma IgG responses were similarly determined using HeLa cells that were used 18 hours after infection with SFV.

Elispot assay.

The frequency of SIV protein-specific interferon-gamma producing cells (IPC) was determined by Elispot for interferon-gamma (IFN-γ). PBMC depleted for CD4 cells (ΔCD4 PBMC) using CD4 Dynabeads (Dynal, Hamburg, Germany) were cultured overnight in 96-well round-bottomed plates (Greiner, Labor Technik, Nürtingen, Germany) in RPMI-1640 supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), 2-mercaptoethanol (10^{-5} M), 10% FBS and 1% pooled serum from naïve macaques (referred to as culture medium). After eighteen hours cells were counted and seeded in 96-well V-bottomed plates (Greiner) at a

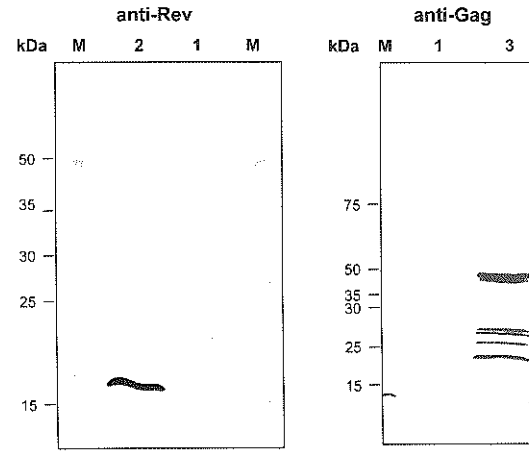

Fig. 1. Western blot analysis of SIV Rev and Gag proteins. Proteins in lysates of CEF infected with wildtype MVA (lane 1) or rMVA expressing the SIVmac protein Rev (lane 2) or Gag-Pol (lane 3) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with mouse monoclonal antibodies against SIV Rev (anti-Rev) or Gag (anti-Gag) proteins. M, lanes with protein standards, molecular masses (in kDa) are indicated by the numbers on the left.

concentration of $1x10^5$ cells/well (pre-challenge) or $0.5x10^5$ cells/well (post-challenge). Autologous herpes papio virus-transformed B cells (B-LCL) were pulsed at a cell density of $1x10^6$ cells/ml with 10 µg/ml of 20-mer peptides with 10 amino acids overlap during one hour at 37°C in a round-bottomed 96-wells plate. Tat-derived peptides (ARP7057.1-12), Rev-derived peptides (ARP7058.1-10) and a selected set of Gag-derived peptides were obtained from Centralised Facility for AIDS Reagents, NIBSC. Gag-derived peptides were selected to contain previously defined CTL epitopes (p26 (ARP714.4-6) p17 (EVA775.2, 5, 11-14, 17 and 21) p15 (EVA776.1-2) (Los Alamos National Laboratory, HIV sequence database). After the pulse, half of the culture medium was replaced by fresh medium without peptide. After over-night incubation at 37°C cells were irradiated (30 Gy) and added to the ΔCD4 PBMC at a 1:2 ratio. Plates were centrifuged briefly, incubated at 37°C for one hour and cells were transferred to Elispot plates coated with mouse monoclonal IFN-γ-specific antibody (U-CyTech, Utrecht, The Netherlands). Six hours later, cells were transfered to a round-bottomed 96-wells plate and maintained in culture medium supplemented with recombinant IL-2 during about ten days. Subsequently, cells were expanded using PHA and xenogenic stimulation for future analysis of specific CTL epitopes. Elispot plates were developed with biotinylated rabbit polyclonal IFN-γ detecting antibody (U-CyTech) followed by streptavidin alkaline phophatase conjugate (DAKO, Glostrup, Denmark) and BCIP/NBT phosphatase substrate (Kirkegaard&Perry Laboratories, Gaithersburg, USA). Developed spots were quantified by light microscopy.

Virus detection.

SIV plasma viraemia was monitored by real-time RT-PCR. We quantified viral RNA using a Taqman assay (forward primer, 5'-CTTGGTCCATGGGGAAAGAA-3'; reverse primer, 5'-TCAGCCCCTGATGCACTTG-3' and FAM/TAMRA-labelled probe, 5'-CCCCGCAATTTCCCCATGGC-3') as previously described [13]. Serial dilutions of SIV_{mac251} viral lysate (SanverTECH, Heerhugowaard, The Netherlands) were spiked into negative plasma and used to calculate the RNA copies/ml. The cell-associated viral load was determined with an infectious center test on c8166 cells as described previously [14].

Statistical analysis.

We used repeated measures analysis of variance (rmANOVA), assuming a compound symmetry structure for the residual (co)variance matrix. For testing the mean differene in plasma load of viral RNA between groups, we used ln-transformed data from day 3 to 109. Values below the detection limit of the real-time quantitative RT-PCR assay were set to 500 copies/ml.

Results

Antibody response against the vectors.

Animals were vaccinated in a prime-boost regimen, with 2 doses of rSFV followed by 2 doses of rMVA, for optimal induction of cellular immunity [15]. Vaccinations were monitored via vector-specific antibodies in the plasma. After each administration, antibody titers increased, reaching similar levels in all groups at the end of the vaccinations (Fig. 2). The kinetics of SFV- and MVA-specific antibody

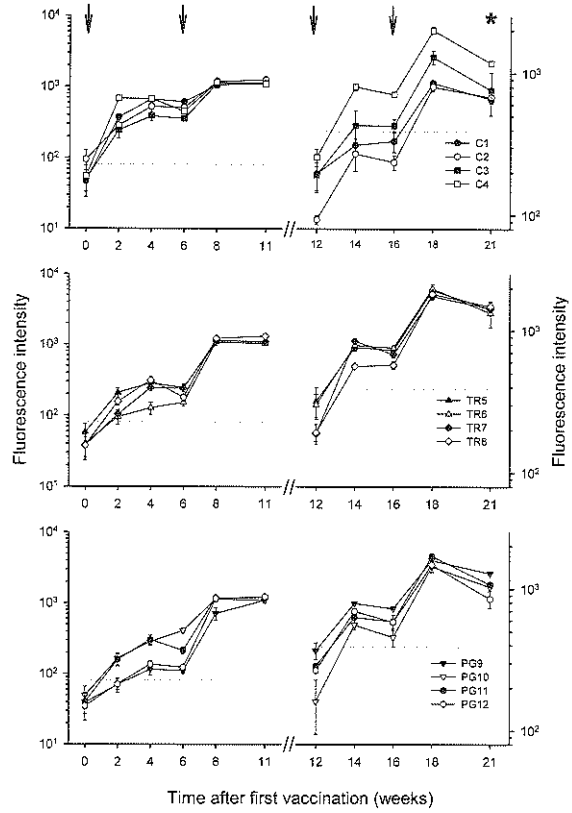

Fig. 2. Development of IgG plasma responses against the vaccine vectors in vaccinated macaques. Left panel, rSFV; right panel, rMVA; Macaques vaccinated with control vectors (top), with Tat and Rev expressing vectors (middle), or Pol and Gag expressing vectors (bottom). The four vaccinations are indicated (arrows) as well as the moment of infection SIV infection (asterisk). The data are presented as fluorescence intensity (average ± SD). An arbitrary cut-off level was chosen at the average + 2xSD (dotted line).

responses were largely similar in all macaques upon primary and secundary vaccination, showing that indeed all the macaques were vaccinated and that re-vaccination boosted the immune response against the respective vectors (Fig. 2). SIV antigens did not seem to interfere with the induction of the immune response, despite reported immuno-modulatory effects for Tat [16].

Cellular immune response against SIV proteins.

The number of SIV-specific IPC was quantified with an Elispot. At the day of challenge no SIV-specific IPC were found in the control animals (Fig. 3). In Tat/Rev-vaccinated animals IPC against Tat and Rev (40-6880 IPC/10^6 ΔCD4-PBMC) could be demonstrated, but not against Gag. Conversely, Gag/Pol-vaccinated animals had developed IPC against Gag (10-2700 IPC/10^6 ΔCD4-PBMC), but not against Tat or Rev.

After challenge, when viral loads declined, control animals had developed a cellular immune response against SIV. This was demonstrated by moderate levels of IPC against all 3 antigens tested (Fig. 3; 60-1220 IPC/10^6 □CD4-PBMC). In Tat/Rev-vaccinated animals, IPC against vaccine antigens were still detectable, but only one animal had developed low numbers of IPC against Gag. Similarly, Gag-specific responses were retained in animals vaccinated with Gag/Pol and 2 animals had acquired low numbers of IPC specific for Tat and Rev.

SIV viral loads.

After challenge with the high dose of infectious virus all animals became infected (Table 1). One control animal became persistently viraemic and 3 had a transient viraemia which is common for this model of lentivirus infection [17]. Two of the Tat/Rev-vaccinated animals remained negative for plasma viraemia (Fig. 4). The other 2 had a short viraemia, with little virus production. By contrast, Gag/Pol-

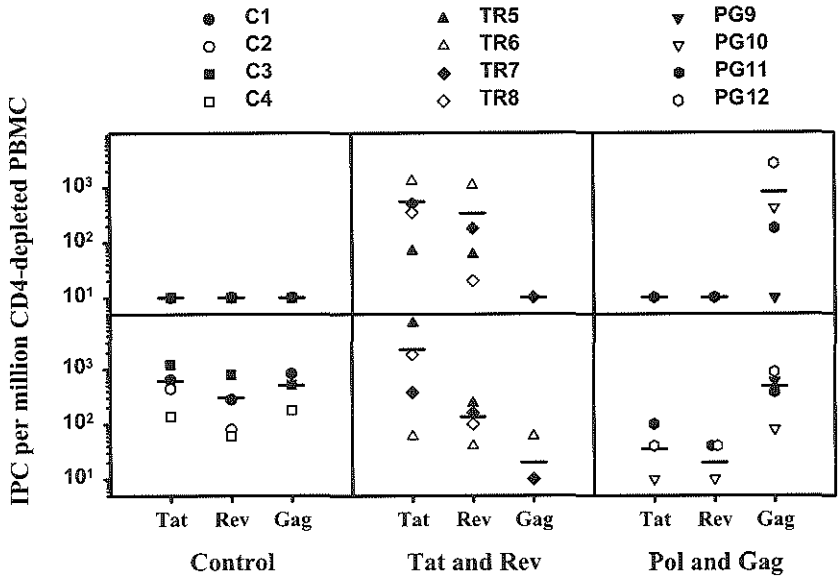

Fig. 3 Numbers of Tat-, Rev- and Gag-specific IFN-γ producing cells (IPC). Numbers of IPC in CD4-depleted PBMC collected at the day of challenge (upper panel) or at day 20* after challenge (lower panel) were assessed by an Elispot assay. Spots were quantified visually by light microscopy. *Occasionally PBMC collected at day 20 were not available, samples from day 27 or 33 were used instead (#TR6 and #TR8: Gag peptides, #PG12: Tat and Rev peptides). The horizontal lines in the graphs indicate the mean value of the group.

vaccinated animals all developed detectable plasma viral loads similar to the control animals and 2 were still positive at the end of the observation period. The differences between the groups were only borderline significant (Tat/Rev vs. Control p=0.058 and Tat/Rev vs. Pol/Gag p=0.089), likely due to the small number of animals tested.

The data from the infectious centre test showed similar trends as the plasma viral loads. From all animals virus could be isolated at some stage after challenge (Table 1). The Tat/Rev-vaccinated animals cleared the virus infected cells faster and only one animal had remained virus positive at the end of the experiment. By contrast, infected cells were persistently detected in 3/4 and 4/4 control and Gag/Pol-vaccinated animals, respectively.

Discussion

Here we compared the effect of vaccination with early versus late SIV proteins on the induction of specific immunity and containment of challenge. The SIV-vaccinated animals, but not the control animals, had developed SIV-specific IPC at the day of challenge. Despite pre-existing immunity, all animals became infected, but 2 Tat/Rev-vaccinated animals with high numbers of Tat-specific IPC remained negative for plasma viraemia. There was, however, a provocative trend in that Tat/Rev vaccinated animals better controlled viraemia.

We and others previously found that CTL against structural proteins are ubiquitous in lentivirus infection, but that Tat and Rev specific CTL correlate with a low viraemia and a benign course of infection [4,17,18]. From these data it could not be inferred whether the beneficial effect of Tat/Rev-specific CTL was the

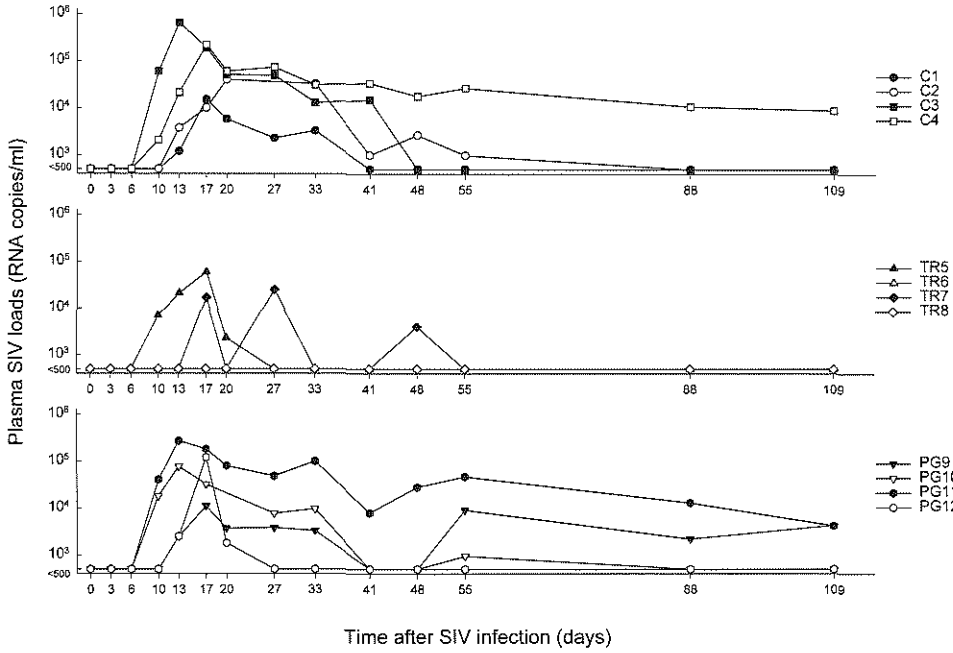

Fig. 4. Detection of plasma viral loads. Plasma viraemia was measured using a real-time quantitative RT-PCR as described previously[13]. The detection cut off was 500 SIV RNA copies/ml.

consequence of a broader response in general, or whether it was intrinsic to Tat and/or Rev-specific CTL. Our current study argues against the breadth of the immune response as an explanation, since each vaccine contained 2 proteins. Moreover, Gag and Pol are much larger proteins and may therefore induce more CTL populations with different specificities. Therefore, we favor the hypothesis that early expression of Tat and Rev during the virus replication cycle contributes to timely elimination of infected cells and more effective reduction of the amount of virus produced (van Baalen submitted).

Of note, a bias in the immune response of the vaccinated macaques was observed after challenge. Whereas control animals developed IPC against all antigens tested, vaccinated animals had a preference for the antigens used in vaccination. This may have resulted from immune-mediated limitation of viral replication, which could hamper sensitization to SIV antigens that were encountered only after vaccination. This explanation is, however, not consistent with the data. Two of the 3 animals that developed IPC against non-vaccine antigen showed no (TR6) or limited (PG12) plasma viraemia after challenge. In all animals with high vireamia an increase in IPC against SIV antigens that were included in the vaccine was observed. On the other hand, the immune system may have become skewed towards the antigens as a result of the vaccination. In that case, and if CTL against early proteins are indeed more protective, vaccination with viral structural proteins may be suboptimal or even counterproductive.

Table 1. Detection of cell-associated viral load.

Day After SIV Chall.	Control				Tat and Rev				Pol and Gag			
	C1	C2	C3	C4	TR5	TR6	TR7	TR8	PG9	PG10	PG11	PG12
3	‡-	-	1	1	1	1	1	-	25	1	-	-
6	-	-	125	1	-	-	-	-	5	-	-	-
10	1	25	≥625	125	125	25	1	1	125	125	≥625	125
13	25	125	≥625	≥625	≥625	≥625	5	25	125	≥625	≥625	≥625
17	25	≥625	≥625	≥625	125	5	≥626	-	≥625	≥625	≥625	≥625
20	≥625	≥625	125	≥625	≥625	125	≥625	1	≥625	≥625	≥625	≥625
27	≥625	≥625	125	≥625	≥625	125	25	5	125	≥625	≥625	25
33	1	125	125	25	25	1	125	-	25	5	25	-
41	1	25	25	≥625	25	-	5	-	25	25	25	-
48	1	25	-	≥625	25	-	5	-	125	25	≥625	-
55	-	5	5	≥625	-	-	25	-	125	5	125	-
72	125	-	25	3125	-	1	5	-	25	1	3125	-
88	5	-	25	3125	-	-	5	-	25	1	3125	1

The number of SIV-infected cells per 10^6 PBMC isolated from blood samples collected at different days after intravenous infection with 50 MID_{50} SIVmac 32H (pJ5) were detected using an infectious center test as previously described[17]. ‡ Less than one infected cell per 10^6 PBMC.

In this study vaccination with Gag/Pol did not result in any noticeable effect on virus replication, whereas in other studies some protection has been reported [10]. It is important to realize these vaccinations have been carried out in different models, which may explain the discrepancy in results. Infection of cynomolgus macaques with SIVmac 32H (pJ5) results in low level, transient viraemia, which is milder than HIV-1 in humans. Other primate models for lentivirus infection give much higher viral loads during primary infection and a more rapid progression to disease and death than HIV-1 infection [11]. It will therefore be useful to perform comparitive studies in these models to further evaluate the effectiveness of different vaccine strategies against HIV.

Collectively, our and other observations point to an advantage of specific immunity directed against the early proteins Tat and Rev in controlling viraemia in lentivirus infections [5,8,9]. Therefore, we emphasize that the use of early regulatory proteins should be given serious consideration in the development of HIV vaccination strategies [4].

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CHAPTER 5

Discussion

Timing in control of HIV by cytotoxic T lymphocytes

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(submitted)

Timing of disease progression and CTL responses in HIV infection

Most individuals infected with human immunodeficiency virus type 1 (HIV-1) develop AIDS, in absence of antiviral drug therapy, in about 10 years (1). A proportion of untreated infected individuals (~10%) develops AIDS within three years, and approximately 10% have no signs of disease progression even after 12 to 15 years(1;2). This variability in rates of disease progression correlates with the quantity of virus in plasma (3), most of which is produced by recently infected cells (4). Virus-specific cell-mediated and humoral immune responses can generally be detected during the early stages of primary infection and persist for many years. Factors that render these responses unable to prevent establishment of chronic infection and disease progression, in most infected individuals, are central in research on the pathogenesis of HIV infection (5-10). It is expected that identification of mechanisms explaining long-term survival may provide insights into possible therapeutic interventions.

The immune response and initial control phase in HIV or SIV infection is dominated by $CD8^+$ cytotoxic T lymphocytes (CTL) (8;11-15). With their T cell receptor (TCR), these cells can recognize fragments of viral proteins that are presented by molecules of the class I major histocompatibility complex (MHC) on the surface of antigen presenting or infected cells (16;17). If mature CTL receive an appropriate signal (18), they can reduce HIV production by releasing factors that lyse infected cells or inhibit virus production in non-lytical ways (19). Inhibition should occur before a significant number of progeny virions is released, in order to control reproduction and spreading of virus (20). Although CTL do not always clear virus after primary viremia, they have been shown to be critical for control of virus reproduction in several viral infections (15;21). HIV differs from other viruses by the level at which propagation continues after primary viremia, and by its ability to persist in and on cells that are central to protective immune responses, including $CD4^+$ T helper cells, macrophages and follicular dendritic cells (10). These characteristics may interfere with induction and maintenance of effective immune responses and offer several possibilities for the virus to resist clearance mechanisms (7;22). Thus, if HIV is not eliminated early, a dynamical balance develops between reproduction of virus in activated immune cells on the one hand (23;24), and induction and maintenance of active immune cells on the other.

Timing in induction and expansion of CTL

Whether CTL can eliminate virus early after infection, and if not, the level at which virus persists, depend on dynamical and qualitative characteristics that govern virus reproduction and CTL responses (21). Before CTL can exert their antiviral activity, they have to be induced, expand, maturate, migrate to infected tissues and decide whether cells are infected or not. Expansion and differentiation of CTL is influenced by several factors, including epitope production levels in infected cells (25;26), the repertoire of TCR and MHC molecules (27-29), cytokines (30;31), and the level of costimulation (32-34). Although innate immune mechanisms can interfere before adaptive immune responses develop (35), quantities of virus and infected cells generally increase exponentially during the period of CTL differentiation and expansion. Once a CTL is differentiated into an effector cell and encounters an infected cell, it can deliver a lethal hit, detach, migrate to another cell, decide whether it is infected, deliver a lethal hit if appropriate, and so on until it dies (18;36). If soluble factors that are released after encountering an infected cell interfere with virus infectivity or with virus production in nearby infected cells, CTL may not be required to attack each cell individually (37).

Factors that influence the outcome of virus-host interactions have been studied in detail for lymphocytic choriomeningitis virus (LCMV) infected mice (21). The dose, route, and replication rate of virus early after infection were shown to determine whether CTL clear virus, mediate immunopathology or become exhausted (21). In adoptive transfer experiments, the number of CTL transferred and the time period required to reach cytolytic effector function, were critical for their ability to control LCMV during the early stages of infection (38-40). If the number or functional state of the transferred cells (i.e. cells obtained from uninfected mice or during acute or memory stages of infection) was insufficient to prevent virus production early, additional time to perform effector function did not improve overall virus control. These data indicate that in order to be effective, CTL must act faster than the virus replicates, that they have to lyse infected cells within a given time window, and that small changes in CTL numbers or speed of killing can have dramatic effects on virus control because of the capacity of virus populations to expand exponentially (38).

Timing of target cell recognition by CTL

Virus infected cells are invisible for CTL during the eclips phase until sufficient viral protein is produced, degraded and processed into peptides that associate with MHC class I molecules (16;17;21). The production rate of MHC-peptide complexes and their half-life on the cell membrane, i.e. their densities, determine whether an infected cell is vulnerable at the moment that their surface is sampled by the TCR of a virus-specific CTL (17;18). Different antigen densities may be required to trigger distinct effector mechanisms (18;41), and some CTL require lower antigen densities than others (see below). These factors are expected to influence the time window that is available for a CTL to attack infected cells before onset of virion release, and thereby its capacity to control viral reproduction.

One of the first studies on the impact of timing on CTL effectiveness demonstrated that infected cells can be lysed before infectious virus progeny is assembled (20). During the initial four hours after experimental infection of cells with vaccinia virus, spleen cells from vaccinia immune mice lysed 91% of the infected cells and reduced virus production by about 1.3 $\log_{10}$, as determined by quantification of plaque forming units (PFU). If the effector cells were added four hours after infection, they also lysed about 90% of the infected cells during the subsequent four hours, but virus production was not affected. These data confirmed an earlier conclusion that cells infected with mousepox virus become vulnerable to CTL at about 3 hours after infection (42). Adoptively transferred vaccinia immune spleen cells that were cytolytic *in vitro*, reduced up to four $\log_{10}$ of PFU in spleens of preinfected recipients within 24 hours (43).

Timing of target cell recognition has also been studied with distinct CTL lines and clones that recognize an epitope from the HIV-1 envelop protein with different avidities. Cells infected with a recombinant vaccinia virus containing this epitope were lysed earlier by high-avidity than by low-avidity CTL (44). Two independent mechanisms were identified: the high-avidity CTL recognized lower antigen densities and they initiated target cell lysis more rapidly at any given antigen density. In agreement with earlier reports on the *in vivo* antiviral capacity of CTL with different avidity (26;45), 10^7 high-avidity CTL injected into mice simultaneously infected with the recombinant vaccinia virus prevented 2 to 3 $\log_{10}$ more virus production in three days than the same number of low-avidity CTL. This could not be compensated by adding 3-fold more cells or by extending the observation period by another 3 days. Because both the high- and low-avidity CTL target the same epitope, which excludes variability due to differences in antigen processing and

expression of presenting MHC molecules, these results demonstrate that kinetic aspects of viral protein production can significantly influence the ability of CTL to control actively replicating virus.

CTL against early or late viral proteins *in vivo*

Production of viral proteins is generally strictly regulated in time. This is expected to cause variation in the time that infected cells are vulnerable to CTL of different protein specificities before release of progeny virus begins. Although studies that directly assess the moment infected cells become vulnerable to CTL are limited, presentation of epitopes from proteins that are available early after infection has been implicated in more effective control by CTL.

Protective immunity to murine cytomegalovirus (MCMV) is provided by CTL against immediate early and early proteins rather than structural proteins that are expressed later in infected cells (46-49). In human cytomegalovirus (HCMV) infection, CTL against immediate early proteins have been proposed to be important for an effective response against latently infected cells during reactivation (50). In cells acutely infected with HCMV, early vulnerability can also be provided by antigen processing of structural proteins, e.g. pp65, of incoming virions (51). The ability of pp65 to reduce MHC expression (52) can limit the time that infected cells are vulnerable, but the *in vivo* significance of this mechanisms is still debated (49). Early after infection of macaques with SIV, CTL directed against the early expressed Tat (transactivator of transcription) were associated with better control of virus replication and provided a growth advantage for virus variants that could no longer be recognized due to mutations in CTL epitopes (53). CTL against the later expressed Gag (group-specific antigen) did not select such variants.

In HIV-1 infection, limiting dilution analyses of peripheral blood mononuclear cells for precursor CTL (CTLp) against Tat and another early protein, Rev (regulator of virion expression), showed their prevalence in individuals with no or slow disease progression (54) (§2.3 this thesis). Simultaneously analyzed samples obtained early after infection from individuals who subsequently progressed to AIDS rapidly, contained CTLp against Gag, RT (reverse transcriptase), Env (envelope) and Nef (negative factor) in frequencies similar to those found in long-term asymptomatics, but levels of Rev- or Tat-specific CTL were undetectable or low (54;55). These findings support the hypothesis that the latter are better able to control HIV by virtue of their specificity for early viral proteins. This neither implies that they are expected to prevent AIDS entirely in all individuals, nor that CTL against other proteins do not affect virus production or that the potential to operate early always leads to better control (see section "consequences for pathogenesis and therapies").

CTL against early or late HIV proteins *in vitro*

Relatively few studies have directly addressed effects of CTL of different protein specificity on HIV-1 reproduction. Analyses of this parameter can add importantly to measurements of effector cell frequencies in peripheral blood, because the most protective CTL may not always be the most abundant (26).

Co-cultivations of CTL directed against Gag, Env or RT with HIV-1 infected cells *in vitro*, have shown that they can lyse HIV-1 infected cells before peak virus production (56), and suppress virus spread in immortalized T-cell lines and PBMC by lytic and non-lytic mechanisms (19;57;58). Levels of inhibition depended on inoculum size and effector-to-target cell ratio. Elimination of virus from infected cell cultures has been documented for one CTL clone only(58). Virus generally continued

to replicate in the presence of specific CTL, albeit at lower levels, even in cultures containing 50% specific CTL and 50% $CD4^+$ cells that had been infected with 10^{-2} $TCID_{50}$ per cell prior to cocultivation (57-59). Addition of dendritic cells further reduced CTL effectiveness, probably because this increased the viral reproduction rate (59).

During the first 36 hours following infection of non-immortalized $CD4^+$ T cells with $HIV\text{-}1_{IIIB}$ (at a high multiplicity of infection), CTL directed against Rev or RT inhibited viral protein production in infected cells that could not yet be lysed (60)(§3.1 this thesis). By 48 hours they had prevented >97% and ~92% of virus release, respectively, as determined by quantification of extracellular RT-activity. This small difference between the two CTL populations early after infection was shown to increase significantly in experiments designed to allow the residual virus to start new rounds of infection. Cultures were initiated at a low multiplicity of infection, i.e. containing mostly uninfected susceptible cells, and Rev-specific CTL had prevented at least 2 log_{10} more virus production than similar numbers of RT-specific CTL by day 10 (61)(§3.4 this thesis). This difference was not compensated for by ten-fold more RT-specific cells.

Several mechanisms that could explain these results were addressed, including escape of virus from CTL recognition by mutation, variations in effector cell numbers over time, amounts of peptides required to trigger cytolytic activity, direct cytolytic capacities of the effector cells, and fractions of infected cells that become vulnerable to CTL (61). The results indicated that these factors are not likely responsible for the higher per cell capacity of the Rev-specific CTL to control viral reproduction. We did not yet directly address possible differences in the number of vulnerable cells that one effector cell can eliminate during its lifetime, the rate of serial killing, or the efficiency of non-cytolytic effector mechanisms. However, reproduction of a recombinant virus that encoded the minimal RT-epitope in the early expressed *nef* gene (62), remained undetectable in presence of the RT-specific CTL for 10 days (61). That they could not efficiently control reproduction of the parental virus can therefore not be explained by insufficient potency of effector mechanisms of the CTL, but is most probably due to their inability to attack enough infected cells before progeny virus started new infection cycles. Further studies are required to determine the average and range of the time period during which cells infected with the parental or recombinant virus are vulnerable before production of progeny virions.

The precise mechanism that underlies the higher susceptibility of cells infected with the recombinant virus for CTL activity against the RT-epitope is presently unclear. The epitope is expected to be expressed earlier and at higher levels, which both would result in earlier vulnerability of the target cells. The epitope insert in the *nef* gene did not affect the ability of Nef to reduce MHC class-I expression (61), since MHC class-I molecules were expressed at similar levels on the surface of cells infected with the recombinant or parental virus. Finally, if Nef-mediated effects on MHC-peptide production delay onset of target cell vulnerabilty, early epitopes are more likely than late epitopes to be presented before Nef can interfere with their presentation. Although the relative contribution of different mechanisms to the timing of target cell vulnerability requires further analyses, our results indicate that it is critical for CTL-mediated control of HIV reproduction and spread.

Mathematical models for timing in control of HIV by CTL

Direct measurement of the interval between the onset of target cell vulnerability and virion release is problematic because it is influenced by multiple factors in infected cell, but also by characteristics of effector cells, including the density of TCR

molecules on the surface, differentiation state, and the potency of effector mechanisms. Moreover, whether a certain interval is sufficient for CTL to control virus spreading will also depend on kinetics of virus reproduction and rates of serial target cell elimination by CTL. As more information on these parameters becomes available, integration of experimentally determined values in mathematical models can help to assess the relative contribution of each parameter to the final outcome.

The dynamics and kinetics of HIV protein production have been studied in several cell types with monoclonal antibodies, primer sets and probes that detect specific proteins or transcripts. The proteins Tat, Rev and Nef have been detected as early as 6 hours following acute infection in T cells, while Env remained undetectable up to 12-16 hours and was first detected at 24 hours (63). The early proteins are translated from multiple spliced transcripts of about 2 kb that are the first to be induced (64;65) and can be detected between 6 to 8 hours after infection of T cells with HIV-1 (66). Tat and Rev regulate viral gene expression (67) and shuttle back to the nucleus, where increased levels of Tat lead to increased transcription. Accumulation of Rev in the nucleus leads to increased export of singly spliced and unspliced transcripts. Translation of singly spliced transcripts produces Env and several accessory proteins, and unspliced transcripts are translated to produce virion structural polyproteins, including Gag and Pol. Levels of unspliced transcripts remain low until 24 hours after acute infection in T cells, followed by a sharp increase which coincides with release of virions during the next 12 hours (64). Also in chronically infected T cells and promonocytes after activation by phorbol esters, induction of multiple spliced transcripts preceeded induction of singly spliced transcripts, followed by increasing levels of unspliced transcripts and concurrent assembly of new virions (68). Ordered appearance of transcripts also occurs in monocyte-derived macrophages following high multiplicity infection with HIV, but with different kinetics. Multiple spliced transcripts were detected at 24 hours after infection, and singly spliced transcripts at 48 hours which coincided with the onset of p24 antigen and infectious virus production (69). Following transmission of virus by fusion of chronically non-productively infected cells to uninfected cells, *gag* and *env* transcripts did not increase significantly for at least 16 hours, and by 24 hours 20-fold and 7-fold higher levels were observed (70). Structural proteins of HIV virions that enter T cells or macrophages during infection are not recognized by CTL and de novo protein production is required (71).

Data on kinetics of protein production in infected T cells have been used in mathematical models to estimate the fraction of cells that are killed by CTL as opposed to virus (72). It was shown that if, for example, elimination of target cells by CTL is an exponential decay process with a rate (α) of 3.06 per day, i.e. 40% lysis in a standard 4 hour chromium release assay, and it starts 0.3 days (7-8 hours) after infection, CTL prevent 99% of virus that would have been produced by the target cells. If it starts 0.9 days (21-22 hours) after infection, CTL would prevent 92% of virus production. Because rates of target cell elimination depend on the numbers of CTL and vulnerable target cells, and virus production *in vivo* is distributed throughout the lymphoid tissue, the distribution of CTL of different protein specificity, and their local density will add importantly to variations in their effectiveness. This makes it difficult to directly address the influence of time that target cell are vulnerable on the capacity of CTL to reduce virus production *in vivo*. But *in vitro* some of these variables can be controlled, and in mathematical models the significance of time for CTL effectiveness can be studied independently from differences in these and other parameters.

The ability of progeny virus to infect new cells is another essential dynamical characteristic in virus propagation. Therefore, we extended the model for antiviral CTL activity by including this parameter (61)(§3.4 this thesis)(Fig. 1). The number of cells that can be infected by the viral progeny of one infected cell during its lifetime, the viral reproduction ratio R_0, has been estimated to be approximately 19 (range 7 to 34) during primary infection *in vivo* (73). From the exponential rise of virus levels in our *in vitro* studies, we estimated R_0 to be between 30 and 35. To extinguish ongoing viral replication, the activity of CTL must result in less than one secondary infected cell arising from each infected cell (R_0<1). Thus, in our *in vitro* experiments, CTL should prevent on average more than 97% of virus production during each replication round. Taking the values from the numerical example outlined above, this implies that the CTL that start 0.3 days after infection can control continual reproduction (and the associated increase in virus titers and numbers of infected cells), but CTL that start 0.9 days after infection cannot, although both eliminate vulnerable target cells with a decay rate of 3.06 per day and significantly reduce virus production during each replication round.

How the outcome would change if CTL eliminate vulnerable cells at different rates, or infected cells become vulnerable at different times, or virus propagation proceeds at different reproductive ratios, is shown in Fig. 2. This analysis reveals a threshold target cell elimination rate of about 2 per day, below which CTL will not prevent ongoing virus replication irrespective of the onset of target cell vulnerability or viral reproduction ratios. Above this threshold relatively low target cell elimination rates can control virus that reproduces at ratios as high as 50, provided that infected cells become vulnerable within approximately 16 hours after infection. After that time, the target cell elimination rate, e.g. the number of effector cells, has to increase considerably as the moment infected cells become vulnerable approaches the onset of virion release, especially if viral reproduction ratios are within the range observed in activated T cells.

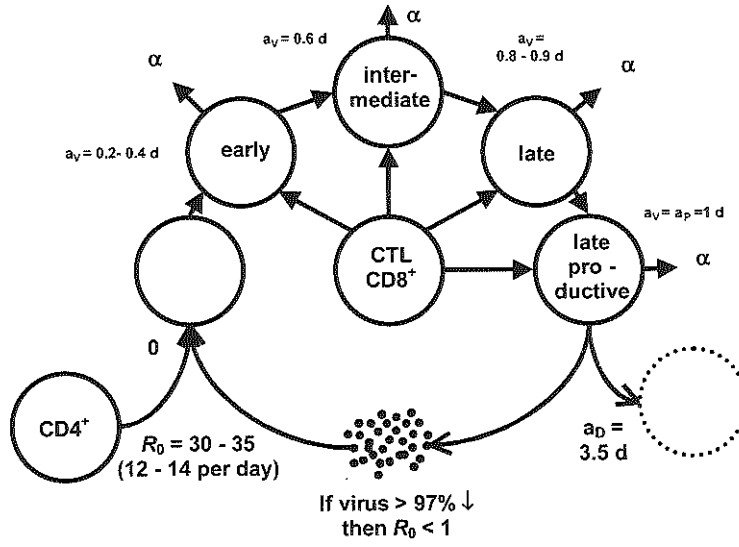

Fig. 1. Dynamics of HIV reproduction and interference by CTL. Infected cells become vulnerable to CTL attack at age a_V, start to produce virus at age a_P and die at age a_D, in absence of CTL. The rate at which infected cells are eliminated from the replication cycle by CTL is α. The reproduction ratio R_0 of the virus in absence of CTL is $R_0 = m(a_D - a_P)$ where m is the rate of infection (estimates for m varied between 12-14 per day *in vitro* [61](§3.4 this thesis)). Reduction of R_0 by CTL depends on a_V and α; for detailed mathematical model and equations see [61](§3.4 this thesis).

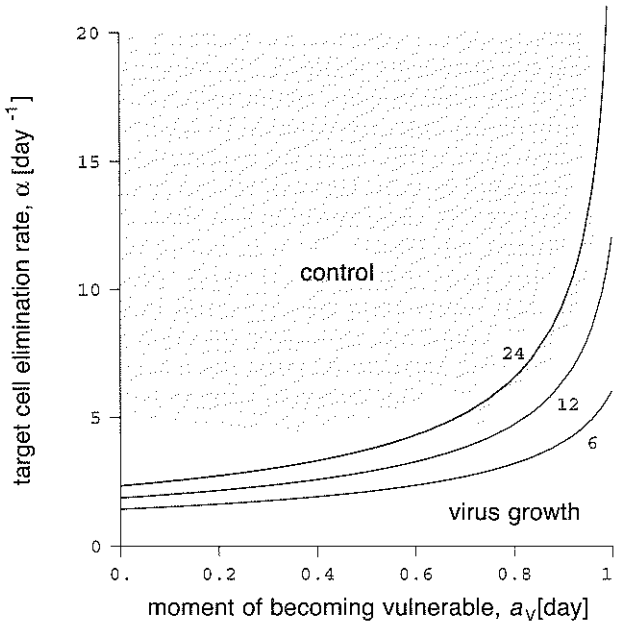

Fig. 2. Control of HIV reproduction by CTL depends on the rate of CTL-mediated target cell elimination and on the period that infected cells are vulnerable before the onset of virion release. Depicted are combinations of the timing of becoming vulnerable (a_V) and the target cell elimination rate (α) that result in control of ongoing virus replication. In the shaded area the virus reproduction ratio R_0 is less than unity for the case where the infection rate m equals twelve; the curves indicate how the area changes with infection rate (m = 6, 12 or 24). Fig. taken from [61](§3.4 this thesis).

Consequences for pathogenesis and therapies

Because the level at which continual virus reproduction proceeds is associated with the severity of pathogenic mechanisms and rates of disease progression, the most important problem in HIV infection appears to be that, in most individuals, CTL and antibodies cannot sufficiently reduce primary viremia. The ability of initial immune responses to control HIV will be influenced by multiple factors, including the dose, host cell range and replication rate of infecting viruses, the route of infection, and mechanisms that influence the quantity and quality of responding CTL. The precise mechanisms responsible for effective initial control, or lack thereof, are therefore likely to differ among individuals.

Even during the early stages of primary infection lack of control may be due to specific characteristics of HIV that interfere with induction, expansion and distribution of CTL, including infection of responding T helper cells, interference with APC function, reduction of MHC expression in infected cells, egress of CTL from main sites of virus production, and exhaustion of CTL clones (7-9;14;74). Escape by mutations in sequences encoding CTL epitopes can also reduce effectiveness of circulating CTL during both early and chronic stages of infection, and contribute to increasing diversity in both virus and CTL populations (9). A diversity threshold has been proposed, above which the immune system can no longer respond to new variants and control is lost completely (75). During the early stages of infection, escape by mutation is predominantly found in epitopes from early or intermediate proteins, indicating that pressure directed against these proteins is higher than pressure directed against late proteins (76). In addition, the frequent detection of

escape variants implies that, in order to retain effectiveness, CTL should be able to recognize epitopes in which variation is not tolerated. This is expected to reduce diversity if complete inhibition cannot be achieved (77).

To date, most studies have addressed CTL responses directed against structural proteins. Induction of more broadly directed responses and higher frequencies of CTL against these proteins have been found in association with lower viral loads and reduced risk for progression to AIDS (8;11-14;78-83). However, these correlates are not consistently observed: in some long-term nonprogressors CTL numbers may be low as a result of low antigen levels (84-87), and high level viremia can persist in many individuals despite large numbers of $CD8^+$ T cells directed against Gag-Pol, Nef and Env (88). The latter could be due, in part, to impaired cytolytic function resulting from chronic interference with T helper cell responses (74;89). During the very early stages of infection, virus levels can also remain relatively high in rapid progressors, while CTLp against Gag, RT, Env or Nef circulate at similar frequencies found in long-term asymptomatic individuals (54;55). By contrast, simultanously analysed CTL responses directed against Rev or Tat were low to undetectable in the rapid progressors and readily detected in all the long-term asymptomatic individuals tested. Together, these studies indicate that CTL against structural proteins are instrumental in limiting virus propagation, at least during the early and asymptomatic stages of infection, and that Rev- and Tat-specific CTL could considerably increase levels of control.

The association of CTL directed against Rev or Tat with slower rates of disease progression may be due to their contribution to a more broadly directed CTL response. But it can also be explained by a higher per cell capacity to control reproduction of HIV. Our data indicate that CTL control reproduction of HIV more effectively if they are able to recognize infected cells earlier after viral entry, i.e. longer before release of progeny virions begins. Although Nef-specific CTL are also likely to operate early, their overall efficacy may be limited because the *nef* gene, unlike those of *tat* and *rev*, does not overlap other open reading frames. Mutations resulting in escape may therefore be more frequently tolerated, which is supported by the dynamical balance between Nef-escape variants and variant-specific CTL responses observed in infected individuals (90), and by the rapid escape from adoptively transfered Nef-specific CTL (91). If virus cannot be eliminated due to its ability to persist in latent forms or infect immuneprivelidged sites, reactivation and new episodes of high level virus production from these sources will also be more effectively controlled by the CTL that have a higher per cell capacity to prevent new infections. Finally, a considerable number of infected cells in circulation and lymphoid tissue express multiple spliced transcripts only. If these cells present antigen, they can only be eliminated by CTL against proteins encoded by these transcripts, i.e. Rev, Tat and Nef.

Although 20-30% of infected individuals mount detectable CTL responses against Rev and Tat, these small regulatory proteins appear to be less immunogenic than the structural proteins (92-94). Some individuals may lack specific MHC molecules that can present epitopes encoded by *rev* and *tat* genes of the infecting virus variants. But even if CTL can respond to the infecting virus, it may take years before they are induced and detectable (95). Studies on immunodominance indicate that it is not a static poperty, but depends in part on the immunologic context that forms after infection (95;96).

Based on the considerations presented sofar it is reasonable to expect that vaccination with Rev and Tat, before infection or during therapy, may induce or boost CTL responses that control virus replication more effectively than those

induced by intermediate or late proteins. Several studies have shown a beneficial effect of vaccination with Rev or Tat in macaque models for AIDS (97-99)(§4.1 this thesis). Variable degrees of success in controlling primary viremia have also been reported with other SIV antigens (15;100). Because animals, viruses and vaccination strategies differ significantly among these studies, they do not allow conclusions on the relative success of early versus late proteins. Recently, we directly compared the ability of four macaques vaccinated with recombinant viral vectors expressing Rev and Tat to control primary SIV infection, with that of four macaques vaccinated with Gag and Pol by the same approach (101)(§4.2 this thesis). CTL responses against vaccine antigens were detected prior to infection, but the Gag/Pol vaccination did not enhance control of viremia. By contrast, in two Tat/Rev vaccinated animals, plasma viral loads remained below the limit of detection, and the other two had shorter and lower viremia compared with the Gag/Pol vaccinated or control animals. Considering the number of proteins, their size and levels of expression, it is unlikely that the higher protection level induced by the Rev/Tat vaccine was due to more broadly directed immune responses.

Conclusions

If HIV is not eliminated early after infection, a dynamical balance develops between propagation of virus in activated immune cells on the one hand, and induction and maintenance of active immune cells on the other. Progression of disease is more rapid if virus levels are higher when this balance is reached. The level of initial control that can be achieved by CTL, will depend on the rate at which they are induced, expand and migrate to infected sites, on the number of effector cells and their per cell capacity to prevent new infections, and on possibilities of the virus to escape. The earlier these factors, in combination, balance the rate of virus production and propagation, the better infection will be controlled. Moreover, the studies discussed indicate that CTL control virus propagation more effectively if they are able to recognize infected cells earlier after viral entry, i.e. longer before release of progeny virus begins. Episodes of high level virus production following reactivation of latent virus will also be more effectively controlled by CTL with a higher per cell capacity to prevent new infections. Vaccination with the early viral proteins Rev and Tat, before infection or during therapy, may induce or boost CTL responses that control virus replication more effectively than those induced by intermediate or late viral proteins. This is supported by vaccination studies in macaques, and studies in HIV infected individuals have been initiated.

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The advantage of early recognition of HIV infected cells by cytotoxic T lymphocytes

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Abstract

Accumulating evidence indicates that cytotoxic T lymphocytes (CTL) play an important role in the clearing of primary and control of chronic HIV infection. Here, we discuss recent findings that indicate that the timing of target cell recognition critically contributes to CTL effectiveness. In this light several problems that have troubled CTL research are discussed. The use of early proteins like Tat and Rev is proposed for future vaccine design.

1. Introduction

Since the recognition of the clinical symptoms of AIDS, and HIV as the etiological agent, the epidemic has spread continuously and an estimated 40 million people are now infected, with >5 million new infections every year. Although HAART has proven successful in reducing virus loads and stopping progression to clinical disease in infected individuals [1], therapy has several important limitations. These include the high price, the difficulty to adhere to therapy and the development of HIV variants that are resistant to the antivirals. Therefore other strategies are urgently needed and vaccine development is an attractive approach given the previous successes in combatting infectious diseases.

Soon after the discovery of HIV, specific immune responses to the virus were identified [2-4]. Inducing or boosting humoral responses against the Envelope was recognized as a promising approach and vaccinations aimed at the induction of antibodies against Env are now in phase 3 clinical trials [5]. However, many AIDS researchers are sceptical about the possibilities of current vaccines based on humoral immune responses [6]. Therefore, the cellular immune response against HIV has recently gained attention as an approach to eliminate infected cells and thus interrupt the production of new infectious viruses [7].

2. CTL in HIV infection

Cytotoxic T lymphocytes (CTL) have been shown to be instrumental in reducing viral loads during primary HIV and SIV viremia. Their appearance in the peripheral blood coincides with the decline of viremia [8-10] and CTL numbers inversely correlate with viral load [11]. Pressure of CTL on the virus was deduced from mutations that accumulate in epitopes recognized by the CTL populations present [10,12,13]. More direct evidence came from experiments in macaque models for AIDS. Depletion of $CD8^+$ cells, before or after infection, results in increased viremia, prolonged depletion of $CD4^+$ T cells and accelerated disease progression [14-16]. However, CTL can be detected in all HIV-infected persons and, despite the antiviral pressure, CTL are generally not capable of eliminating HIV from the body or even in suppressing the virus to latency, like in other chronic infections [17,18].

There may be a few exceptions, where exposed individuals seem to have cleared the virus [19]. Of particular interest, a small percentage of the prostitutes in Nairobi have not become infected despite prolonged high exposure to the virus, via unprotected sex [20]. HIV-specific CTL were found in these women, which could indicate that the cellular immune response was capable of eliminating HIV in these exceptional cases. More recently it was found that protection in these women was dependent on regular contacts. The explanation given for this observation, was that the cellular immune system needed frequent restimulation [21].

3. Effectiveness of CTL

The question which factor(s) are responsable for successful control of HIV infection has not been answered yet. Circumstancial evidence has been derived from groups with different courses of HIV infection, fast versus slow. Broad cellular immune responses, i.e. more CTL with different specificities, were found in people that progress slowly to AIDS [22,23]. But does this mean that more is better or is a critical component included in these broad response?

We found CTL directed against Tat and Rev in long-term asymptomatics only. People that progressed rapidly to AIDS did have comparable CTL responses against other HIV proteins, but not against Tat and Rev [24]. Using a different experimental approach Tat-CTL were found in higher numbers, targeting more epitopes in people that control HIV infection [25]. Furthermore, after experimental infection of cynomolgus macaques the appearance of Tat- and Rev-CTL correlates with the rapid elimination of SIV from the peripheral blood [26].

4. Tat and Rev as vaccines

After our initial findings we started testing Tat and Rev as candidate vaccines. We used a rSFV prime, rMVA boost approach and found efficient control of the challenge virus in cynomolgus macaques [27]. The group of Ensoli tested various vaccination strategies, including tat DNA alone or with a Tat protein boost, and found partial protection in some of the rhesus macaques [28]. Pauza et al based their approach on the possibility that extracellular Tat may be neutralized by antibodies, but they likely also induced CTL reaction against the inactivated Tat protein [29]. They also reported reduced viremia and attenuated disease after challenge. Together these studies indicate that vaccinations with Tat and Rev may be helpful in efficient combatting primary viremia.

However, variable degrees of success in controlling primary viremia have also been reported with other SIV antigens in vaccination studies [5]. The relative success of vaccinations is difficult to compare, because different animals, viruses and vaccination stratagies are used the various studies [30]. Therefore we directly compared vaccination with Tat/Rev and vaccination with Gag/Pol, in one experiment [31]. Prime-boost vaccinations lead to detectable CTL responses against the specific antigen in all animals. In the cynomolgus macaques the Gag/Pol vaccination did not result in decreased viremia after challenge compared with controls. In the animals vaccinated with Tat/Rev, however, two animals with the highest Tat-CTL response had no detectable viremia and the other 2 animals, with lower levels of Tat- and Rev-CTL, had a shorter and lower viremia compared with Gag/Pol vaccinated or control animals [31].

These vaccines, which each contain 2 proteins expressed at similar levels, result in different protection levels. Therefore, the breadth of the immune response is probably not the only explanation, although it may still contribute to a higher antiviral pressure. What makes vaccines based on the small Tat and Rev proteins more potent? Both are small regulatory proteins, essential for virus replication [32,33]. Their open reading frames overlap in part with each other and with the envelope. This may limit the number of mutations that are tolerated and consequently the possibilities to escape CTL-mediated pressure. Allen et al. found that SIV replication was best controlled in macaques that mounted CTL responses against Tat. Nevertheless, rapid accumulation of mutations in Tat epitopes was

found (Rev was not analysed), when Tat-CTL became detectable during primary SIV viremia [34]. At the same time Gag-CTL were detected but their presence did not lead to mutations in their specific epitopes. Therefore it was concluded that Tat-CTL put more pressure on the virus than Gag-CTL.

5. Early expression as an explanation

What determines the pressure exerted by CTL of different specificity on HIV- or SIV-infected cells? We recently developed, in collaboration with M van Baalen, a model in which we addressed 2 main components of CTL pressure [35]. The first factor (α) is defined by the CTL and describes the rate at which CTL eliminate target cells, which depends on number of CTL present. It is difficult to understand why Tat-CTL would generally have a higher factor α than Gag-CTL. One could argue that more potent CTL are induced because Tat is expressed at lower levels than Gag. But other antigens like RT are also expressed at low levels and RT-CTL have not been found to put high pressure on the virus. The other factor is the time (a_V) at which infected cells become vulnerable, which determines when and how long CTL can attack virus infected cells. Because Tat and Rev are expressed early in the replication cycle of the virus, the timing of recognition may be a better explanation for the results in the various experiments described above.

It may seem difficult how a small difference in recognition time can have large consequences. Taking values from literature Klenerman et al calculated that CTL against an early protein on average have 3.2 days to trace and kill an infected cell, whereas CTL against a late protein have a mean of 2.6 days [36]. We developed a dynamical model, based on the static model of Klenerman, which simulates CTL-target cell interactions over several generations [35]. The small difference in CTL pressure observed in a single round of interaction then acummulates and rapidly becomes important. Early recognition becomes a critical factor, because more cells are eliminated before the production of infectious virus particles starts. Thus, less new infections can occur and spreading of virus is controlled more effectively [35]. The predictions of the model confirm the results from in vitro co-cultures, where Env- and Rev-CTL can suppress virus production completely and Gag and RT-CTL only partially [35,37]. However different CTL clones may also have differences in the antiviral factor α, as has been described [38]. Therefore, HIV recombinants with Nef-associated early expression of the late RT epitope have been constructed [39]. Cells infected with these recombinants have been co-cultured with the same RT-CTL (which excludes α as a variable) and have confirmed the importance of early recognition for CTL effectiveness [35].

6. Consequences for in vivo observations

These findings may help to explain the difficulties that have been met when searching for escape to dominant CTL responses in vivo (see [40] for an excelent review). Whereas pressure by anti-virals invariably, and even reproducibly, results in escape, CTL pressure rarely seems to do so. In most studies HIV containing the index epitope could replicate, despite the presence of high numbers of virus-specific CTL (see table 1). Only few patients acquire mutations in the epitope, which escape CTL recognition by the dominant Gag-CTL population present [41,42]. Furthermore, this usually only takes place after years of infection and virus replication under CTL

Table 1: Evolution of CTL epitopes in the presence of their cognate CTL in vivo

Protein	epitope	MHC	stage	escape	ref
late					
Gag	KRWIILGLNK	B27	chronic	no	Meyerhans [47]
	GGKKQYKL	B8	acute	no	Price [13]
	SLYNTVATL	A0201	chronic	no	Goulder [43]
	SLYNTVATL	A0201	chronic	no	Brander [48]
	SLYNTVATL	A0201	chronic	no	Kostense [49]
	EIYKRWII	B8	chronic	no	Kostense [49]
	IRLRPGGKK	B27	transmission	no	Goulder [44]
	KRWIILGLNK	B27	chronic/late	yes 2/6	Goulder [41]
	KRWIILGLNK	B27	chronic	yes 5/12	Kelleher [42]
Pol	SPAIFQSSM	B7	acute/chronic	no	Hay [50]
intermediate					
Env	RPNNNTRKSI	B7	chronic	yes	Wolinsky [51]
	SFNCGGEFF	Cw4	chronic	yes	Wolinsky [51]
	AENLWVTVY	B44	acute	yes	Borrow [10]
	IPRRIRQGL	B7	acute/chronic	no	Hay [50]
	several epitopes	Mamu	chronic	yes	Evans [52]
early					
Nef	QVPLRPMTYK	A3.1	transfer/acute	yes	Koenig [53]
	several epitopes	A2/B7	chronic	yes	Haas [12]
	FLKEKGGL	B8	acute	yes	Price [13]
	several epitopes	Mamu	chronic	yes	Evans [52]
Tat	STPESANL	Mamu A01	acute	yes	Allen [34]

pressure. In other people, with the appropriate MHC haplotype, escape mutations in the major Gag epitopes have been found [43,44]. However, these mutations occured in the absence of detectable CTL effectors against the epitope. It was explained as a result of fixation after CTL pressure, although no direct evidence could be given.
Several other explanations have been put forward to explain the absence of CTL pressure and virus escape, inculding MHC downregulation by viral proteins, killing or dysfunction of CTL by HIV-infected cells and sequestration of infectious virions [7]. Furthermore mutations may be difficult to tolerate in certain epitopes, although mutations have been observed in conserved epitopes [43,44]. Therefore, we favor the explanation that CTL pressure by late CTL is limited and that escape may occur, but virus can continue to replicate despite these CTL.

By contrast, escape is readily observed in other studies of HIV infection and after experimental SIV infection (see table 1). In all these cases CTL were directed against early (Nef, Tat) or intermediate (Env) proteins. In a xeno-GvHD mouse model, we observed that, under pressure of Rev-CTL, HIV remained undetectable unless escape by mutaion occured [45]. Furtermore, after infection of macaques with a clonal SIV

isolate, CTL escape was observed in Tat, and to a lesser extent in Rev Env and Vpr, all early or intermediate proteins [46].

7. Conclusions

We propose that early recognition is a critical factor in CTL effectiveness against HIV. It puts higher pressure on the virus because more infected cells can be elimated, before progeny virus is released. In natural infection, the CTL pressure has been deduced from mutations in their specific epitopes. Epitopes in early and intermediate proteins accumulate mutations faster and more frequently than epitopes from late proteins. These mutations often deminish or abolish CTL recognition, which reduces CTL pressure. Pre-existing and broad cellular immune responses against early (and to a lesser extent ,intermediate) proteins may therefore give better immune control of HIV infection resulting in lower viral loads. This is supported by results of preliminary vaccinations experiments. Therefore we emphasize that vaccination strategies should include early antigens, especially Tat and Rev.

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CHAPTER 6

Summary

6.1. Summary

Most HIV-1 infected individuals develop AIDS, in absence of antiviral drug therapy, in about 10 years. A proportion of untreated infected individuals (~10%) develops AIDS within three years, and approximately 10% have no signs of disease progression even after 12 to 15 years. If HIV-1 is not eliminated early after primary viremia, a dynamical balance develops between propagation of virus in activated immune cells on the one hand, and induction and maintenance of active immune cells on the other. Slower rates of disease progression correlate with lower levels of virus in plasma after primary viremia, and reduction of virus results, at least in part, from virus-specific cell-mediated and humoral immune responses. Characteristics of these responses in the few infected individuals that do not, or slowly progress to AIDS, may hold clues for developing effective vaccination strategies.

The focus of this thesis is on characteristics of cytotoxic T lymphocyte (CTL) responses. With their T cell receptor (TCR), these cells can recognize fragments of viral proteins that are presented by molecules of the class I human leukocyte antigen (HLA) complex on the surface of antigen presenting or infected cells. If mature CTL receive an appropriate signal, they can reduce HIV production by releasing factors that lyse infected cells or inhibit virus production in non-lytic ways. Inhibition should occur before a significant number of progeny virions is released, in order to control reproduction and propagation of virus. The level of control that can be achieved by CTL, will depend on the rate at which they are induced, expand and migrate to infected sites, on the number of effector cells and their per cell capacity to prevent new infections, and on possibilities of the virus to escape. The earlier these factors, in combination, balance the rate of virus production and propagation, the better infection will be controled.

Several aspects of CTL responses in relation to rates of disease progression are described in **chapter 2**. In long-term asymptomatics, persistent HIV-1 Gag-specific CTL responses and very low numbers of HIV-1-infected $CD4^+$ T cells coincided with normal and stable $CD4^+$ cell counts and preserved CD3 mAb-induced T cell reactivity for more than 8 years (**§2.1**). In five out of six rapid progressors Gag-specific CTLp were also detected. However, early in infection the number of circulating HIV-1-infected $CD4^+$ T cells increased despite strong and mounting Gag-specific CTL responses.

Other studies have indicated that, later in infection, Gag-specific CTL do exert antiviral pressure. This was inferred from the outgrowth of virus variants that escaped recognition by mutations in or around epitope encoding regions in the viral genome. Escape by mutations in the viral envelop proteins and Nef has also frequently been observed (**§5.2**). Thus, to retain their antiviral capacity, CTL should be directed against sequences in which variation is not easily tolerated. Such sequences are most likely conserved among virus variants. Fine-specificities of CTL which recognize conserved epitopes derived from Gag were studied in seven long-term asymptomatic individuals (**§2.2**). Several specificities identified corroborated results from other studies and the minimal sequence of the first HLA-A25 restricted CTL epitope, derived from p24, was defined as $p24_{203\text{-}212}$ ETINEEAAEW. This sequence predominates in clade B and D virus strains, while viral sequences from other clades differ frequently at the 1st, 2nd, and 9th residue. Corresponding variant peptides could not be recognized by CTL directed against the index sequence,

suggesting that viruses may escape if they mutate accordingly. However, no variation in this epitope sequence was observed in viruses isolated at 84 and 90 months follow-up of the individual with $p24_{203-212}$-specific CTL. The viral genomes analysed showed considerable variation in the variable regions 1, 2 and 3 of the viral envelop. Thus structural constraints may limit variation in $p24_{302-212}$ of clade B and D viruses, and CTL directed against this sequence may have been instrumental in delaying disease progression. An alternative explanation would be that the CTL directed against this epitope exerted little pressure on reproduction of the virus, and other factors accounted for the long-term asymptomatic period.

The protein specificity of CTL responses in rapidly or slowly progressing individuals showed striking differences during the early stages of infection (**§2.3**). Precursor CTL (CTLp) directed against the proteins Rev and Tat, which both have indispensable regulatory functions and are produced early after virus enters a cell, were prevalent in individuals with no or slow disease progression. Simultaneously analyzed samples obtained from more rapidly progressing individuals, contained CTLp against Gag, RT, Env and Nef in frequencies similar to those found in long-term asymptomatics, but levels of Rev- or Tat-specific CTL were undetectable or low. These findings support the hypothesis that the latter are better able to control HIV by virtue of their specificity for early viral proteins. This neither implies that they are expected to prevent AIDS entirely in all individuals, nor that CTL against other proteins do not affect virus production or that the potential to operate early always leads to better control. In fact, if Rev- and Tat-specific CTL contributed to slower disease progression this could be due to a mere broadening of the CTL response, which has been suggested to be beneficial in itself.

Previous studies indicate that the time required for virus infected cells to become vulnerable for the activity of CTL, can be of significance for the capacity of CTL to control viral reproduction. Whether this applies to the effectiveness of HIV-1 specific CTL was the central theme of the studies presented in **Chapter 3**. During the first 36 hours following infection of non-immortalized $CD4^+$ T cells with HIV-1IIIB (at a high multiplicity of infection), CTL directed against Rev or RT inhibited viral protein production in infected cells that could not yet be lysed (**§3.1**). By 48 hours they had prevented >97% and ~92% of virus release, respectively, as determined by quantification of extracellular RT-activity. This small difference in effectiveness between the two CTL populations early after infection was shown to increase significantly in experiments designed to allow the residual virus to start new rounds of infection (**§3.4**). Cultures were initiated at a low multiplicity of infection, i.e. containing mostly uninfected susceptible cells, and Rev-specific CTL had prevented at least 2 $\log_{10}$ more virus production than similar numbers of RT-specific CTL by day 10. This difference was not compensated for by ten-fold more RT-specific cells.

Several mechanisms that could explain these results were addressed (see **§3.4** and **§5.1**). To assess the contribution of antigen production kinetics independently from variability in the potency of effector mechanisms of CTL populations of different protein specificity, a recombinant virus was constructed that encoded the minimal RT-epitope in the early expressed *nef* gene (**§3.3**). Reproduction of this virus remained undetectable in presence of the RT-specific CTL for 10 days. That they could not efficiently control reproduction of the parental virus can therefore not be explained by insufficient effector mechanisms of the CTL, but is most probably due

to their inability to attack enough infected cells before progeny virus has started new infection cycles.

To study the contribution of parameters that cannot be independently assessed *in vitro*, we developed a mathematical model that describes CTL-target cell interactions and viral reproduction dynamics (**§3.4**). The results show that if CTL eliminate infected T cells from the reproduction cycle with an exponential decay rate of approximately 2 to 3 per day, they can control the infection, provided that infected cells become vulnerable within approximately 16 hours after infection. After that time, the target cell elimination rate, e.g. the effector cell number, has to increase considerably as there is less time before release of progeny virus begins. Together, the results presented in **§3.1**, **§3.3** and **§3.4** indicate that CTL can control reproduction more effectively if they are able to recognize infected cells earlier after viral entry.

The high capacity of Rev-specific CTL to control reproduction of HIV-1 also became evident by adoptive transfer into irradiated mice that had been reconstituted with a high dose of human peripheral blood mononuclear cells and were infected with molecularly cloned primary isolates of HIV-1 (**§3.2**). Six days after transfer, no infected cells were detected unless virus had mutated in the minimal epitope sequence. Furthermore, a macrophage-tropic HIV-1 clone escaped more efficiently from specific CTL pressure than its non-macrophage-tropic counterpart. Thus CTL may favor the selective outgrowth of macrophage-tropic HIV-1 variants also in cases where the mucosa is bypassed as port of entry during transmission, e.g. after blood transfusion, needle-stick accidents, or intravenous drug abuse. Together, the studies in chapter 3 indicate that CTL-mediated control of virus reproduction would be most effective if the CTL recognize epitopes derived from early viral proteins in which variation is not tolerated. In this respect Rev and Tat may be more efficacious than Nef because the open reading frames of *rev* and *tat* overlap each other and *env*, while *nef* does not overlap other open reading frames.

Although 20-30% of infected individuals mount detectable CTL responses against Rev and Tat, these small regulatory proteins appear to be less immunogenic than the structural proteins. Some individuals may lack specific HLA molecules that can present epitopes encoded by *rev* and *tat* genes of the infecting virus variants. But even if CTL can respond to the infecting virus, it may take years before they are induced and detectable. Studies on immunodominance indicate that it is not a static poperty, but depends in part on the immunologic context that forms after infection.

Based on the studies presented in chapters 2 and 3, it is reasonable to expect that vaccination with Rev and Tat, before infection or during therapy, may induce or boost CTL responses that control virus replication more effectively than those induced by intermediate or late proteins. Studies on this issue are in presented in **chapter 4**. A clear beneficial effect of vaccination with Rev and Tat was observed during 36 weeks of follow-up after infection of macaques with SIV (**§4.1**). Variable degrees of success in controling primary viremia have also been reported with other SIV antigens. But because animals, viruses and vaccination strategies differ significantly among these studies, they do not allow conclusions on the relative success of early versus late proteins. In the study presented in (**§4.2**) we directly compared the ability of four macaques vaccinated with recombinant viral vectors expressing Rev and Tat to control primary SIV infection, with that of four macaques vaccinated with Gag and Pol by the same approach. CTL responses against vaccine antigens were detected prior to infection, but the Gag/Pol vaccination did not

enhance control of viremia. By contrast, in two Tat/Rev vaccinated animals, plasma viral loads remained below the limit of detection, and the other two had shorter and lower viremia compared with the Gag/Pol vaccinated or control animals. Considering the number of proteins, their size and levels of expression, it is unlikely that the higher protection level induced by the Rev/Tat vaccine was due to more broadly directed immune responses.

In conclusion, the studies on CTL responses in rapidly and slowly progressing HIV-1 infected individuals (**chapter 2**), combined with the studies on the capacity of CTL to control viral reproduction *in vitro, in vivo* and *in silico* (**chapter 3**), and the vaccination studies in the SIV macaque model (**chapter 4**), provide rationale (discussed in **chapter 5)** for immunization strategies that aim at inducing, boosting or skewing CTL responses to the early regulatory HIV proteins Rev and Tat in AIDS vaccine development.

6.2. Samenvatting

De meeste HIV-1 geïnfecteerde mensen ontwikkelen zonder behandeling met antivirale middelen na ongeveer 10 jaar AIDS. Een aantal (~10%) krijgt, zonder behandeling, binnen drie jaar AIDS, terwijl ongeveer 10% zelfs na 12 tot 15 jaar geen symptomen heeft ontwikkeld. Langzame ziekteontwikkeling correleert met lage hoeveelheden virus in het bloed, en reductie van de hoeveelheid virus wordt, ten minste voor een deel, veroorzaakt door virusspecifieke afweerreacties. Indien het virus niet kort na infectie opgeruimd wordt, vormt zich een dynamische balans tussen virusvermenigvuldiging in geactiveerde $CD4^+$ afweercellen aan de ene kant, en activatie en instandhouding van geactiveerde afweercellen aan de andere. Onderzoek naar karakteristieken van afweerreacties bij HIV-geïnfecteerde mensen die niet, of heel langzaam, ziek worden zou informatie kunnen opleveren voor het ontwikkelen van doeltreffende vaccinatie strategieën.

De aandacht in dit proefschrift is vooral gericht op karakteristieken van de responderende cytotoxische T lymfocyten (CTL). Deze witte bloedcellen hebben een T cel receptor (TCR) waarmee zij fragmenten (epitopen) van virale eiwitten kunnen herkennen als deze gepresenteerd worden door moleculen van het klasse I histocompatibiliteitsantigenen complex ('human leukocyte antigen', HLA) op de buitenkant van geïnfecteerde cellen. Indien CTL een passend signaal ontvangen kunnen zij virusproductie onderbreken door factoren uit te scheiden die geïnfecteerde cellen doden of die virusproductie in de cel remmen. Om doeltreffend te zijn moet deze onderbreking plaatsvinden voordat de hoeveelheid nieuw virus voldoende is om de populatie geïnfecteerde cellen in stand te houden. De mate waarin CTL nieuwe infectieronden kunnen voorkomen zal mede afhangen van de snelheid waarmee zij geïnduceerd worden, in aantal toenemen, en migreren naar plaatsen waar virus zich vermenigvuldigd. Tevens zal het vermogen per CTL om nieuwe infecties te voorkomen, en de mogelijkheden van het virus om aan herkenning te ontsnappen een rol spelen. Hoe eerder deze factoren gezamelijk de hoeveelheid virus en de snelheid van virusverspreiding verminderen, hoe beter de infectie gecontroleerd kan worden.

Na een algemene inleiding in **hoofdstuk 1** worden in **hoofdstuk 2** diverse aspecten van CTL responsen beschreven in relatie tot de snelheid van ziekteontwikkeling. Bij mensen met langzame ziekteontwikkeling blijft gedurende meer dan 8 jaar het aantal CTL gericht tegen het virale eiwit Gag langdurig op peil (**§2.1**). Het aantal HIV-geïnfecteerde $CD4^+$ T cellen bleef laag en het aantal T helper cellen en de T cel reactiviteit stabiel en normaal. In vijf van de zes onderzochte mensen met een snelle ziekteontwikkeling werden echter ook CTL gericht tegen Gag gevonden. Ondanks sterke en toenemende CTL responsen steeg het aantal HIV-geïnfecteerde $CD4^+$ T cellen ook vroeg na infectie.

Andere studies hebben laten zien dat, met name later in de infectie, Gag-specifieke CTL wel degelijk antivirale druk kunnen uitoefenen. Dit werd afgeleid uit de toename van virusvarianten met genetische veranderingen in of rond de gebieden die door de CTL herkend werden. Dit type van ontsnapping is ook gevonden voor CTL die fragmenten van de virale envelop (Env) en Nef eiwitten kunnen herkennen (**§5.2**). Om hun antivirale vermogen te behouden zouden CTL daarom idealiter gericht moeten zijn tegen virale eiwitfragmenten die niet kunnen veranderen zonder nadelige effecten voor het virus. Het ligt voor de hand dat dergelijke epitopen

geconserveerd zijn in het genoom van verschillende virusvarianten, en identificatie hiervan kan bijdragen aan het ontwikkelen van effectieve vaccins.

In zeven mensen met langzame ziekteontwikkeling werd de epitoop-specificiteit onderzocht van CTL die geconserveerde delen in Gag herkennen (**§2.2**). Naast een aantal eerder beschreven epitopen werd een nieuw epitoop geïdentificeerd en gekarakteriseerd: $p24_{203\text{-}212}$ ETINEEAAEW, het eerst beschreven epitoop waarvan aangetoond werd dat het door HLA-A25 moleculen gepresenteerd kan worden. Dit epitoop komt vooral voor in de HIV stamgroepen B en D, terwijl sequenties in andere stamgroepen vaak verschillen in het 1e, 2e en 9e residu. Epitopen met de beschreven variaties werden niet herkend door CTL gericht tegen de oorspronkelijke variant. Het virus zou dus kunnen ontsnappen door op overeenkomstige wijze te veranderen. Er werd echter geen variatie gevonden in het oorspronkelijke epitoop van virus dat geïsoleerd werd op 84 en 90 maanden na begin van de studie van de persoon met $p24_{203\text{-}212}$-specifieke CTL. De virale genomen varieerden wel in andere gebieden. Deze gegevens kunnen betekenen dat virussen van de stamgroepen B en D niet kunnen veranderen in de regio $p24_{203\text{-}212}$, en dat de CTL die dit epitoop herkennen langdurig hebben kunnen bijdragen aan het vertragen van ziekteontwikkeling. Een alternatieve verklaring kan zijn dat deze CTL slechts weinig antivirale druk uitoefenden, waardoor er geen selectie van varianten plaats vond. In dat geval hebben waarschijnlijk andere factoren aan de langzame ziekteontwikkeling bijgedragen.

In een studie naar het aantal en de aard van de virale eiwitten waartegen zich vroeg na infectie een CTL respons ontwikkelde (**§2.3**), bleek dat er verschillen waren voor snelle en langzame ziekteontwikkeling. Naast CTL responsen gericht tegen de virale eiwitten Gag, RT, Env en Nef, die vergelijkbaar waren tussen de twee groepen, werden CTL responsen gericht tegen Rev en Tat vrijwel uitsluitend gevonden in associatie met langzame ziekteontwikkeling. Rev en Tat behoren tot de eerste eiwitten die in geïnfecteerde cellen gemaakt worden en reguleren de productie van nieuwe virale genomen en eiwitten, waaronder Gag, RT en Env. Het is daarom aannemelijk dat het vermogen van Rev- en Tat-specifieke CTL om de reproductie van HIV te beperken groter is dan dat van andere CTL, omdat zij eerder zouden kunnen ingrijpen tijdens het proces dat nieuwe generaties infectieus virus voortbrengt. Dit hoeft niet te betekenen dat zij AIDS volledig kunnen voorkomen in alle geïnfecteerde mensen, en ook niet dat CTL gericht tegen andere eiwitten geen antiviraal effect hebben, of dat de potentie om vroeg in te kunnen grijpen altijd doeltreffender is (Nef is bijvoorbeeld ook een vroeg eiwit). Het zou zelfs zo kunnen zijn dat de bijdrage van Rev- en Tat-specifieke CTL aan een doeltreffender controle van de infectie zich beperkt tot een verbreding van CTL respons (meer verschillende epitopen) die op zichzelf gunstig zou kunnen zijn.

Uit recente studies met andere virussen is gebleken dat het antivirale vermogen van CTL groter is naarmate zij geïnfecteerde cellen eerder na binnenkomst van het virus kunnen uitschakelen. De studies in **hoofdstuk 3** hebben betrekking op het antivirale vermogen van HIV-specifieke CTL. In het algemeen is hierover nog weinig bekend. Gedurende de eerste 36 uur na infectie van $CD4^+$ T cellen met HIV-1 werd productie van virale eiwitten onderdrukt door CTL gericht tegen Rev (vroeg) en RT (laat) zonder dat zij de geïnfecteerde cellen konden lyseren (**§3.1**). Na 48 uur hadden ze de productie van respectievelijk >97% en ~92% nieuwe virusdeeltjes verhinderd.

Dit kleine verschil in effectiviteit tussen de twee CTL populaties vroeg na infectie bleek vervolgens sterk toe te nemen wanneer de nieuwe virusdeeltjes nieuwe infectieronden konden beginnen (**§3.4**). Na 10 dagen hadden Rev-specifieke CTL ten minste 100 keer meer virusproductie verhinderd dan een vergelijkbare hoeveelheid RT-specifieke CTL. Dit verschil werd niet opgeheven door met 10 keer meer RT-specifieke CTL te beginnen.

Verschillende mechanismen die deze resultaten zouden kunnen verklaren werden onderzocht (**§3.4** en **§5.1**). Om de bijdrage van de kinetiek (vroeg vs. laat) van antigeenproductie te onderzoeken in afwezigheid van andere verschillen tussen CTL populaties, werd een recombinant virus gemaakt dat het RT-CTL epitoop ook vroeg kan aanmaken, gelijktijdig met het Rev-CTL epitoop. Dit virus werd door de RT-specifieke CTL minstens zo effectief onderdrukt als het oorspronkelijke virus door Rev-specifieke CTL. Deze resultaten tonen aan dat de beperkte effectiviteit van de RT-specifieke CTL tegen het oorspronkelijke virus niet verklaard kan worden door onvoldoende efficiëntie van de antivirale effector mechanismen van de CTL. Waarschijnlijker is dat zij onvoldoende geïnfecteerde cellen kunnen uitschakelen voordat er nieuwe infecties plaatsvinden, vanwege hun specificiteit voor een eiwit waarvan de productie relatief snel gevolgd wordt door het vrijkomen van nieuwe virusdeeltjes.

Er werd een mathematisch model ontwikkeld om de bijdrage te onderzoeken van parameters die *in vitro* niet onafhankelijk van elkaar gemeten kunnen worden (**§3.4**). Dit model beschrijft de virale reproductie dynamiek en interacties tussen CTL en geïnfecteerde cellen. De resultaten tonen aan dat CTL de voortdurende reproductie van HIV kunnen uitdoven wanneer zij geïnfecteerde cellen verwijderen uit de reproductiecyclus met een exponentiele vervalsnelheid van 2 tot 3 per dag. Hiertoe moeten ze dan wel binnen 16 uur na infectie kunnen beginnen. Als geïnfecteerde cellen pas later gevoelig worden moet de verwijderingsnelheid sterk toenemen, bijvoorbeeld door een sterk toenemend aantal CTL, naarmate er minder tijd is voordat er nieuwe virusdeeltjes vrijkomen. Gezamenlijk geven de in **§3.1**, **§3.3** en **§3.4** beschreven studies aan dat HIV-specifieke CTL virale reproductie doeltreffender kunnen beperken naarmate zij geïnfecteerde cellen eerder na de start van virale replicatie kunnen herkennen.

Het grote vermogen van Rev-specifieke CTL om HIV reproductie te beperken werd ook duidelijk in experimenten waarbij de CTL werden geïnjecteerd in bestraalde muizen die na de bestraling een hoge dosis humane perifere bloed mononucleaire cellen hadden gekregen en geïnfecteerd waren met HIV (**§3.2**). Zes dagen nadat de CTL werden toegediend werd geen virus meer terug gevonden, tenzij er virusvarianten met een veranderd CTL epitoop waren ontstaan. Verder werden veranderde CTL epitopen vaker gevonden als het virus naast T cellen ook macrofagen kon infecteren. Deze resultaten geven aan dat CTL mogelijk een rol spelen bij de selectie van macrofaag-trope virussen ten opzichte van niet-macrofaag-trope virussen, die in veel geïnfecteerde mensen gevonden is.

De resultaten beschreven in **hoofdstuk 3** geven aan dat de antivirale werking van CTL het meest effectief zal zijn indien zij gericht zijn tegen epitopen die afkomstig zijn van vroege virale eiwitten en die niet of moeilijk kunnen veranderen. In dit opzicht is het niet onverwacht dat Rev- en Tat-specifieke CTL effectiever blijken te zijn dan Nef-specifieke CTL; de mogelijkheden voor veranderingen in het *rev* gen en

het *tat* gen zijn meer beperkt doordat zij elkaar en het *env* gen overlappen, terwijl het *nef* gen geen ander gen overlapt.

Hoewel 20-30% van de geïnfecteerde mensen meetbare CTL responsen gericht tegen Rev en Tat aanmaken, lijken deze kleine regulatoire eiwitten minder immunogeen te zijn dan de grote structurele eiwitten. Sommige mensen missen wellicht specifieke HLA moleculen die fragmenten van de Rev en Tat eiwitten van de infecterende virusstammen kunnen presenteren. Maar zelfs in gevallen waar CTL het binnenkomende virus wel kunnen herkennen, kan het jaren duren voordat ze geïnduceerd worden en meetbare hoeveelheden bereiken. Studies naar mechanismen die de immunodominantie van bepaalde antigenen kunnen verklaren, geven aan dat het niet een statische eigenschap van antigenen is, maar dat het deels afhangt van de immunologische context die zich na een infectie vormt.

Gebaseerd op de voorgaande studies en overwegingen kan verwacht worden dat vaccinatie met Rev en Tat zal bijdragen aan het induceren of versterken van CTL responsen die doeltreffender zijn dan vaccin-geïnduceerde CTL responsen gericht tegen late virale eiwitten. In **hoofdstuk 4** worden twee studies beschreven waarbij makaken gevaccineerd werden en vervolgens geïnfecteerd met simian immunodeficiëntie virus (SIV). Uit de eerste studie bleek dat vaccinatie met Rev en Tat een beschermend effect had gedurende de 36 weken na infectie waarin de dieren gevolgd werden (**§4.1**). Door andere onderzoekers is aangetoond dat vaccinatie met andere antigenen ook beschermend kan werken. Maar de resultaten van de verschillende studies kunnen niet gebruikt worden om conclusies te trekken ten aanzien van het relatieve succes van de verschillende antigenen; er zijn namelijk ook grote verschillen in gebruikte virusstammen, diersoorten en vaccinatiestrategieën. Daarom werd in de studie beschreven in **§4.2** het effect van vaccineren met Rev en Tat (4 dieren) direct vergeleken met dat van vaccineren met Gag en Pol (4 dieren), waarbij productie en toediening van vaccins en de daaropvolgende infectie met SIV op dezelfde wijze uitgevoerd werden bij alle dieren. Vóór infectie werden CTL responsen gevonden tegen de vaccin-antigenen, maar de Gag/Pol vaccinatie leverde qua bescherming geen duidelijk voordeel op in vergelijking met de controle vaccinatie (4 dieren). Daarentegen kon bij twee dieren in de Rev/Tat gevaccineerde groep geen virus in het plasma worden aangetoond, één dier had enkele korte periodes met lage virus hoeveelheden en één was vergelijkbaar met de beste van de controle dieren. Uitgaande van het aantal eiwitten, hun grootte en productieniveau is het niet waarschijnlijk dat de betere bescherming door het Rev/Tat vaccin veroorzaakt werd door breder gerichte immuun responsen.

In conclusie, CTL gericht tegen HIV of SIV eiwitten die vroeg na binnenkomst van het virus in geïnfecteerde cellen aangemaakt worden, waren in tegenstelling tot CTL gericht tegen late virale eiwitten, geassocieerd met een betere prognose, een effectievere beperking van voortdurende virusreproductie en een grotere capaciteit om nieuwe infecties onder controle te brengen. Er werden diverse aanwijzingen gevonden voor - en vooralsnog geen tegen - de hypothese dat CTL gericht tegen genoemde vroege virale eiwitten effectiever zijn doordat zij meer geïnfecteerde cellen kunnen uitschakelen voordat er nieuwe generaties virusdeeltjes en geïnfecteerde cellen ontstaan. De resultaten pleiten ervoor om bij de ontwikkeling van vaccins tegen AIDS te investeren in immunisatiestrategieën die leiden tot inductie en versterking van CTL responsen gericht tegen de vroege regulatoire HIV eiwitten Rev en Tat.

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Curriculum vitae

De auteur van dit proefschrift werd geboren op 23 april 1965 te Utrecht. In 1985 werd het diploma VWO behaald aan het Montessori Lyceum te Amsterdam. In datzelfde jaar werd gestart met de studie Biologie aan de Universteit Utrecht. Tijdens de specialisatiefase werden twee stages uitgevoerd: moleculaire microbiologie bij de afdeling Moleculaire Microbiologie van de Universiteit Utrecht (Prof.dr. W.P.M. Hoekstra) en immunologie bij het Laboratorium voor Immunobiologie van het Rijksinstituut voor Volksgezondheid en Milieu, RIVM (Prof.dr. A.D.M.E. Osterhaus). Het doctoraal examen werd behaald in 1991. Als wetenschappelijk medewerker van het Laboratorium voor Immunobiologie van het RIVM werd in 1991 gestart met het onderzoek aan cytotoxische T cel responsen gericht tegen HIV. Dit onderzoek werd in 1994 voortgezet als wetenschappelijk medewerker van de afdeling Virologie van de Erasmus Universiteit Rotterdam onder leiding van Prof.dr. A.D.M.E Osterhaus en Dr. R.A. Gruters.

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