Role of cytotoxic T lymphocyte responses in the control of primate immunodeficiency viruses

De rol van cytotoxische T cel responsen in the controle van primaten immunodeficientie virussen

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Overige leden: Prof. Dr E. Claassen
Prof. Dr H.A. Verbrugh
Dr B. Wahren

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Per Benedict

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1.3 Can the immune response control HIV infection?

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Kinetics and specificities of the T helper-cell response to gp120 in the asymptomatic stage of HIV-1 infection. *Scandinavian Journal of Immunology (1994) 39:355-362.*

Towards an HIV-1 vaccine: lessons from macaque models. *Vaccine, invited review. (In press).*

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Properties of Primate Lentiviruses

The acquired immunodeficiency syndrome (AIDS) was recognised as a new clinical entity in 1981, when an unusual clustering of Kaposi's sarcoma and Pneumocystis carinii pneumonia was observed in young homosexual males. Epidemiological studies implicated an infectious agent transmitted during sexual intercourse, through intravenous drug abuse, by blood and blood products, and vertically from mother to child (1). The first isolation from an AIDS patient of a retrovirus later designated human immunodeficiency virus type I (HIV-1) (Table 1) was made in 1983 by Luc Montagnier and Barre-Sinoussi (at the Pasteur Institute in Paris) and confirmed by Robert Gallo (at the National Institute of Health in Bethesda, USA) immediately thereafter (2). In 1985, simian immunodeficiency viruses (SIV) were isolated from captive Asian macaques with a disease closely resembling human AIDS (3). These viruses include SIV from rhesus macaques (SIVmac), nemestrina macaques (SIVmne) and stump-tailed macaques (SIVstm). A second human immunodeficiency virus (HIV-2) was isolated in 1986 from mildly immunocompromised patients in West Africa; although both HIV-1 and HIV-2 cause AIDS, individuals infected with HIV-2 exhibit a longer clinical latency and lower morbidity (4). Additional genetically distinct SIV strains have been isolated in Africa from several nonhuman primate species, including African green monkeys (SIVagm), sooty mangabeys (SIVsmm), mandrils (SIVmnd) and sykes (SIVsyk). In addition, a close relative of HIV-1, SIVcpz, has been found in chimpanzees. These simian viruses are endemic to the respective African monkey species and do not appear to induce disease in their natural host (5). Remarkably, certain SIV strains share a high degree of sequence homology with several HIV-2 isolates from West Africa (6), whereas HIV-1 is more similar to SIVcpz (7) (Figure 1). Although at present the origin of HIV-1 and HIV-2 remains obscure, phylogenetic analyses suggest that both viruses arose from multiple zoonotic transmissions between nonhuman primates and humans of lentiviruses originated from a common ancestor (8).

Classification

HIV and SIV are members of the lentivirus genus of the *Retroviridae* family by virtue of their morphologic and biochemical characteristics, Mg^{2+}-dependent reverse transcriptase activity, genomic RNA organization, and genetic homology. Lentiviruses are not oncogenic; rather, they cause persistent infections resulting in a variety of chronic conditions with insidious onset and slow progression (Latin *lentus*: slow). Human lentiviruses are grouped into two types, HIV-1 and HIV-2, on the basis of serologic and genetic properties (9). Due to the extreme plasticity of the HIV genome, a high degree of variability exists among independent isolates. The greatest sequence variation, consisting in nucleotide changes, as well as small deletions and

Table 1. *Classification of retroviruses infecting primates*

Subfamily	Disease
Oncovirinae	
HTLV-I	Adult T-cell leukaemia, lymphoma, tropical spastic paraparesis (humans)
HTLV-II	Hairy cell leukaemia (humans)
Spumavirinae	Unapparent persistent infection (primates and other animals)
Lentivirinae	
HIV-1	AIDS. The virus can infect chimpanzees.
HIV-2	AIDS. Less pathogenic than HIV-1. The virus can infect primates.
SIV	Simian AIDS in Asian monkeys. No disease in natural hosts.

HTLV: Human T-cell leukaemia virus

insertions, is displayed by the *env* gene, which encodes the envelope glycoprotein (Env) (10). Classification based mostly on *env* sequences identifies nine genetic subtypes or clades (A through I) and a more divergent set of strains (the O group) of HIV-1, and five subtypes of HIV-2 (A through E) (6, 11). Some areas of the world harbor predominantly a single subtype, whereas two or more subtypes may be prevalent in other populations, reflecting different patterns of virus migration (12). The B subtype of HIV-1 is endemic to the developed countries of North America and Europe, but is rarely found in those African and Asian countries where HIV-1 infection is spreading at alarming rates (11). Up to 25% of the amino acids encoded by *env* may vary in HIV-1 strains from geographically separated locations (interpatient variation). Emergence of *env* variants also occurs in HIV-infected individuals and SIV-infected monkeys (intrapatient variation) to produce populations of closely related but distinct viral genomes designated quasispecies (13). Furthermore, there is evidence that a significant proportion of HIV-1 strains are the result of recombination events between viruses of distinct subtypes (14).

The high degree of variability displayed by Env is believed to be the product of at least two selective pressures: adaptation to infection of different cell types and escape from antiviral immune responses (15, 16). Amino acid differences in Env appear to have significant implications for certain critical functions such as CD4 binding, cell tropism, fusogenicity and cytopathicity (1). Evidence has been provided that while initial HIV-1 infection may be limited to macrophage-tropic virus, usually incapable of causing syncytia in vitro (nonsyncytium-inducing variants), over the course of infection subtly altered viruses emerge showing increased tropism for lymphocytes and syncytium-inducing ability (17). Amino acid differences in Env underlie many of these progressive changes in virus phenotype and tropism, whose importance is underscored by the finding that the appearance of T-cell tropic variants often coincides with heightened virus replication rates and rapidly progressing disease (18).

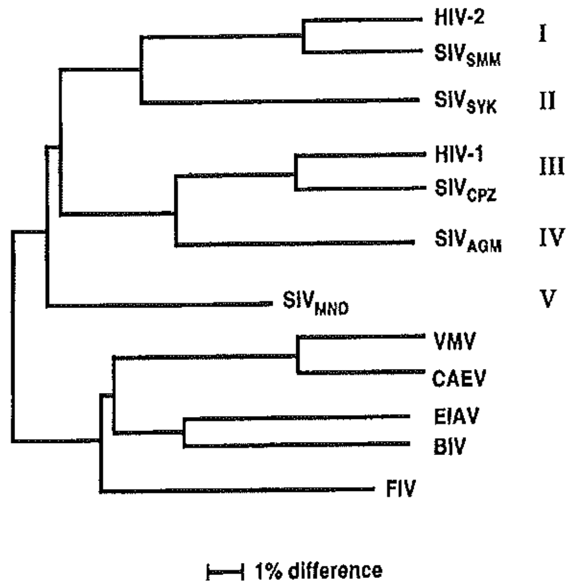

Figure 1. Phylogenetic analysis of lentiviruses. Representative lentiviruses are compared using *pol* gene sequences. **VMV**: Visna-Maedi virus (sheep); **CAEV**: caprine arthritis-encephalitis virus (goats); **EIAV**: equine infectious anaemia virus (horses); **BIV**: bovine immunodeficiency virus (cows); **FIV**: feline immunodeficiency virus (cats). The scale indicates the percentage of estimated nucleotide substitutions (9).

Recently, it has been shown that primary HIV-1 strains can be dual-tropic, as they can infect macrophages much more efficiently than laboratory strains adapted to growth in T-cell lines (19). Such dual-tropic strains may represent an intermediate between macrophage-tropic viruses, which are predominant during the early stage of infection, and T-cell-tropic viruses, which increase just before accelerated T-cell depletion and progression to AIDS.

Morphology and structure

HIV-1, HIV-2 and the various SIV strains share a similar morphology and composition. Typical virions have an overall spherical shape, are about 110 nm in diameter, and consist of a dense, cone-shaped icosahedral core surrounded by a membraneous lipid envelope acquired during budding (Figure 2). The envelope is studded with approximately 72 spikes, 9-10 nm long, representing tri- or tetramers of glycoprotein complexes. Each spike is composed by a surface subunit designated either gp120 (HIV-1) or gp130 (HIV-2 and SIV) that interacts non covalently with a smaller transmembrane subunit designated gp41 (1).The two subunits are generated by cleavage of a common precursor, gp160, encoded by the *env* gene. Within the surface subunit, a domain recognises and binds the CD4 receptor on host cells. In addition, both subunits contain epitopes targeted by virus neutralising antibodies. The most extensive sequence heterogenicity of HIV-1 Env is found clustered in five hypervariable regions (V1-V5) of gp120, which are interspersed with six

conserved regions (C1-C6) (20). Sequence analyses of HIV-2 and SIV isolates supports a similar model for the surface subunit of these two viruses (13). The gp41 subunit shows less heterogenicity and is more highly conserved (20). The envelope also contains cellular proteins acquired during virus budding, including ICAM-1, β_2-microglobulin, and the α and β chains of the human major histocompatibility complex (MHC) class II DR antigen (1, 21, 22). Whether these and other cellular proteins incorporated in the virion play a role in virus replication or pathogenesis is currently under investigation (23).

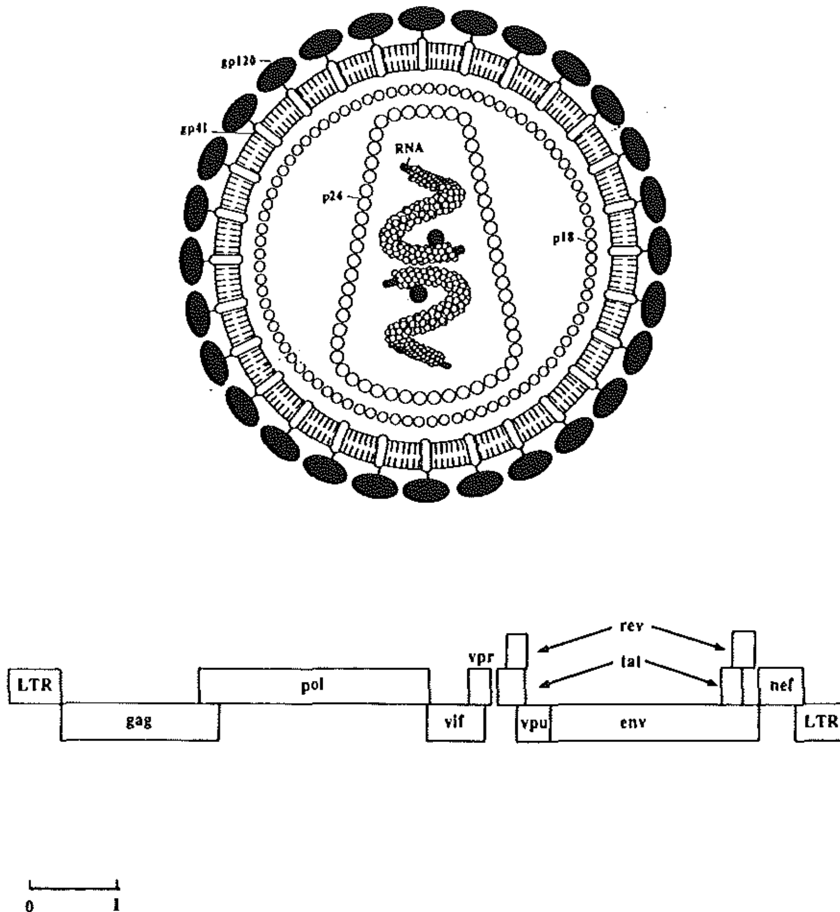

Figure 2. A schematic diagram of the HIV-1 virion and its genomic organization. The gene length is expressed in kilobases.

The virion core contains three major structural proteins: the matrix protein, presumably located between the envelope and the nucleocapsid, the capsid protein, which forms a 5 nm thick capsid shell, and the nucleocapsid protein, which is bound to the viral RNA genome (1). These proteins are derived from a common precursor encoded by the *gag* gene. Several enzymes, derived from a precursor encoded by the *pol* gene, are also packaged into virions; these are protease (PR), reverse transcriptase (RT), ribonuclease (RNase H), and integrase (IN), all presumably contained within the nucleocapsid. PR is essential for the processing of the viral polyproteins; inactivation of the enzyme leads to the production of immature, noninfectious viral particles (1). RT is an RNA-dependent DNA polymerase which synthesizes DNA from RNA as well as DNA templates. RNase H also functions in reverse transcription by degrading the RNA molecules of RNA/DNA hybrids. Both enzymes are indispensable for virus replication. RT is not an accurate enzyme; its errors ('misincorporations'), the lack of a proof reading system and recombination events, are responsible for the generation of viral genetic diversity. For the HIV-1 genome, the in vivo error rate is estimated to be 1-3 misincorporations per replication cycle (24). Finally, IN is an enzyme possessing both DNA cleavage and joining activities, which mediates covalent linkage of viral DNA into the cell genome.

The viral genome consists of two single-stranded RNA molecules with plus strand polarity of approximately 9.2 kb. Lentiviruses have a genomic structure of unique complexity among retroviruses (Table 2): in addition to the structural *env*, *gag* and *pol* genes common to all retroviruses, the RNA genome encodes the two regulatory proteins Rev ('regulatory of viral expression') and Tat ('transcriptional transactivator'), which are essential for viral replication (1). The HIV-1 genome also contains a number of genes encoding factors that are dispensable for virus growth in many in vitro systems and are therefore commonly called accessory genes. Nevertheless, there is increasing evidence that these highly conserved products fulfill crucial functions in vivo (25, 26). The accessory genes of the HIV-1 genome are designated *nef, vif, vpr,* and *vpu*, whereas HIV-2 and SIV encode *nef, vif, vpx* and/or *vpr*.

Replication

The life cycle of primate lentiviruses follows the general outline of other retroviruses. Virus replication begins with the steps of receptor binding, entry, and reverse transcription. This is followed by integration of the viral DNA into a chromosome of the infected cell, a step required for efficient replication of the retroviral genome. The second half of the replication cycle involves the steps of provirus transcription, mRNA processing, protein synthesis and processing, virus assembly, and budding. Attachment of the virion to

Table 2. *Genes and proteins of primate lentiviruses*

Gen	Protein	Function and site of influence
env	gp160	Envelope glycoproteins; virus attachment, entry, and release; CD4
gag	$Pr55^{gag}$	Matrix, capsid and nucleocapsid proteins; virus assembly
pol	$Pr160^{gag\text{-}pol}$	Virion enzymes
rev	p19	Regulator of viral expression
tat	p14	Transcriptional transactivator
vif	p23	Viral infectivity factor; early events after virus entry, before or
vpr	p15	Nuclear import of preintegration complex; cell cycle arrest in G2
vpx	p16	HIV-2 and SIV only; homologous to Vpr
vpu	p16	HIV-1 and SIVcpz only; virus release; CD4 downregulation
nef	p27	CD4 downregulation; virus infectivity; T-cell function

the host cell is mediated by a high affinity interaction between the surface subunit (gp120 or gp130) of Env and the first immunoglobulin-like domain of the CD4 receptor (27-30). The latter is expressed on most T helper lymphocytes, on many cells of the monocyte-macrophage lineage, and on some other cell types. A variety of cell types appear to have at least a limited susceptibility to HIV, including some Epstein-Barr virus (EBV)-transformed B cells, Langerhans' cells and other dendritic cells, follicular dendritic cells, glial cells, gut epithelium, and bone marrow progenitors (41).

The interaction of gp120 with the CD4 receptor triggers a conformational change in Env which promotes fusion of viral and cellular membranes (31). Recently, a number of chemokine receptors have been shown to cooperate with the CD4 receptor to facilitate virus entry through an interaction with gp120 (32-34). The α-chemokine receptor fusin CXCR4 (also known as LESTR or HUMSTR) and the ß-chemokine (RANTES, MIP-1 α and MIP-1 ß) receptor CCR5 serve as entry cofactors for T-cell tropic and macrophage tropic HIV-1 strains respectively. Some macrophage-tropic and dual-tropic strains can also use other ß-chemokine receptors such as CCR1, CCR2b and CCR3, but whether these play a major role in virus transmission and pathogenesis is at present unclear. Both primary and laboratory adapted T-cell tropic HIV-1 isolates have been shown to efficently employ CXCR4 as a co-receptor in cells expressing high levels of CD4. In contrast, cooperative binding of macrophage-tropic HIV-1 onto CCR5 and CD4 is thought to enhance infectivity for cells that express only traces of CD4 (34). The finding that macrophage-tropic strains are those involved in sexual transmission in about 90% of documented cases, highlights the importance of CCR5 in HIV-1 transmission. The important role played by these co-receptors

in infection and pathogenesis is reinforced by the recent finding that co-receptor gene mutations causing lack of protein expression on the cell surface, confer protection against HIV-1 infection and disease in vivo (35-37). Like HIV-1, at least some strains of SIV have been shown to use CCR5 as co-receptor for entry, whereas another coreceptor is thought to be the counterpart of CXCR4 (38). While the current model for virus entry involves therefore the steps of CD4 binding, interaction with CCR5 or CXCR4 (or its SIV counterpart), and then fusion, other factors may facilitate virus entry, including the Fc and complement receptors (39) and soluble CD4 (40). In addition, galactosyl ceramide on glial cells and colorectal cells, leukocyte function-associated antigen 1, and integrins have been proposed as potential mediators of virus attachment and entry into several cell types lacking detectable CD4 (1, 41).

Immediately after entry, viral particles are uncoated to produce a large nucleoprotein complex, followed by activation of RT to initiate the synthesis of a DNA strand of negative polarity from the genomic viral RNA template. Subsequently, RNase H degrades RNA in the DNA/RNA hybrid and a second strand of DNA with plus strand polarity is synthesised, whose integration in the host genome is mediated by IN. Although integration does not target specific sequences, it is not random. HIV-1 for instance preferentially inserts into or near two classes of repeated DNA elements in the human genome, the L1 and Alu elements. These elements are termed retroposons and share properties with retroviruses (42). Following initial infection, the virus may undergo a variable period of absent or low level replication with low or not detectable expression of viral genes. Although the mechanism of latency is not completely understood, provirus expression appears to be dependent on the combined action of multiple cellular and viral factors (43). Like other lentiviruses, HIV and SIV may bind to and enter quiescent cells. In terminally differentiated macrophages and cells arrested in the G1/S or G2 phases of the cell cycle, virus replication can occur after proviral integration. In contrast, integration does not occur within lymphocytes that are in the G0 phase, and until and unless the cell is activated, proviral integration and virus production do not occur. Paradoxically, CD4 $^+$T-cell activation in the course of immune stimulation create favourable conditions for virus integration, replication and dissemination (44).

Extrachromosomal replicative intermediate remain unintegrated for a limited period of hours or days, and may even serve as templates for the production of core and envelope proteins (45), although the data are controversial (46). In contrast, integrated genomes could potentially remain for the lifetime of the quiescent cell. In peripheral blood mononuclear cells (PBMC), advancing disease is characterised by a relative change in the proportion of cells carrying latent genomes and of those actively replicating virus. In special

circumstances, some integrated genomes may be defective and replication incompetent. In at least one instance, such defects have been associated with a prolonged asymptomatic state and mapped to a mutation in the *nef* gene (47).

Several cellular factors, including the NF-kB family of enhancer elements (48, 49), NFAT, AP-1, SP-1 and *tat*-binding proteins (50) influence viral RNA synthesis from the integrated DNA. Certain cytokines, such as interleukin-1 or TNFα (51) can also directly or indirectly influence virus replication. In some cases direct transactivation of latent genomes can be induced by other viruses, such as human herpesvirus 6 (HHV-6) (52) and cytomegalovirus (CMV) (53). A unique aspect of the viral replication cycle also lies in the various viral-encoded factors that regulate the level of provirus expression. The regulatory genes *tat* and *rev* control viral gene expression at the transcriptional and posttranscriptional levels respectively, and are believed to play a crucial role in both viral latency and activation by controlling differential viral gene expression (1). The role of the Nef protein is still debated. The protein makes up to 80% of the early viral transcripts, although it is produced at all stages of viral gene expression. It was initially reported to inhibit HIV replication and downregulate transcriptional activity; however, subsequent reports have failed to confirm this negative effect (25). Recent findings indicate that in fact the protein augments virus replication and markedly affects T-cell function, therefore playing an essential role in pathogenesis in vivo (54, 55).

The current model for regulation of viral gene expression predicts that multiple spliced viral mRNAs encoding Tat, Rev and Nef are produced during the early phase, whereas the late phase is characterised by accumulation of unspliced and singly spliced mRNAs encoding the precursors of the virion proteins Env, Gag and Pol. Cells harbouring latent HIV-1 contain multiply spliced viral transcripts encoding Tat, Rev and Nef (43). Once initiated, the various signals leading to viral replication will drive a self-perpetuating and progressive loop (1). The first event in virion assembly is presumed to be an interaction between Gag and Gag/Pol precursors, host cell membrane and viral RNA genome. This is followed by processing of the polyproteins by the viral protease, final assembly and budding. The *vif* and *vpu* gene products appear to be essential in the late stage of virus maturation and release (25).

Pathogenesis of Infection With Primate Lentiviruses

Target organs

Immediately after infection, high levels of cell-free and cell-associated viraemia

occur in peripheral blood, allowing systemic dissemination of the virus. Thereafter, the absolute levels of virus in blood become lower, to increase again during disease progression (56). The lymphoreticular, haematopoietic and nervous systems are the major targets of primate lentiviruses (41). The mechanisms of virus-induced damage of lymphoid and haematopoietic cells are probably multifactorial and may include both direct and indirect mechanisms (1, 41), such as perturbation of cell cycle (57), apoptosis (58), accumulation of unintegrated proviral DNA, increased permeability and disruption of cell membrane, cell fusion,, by-stander effects induced by infected cells, autoimmunity (50), and complex host adaptive responses to infection (59). Critical for HIV and SIV pathogenesis is the ability of the viruses to induce multiple immunological abnormalities, particularly a progressive dysfunction and depletion of $CD4^+$ T-cells, $CD8^+$ T-cells, monocyte/macrophages and dendritic cells (41) (Table 4). Marked defects in $CD4^+$ T helper cell function commonly precede substantial declines in $CD4^+$ T-cell numbers (60). An early sign of these defects is the loss of proliferative responses to recall antigens, followed by a sequential loss of reactivity to alloantigens and mitogens. In contrast with proliferative responses, the ability to produce interleukin-2 (IL-2) upon antigenic stimulation is initially preserved in infected persons (61), although the response is eventually lost in advanced disease.

Table 4. *Major immunological abnormalities associated with HIV infection*

Dysfunction and depletion of $CD4^+$ T-cells
Decreased helper function
Decreased B- and T-cell proliferative responses
Impaired delayed-type hypersensitivity reactions
Cytokine network disruption
Polyclonal B-cell activation
Polyclonal hypergammaglobulinaemia
Increased immune complex formation
Decreased humoral responses to immunization
Decreased cytotoxic T-cell responses
Decreased natural killer cell activity
Decreased monocyte chemotaxis
Impaired killing of intracellular pathogens
Decreased antigen presenting function

The mechanisms which account for the compromised T helper function in the early stages of infection are unclear; preferential infection and loss of certain T cell subsets (62, 63), defective antigen presentation due to virus-induced dysfunction of dendritic cells (64),

and immunosuppressive effects of gp120 (65), are among the factors that may compromise T-cell responsiveness. The pathogenesis of neurological damage is also obscure (50). The predominant cell type that is infected within the nervous system is of monocyte-macrophage lineage, although other cell types such as endothelial cells, astrocytes and possibly neurons may infected (41). Release of cytokines and viral proteins by infected macrophages may be either directly neurotoxic or may induce further inflammatory events (66). Alternative mechanisms include direct or indirect (e.g. immune-mediated) destruction of glial cells or neurons, and co-infection with cytomegalovirus or JC papovavirus. Recent data indicate that HIV-1 infection may be established in the nervous system by viruses present early after infection, that some of these viruses are particularly tropic for the microglia and that adaptation to this cell type can result in the selection of a pool of predominantly neurotropic (microglia-tropic) viruses (67).

Clinical features

Primary HIV-1 infection may be asymptomatic or may present as an acute an acute mononucleosis-like syndrome, commonly observed 1-6 weeks after exposure (68). Occasionally, more serious symptoms including encephalitis are observed. At present, there are no data to clearly indicate that the long-term prognosis in patients who experience symptomatic primary infection is any different from that of asymptomatic cases. Seroconversion to HIV antibody positivity usually occurs within 3-12 weeks. After seroconversion and resolution of any symptoms, an asymptomatic period generally ensues. Persistent generalised lymphadenopathy, which occurs in some individuals, may be the only clinically apparent abnormality present during this stage. After a variable period of time, usually measured in years (69), infected persons may begin to develop a spectrum of clinical conditions typically associated with a decline of $CD4^{+}$ T-cells, such as skin problems, oral Candida, and oral hairy leucoplakia. Systemic upset with fever, weight loss and diarrhea may also become evident. As immune function declines, the occurrence of opportunistic infections and malignancies increases. The clinical manifestations of AIDS can include systemic, neurological, gastrointestinal, infectious and malignant complications (68), although the relative frequencies of symptoms and signs varies considerably in different patient groups. The spectrum of opportunistic infections, particularly, tends to reflect the pattern of infectious organisms present in a given geographical location. Once a patient develops overt disease, average survival is two years. The clinical course, however, is highly variable and even after AIDS is diagnosed ,infected persons may experience long symptom-free periods between episodes of opportunistic infections. Cohort studies among homosexual men and haemophiliacs in developed countries indicate that about 50% of infected persons

develop AIDS after 10 years. Some persons however progress relatively quickly (less than three years, rapid progressors), whereas others may remain asymptomatic for 16 years or more (long-term nonprogressors) (70, 71).

The asymptomatic period of 'clinical latency' that intervenes between infection and the development of AIDS, does not imply an absence of virus replication (72). Although infectious virus titres and levels of p24 antigen or viral nucleic acid show a positive correlation with clinical stage, active virus replication persists throughout the disease course (73). Indeed, even when detectable virus in the periphery is at a nadir, high levels of virus replication and of 'trapped' extracellular virus (e.g., on follicular dendritic cells) are found within lymphoid organs (74, 75). Over time, changes in total body virus load are determined by the rate of clearance of productively infected cells, the rate of virus release from these cells, and the rate by which cells with latent genomes are cleared (76). Recent experiments studying the kinetics of virus turnover have estimated that in patients with CD4 counts of 500 or less, 10^7-10^9 virions are produced per day, resulting in a peripheral viremia of 10^4-10^7 RNA molecules/ml and in the destruction of approximately 2×10^9 $CD4^+$ T-cells/day (77, 78). Most of the plasma virus is apparently produced within recently infected cells. On the basis of these studies, the possibility has been raised that the kinetics of viral and cellular turn over found in late-stage disease may persist from the outset, leading ultimately to the collapse of the immune system and overt disease (79). Although the hypothesis of 'virological mayhem' (80) is attractive and may be correct, at present it remains an inference from observations made in late-stage disease after perturbation of the steady state with antiretroviral therapy. In fact, recent analyses of T-cell telomere length, supposedly a marker for cellular replicative history, suggest that turnover in the course of HIV infection is increased considerably in $CD8^+$ T-cells, but not in $CD4^+$ T-cells (41).

Infection of macaques with SIV induces an immunodeficiency syndrome closely resembling human AIDS. Main features of simian AIDS include $CD4^+$ T cell loss, constitutional symptoms, neurological disorders, opportunistic infections and neoplasias. The time scale of the infection is faster than in humans, as the average time from infection to disease is 1-2 years (82). As in humans, however, the course of the infection is highly variable in macaques: after virus inoculation, some animals rapidly progress to overt disease, whereas others may remain asymptomatic for more than three years, thus resembling long-term nonprogressors with HIV-1 infection.

Can the Immune Response Control HIV Infection?

The viral, host, or environmental factors underlying the variable rates of disease

progression among HIV-infected individuals or SIV-infected monkeys are currently under investigation. The bulk of evidence indicates that virus replication plays a major role in driving pathogenesis and measurement of steady-state virus load after acute infection clearly predicts disease progression. These observations suggest that some long-term asymptomatic individuals may have been infected with an attenuated virus of limited replication competence or pathogenicity (47, 83). However, many lon-term nonprogressors with HIV-1 infection appear to have been infected with a virus that they have controlled (84). In addition, divergent patterns of disease progression have been observed after infection from the same source (85). A recent study in perinatally infected infants has also indicated that a single virus variant initiated the infection in both rapid and slow progressors (86). While no definitive explanation yet exists to explain these findings, increasing evidence indicates that slow rates of virus replication and disease progression are intimately connected with host genetic factors and the normal functioning of the immune system (87). Among host-genetic factors, defects of coreceptor genes resulting in strong resistance to infection in homozygotes and to disease progression in heterozygotes are currently attracting considerable interest (35-37). In addition, certain MHC genotypes have been found to be associated with the degree of resistance to infection. In particular, the B35 or the A1-B8-DR3 haplotypes have been associated with progression, whereas other haplotypes such as B27-A24-DR1 have been found in long-term nonprogressors (88). These data strengthen the case for a genetically determined ability of the immune system to control HIV infection.

The inference that protective immune responses occur quite commonly in HIV-infected persons may be drawn from studies indicating the difficulty in superinfecting primates (82). Infection of primates with attenuated viruses appears to result in a state of resistance against subsequent challenge with fully virulent viruses. The most striking example is the resistance of macaques that have been infected with SIV defective in the *nef* gene (*nef*-deleted SIV) to challenge with large amounts of fully virulent virus (89, 90). The animals contain DNA sequences of the attenuated virus, indicating the ability of the attenuated viruses to establish persistent infection; virus burden however is very low. The human counterpart can be found in persons infected with the less pathogenic HIV-2, who appear to have a reduced risk of infection with HIV-1 despite potential exposure (91). Although the nature of the resistance has not been determined, it is likely to include an immunological component. This conclusion is supported by the finding that attenuated SIV viruses are fully pathogenic in immunologically noncompetent neonate macaques (92). However, several aspects of the apparent protection still require investigation, including the long time required for the resistance to develop, the nature of the potentially protective immune responses, and the apparent paradox of a response powerful enough to prevent

infection by a virulent virus but unable to fully rid the host of an infection with an attenuated virus. In this respect, it seems possible that an effective immune response that fails to eliminate existing virus, may nonetheless be effective in limiting virus replication and spread, particularly in cells in which virus turns over quite rapidly. By contrast, immunity may lack effectiveness against cellular or compartments that act as virus reservoirs, such as the macrophage lineage in the brain.

Despite the devastating effects of HIV-1 on the host immune system, most infected individuals develop both humoral and cellular immune responses against multiple HIV antigens. Neutralizing antibodies are produced by most infected individuals and are generally directed against Env (41). Epitopic sites in Env hypervariable regions, such as the V3 loop, induce antibodies that are extremely type-specific, whereas those in conserved areas induce group-specific responses. Some investigators have observed a correlation between low neutralizing antibody titres and rapid disease progression, whereas other have not (13). Reactivity to the p24 Gag product is also detectable in infected persons, and it appears to mirror progression of infection. As a marker of prognosis, diminution of circulating anti-p24 antibodies and the appearance of p24 antigen often correlate with marked CD4+ T-cell loss, progression of immune dysfunction and development of AIDS (41). Of note is that HIV-1 genetic subtypes, which are based on primary sequence analyses, do not usually correspond to neutralization serotypes (32). As an important target of cross-subtype neutralization, attention is currently focused on the putative three-dimensional structure of gp120, where conserved binding sites for both CD4 and CCR5 or CXCR4 must be located. A role for specific antibodies in mediating enhancement of virus entry and infection has also been suggested (93, 94) and is currently under investigation.

As cellular immune responses are a critical part of the host defence against viruses, their role in the control of HIV infection is the focus of extensive investigation. Measurement of T-cell proliferation upon antigen stimulation is widely used to test T-helper cell reactivity in vitro. However, in HIV-1-infected persons measurement of cytokine release appears to be more sensitive and informative (61). Proliferative responses and IL-2 release to stimulation with synthetic peptides spanning gp120 have been demonstrated in asymptomatic HIV-1 infected persons with $CD4^+$ T-cell counts >400 (61). In HIV-1 infected humans, as also in immunised macaques (95), responses target multiple epitopes scattered throughout Env. Production of IL-2 in response to in vitro PBMC stimulation with peptides spanning defined regions of Env have also been detected in persons exposed to HIV-1 but lacking evidence of infection (96). Whether exposure to viral antigens, rather than replication-competent virus, is sufficient to prime the $CD4^+$ T-cell responses and whether these responses are markers of exposure or of actual protective immunity remains currently

unclear. Initial reports that helper T-cells of the Th1 phenotype, that is secreting predominantly IL-2 and IFNγ on antigen stimulation, may be associated with protection from infection or maintenance of the asymptomatic status also await confirmation (97, 98).

Antiviral $CD8^+$ T-cells form a primary component of the cellular immune response against viral infections. They may contribute to virus containment through two effector mechanisms. Classically, $CD8^+$ cytotoxic T lymphocytes (CTL) lyse infected cells displaying on their surface viral peptides in the context of MHC class I molecules. Lysis of target cells is induced either be the secretion of soluble lytic proteins (perforin and granzymes) from specialised secretory granules, or by the engagement of the Fas molecule resulting in apoptosis (99). Recently, evidence is emerged that $CD8^+$ T-cells can also actively suppress virus replication by producing antiviral lymphokines. This suppressive activity is not MHC restricted, although it can follow MHC-restricted T-cell stimulation , is mediated, at least in part by a novel $CD8^+$ T-cell antiviral factor (CAF), and has been demonstrated in both HIV-infected humans and SIV-infected macaques (100). Recent data have demonstrated that HIV-1-specific CTL can mediate virus control by both cytolytic and noncytolytic mechanisms (101). The contribution of CAF, of chemokines such as RANTES, MIP-1 α and MIP-1 ß which inhibit the replication of macrophage tropic strains interacting with CCR5 (102), and cytokines such interleukin-16 (103) to the control of virus replication has recently become the subject of intense investigation. Remarkably, the detection of viral suppressive factors have been shown to correlate with protection of immunised macaques against mucosal SIV infection (104).

Cytotoxic T lymphocytes

The best characterized virus-specific CTL are $CD8^+$ T-cells that recognize viral proteins processed in the cytoplasm of infected cells and displayed as peptide fragments on the cell surface in association with class I MHC molecules. Peptides of the appropriate length (mostly 8 or 9 amino acids) are generated in the cytosol following cleavage by proteasomes, and transferred by specialised peptide transporters to the endoplasmic reticulum, where assembly with MHC class I dimers (class I heavy chains associated noncovalently with β2-microglobulin) takes place (105). The stable trimeric complex is then transported through the Golgi apparatus to the cell surface and the peptide is presented to CTL. Chaperon molecules associated with the peptides and the class I molecules at various stages prior to complete assembly of the trimeric complex. MHC class II restricted $CD4^+$ lymphocytes may also display cytotoxic activity against a number of viruses, including HIV-1 (106).

Specific CTL have been detected not only in peripheral blood, but also in lymph

nodes, spleen, bronchoalveolar lavage, genital tract and cerebrospinal fluid of individuals infected with HIV-1 (107-111). Responses are generally multispecific, as they are directed against a relatively large number of epitopes both on external and internal viral proteins (112-115). The recognition that CTL might be important immunological correlates for protection against HIV infection or disease has gradually evolved. To begin with, a role for CD8+ MHC class I restricted CTL can be inferred from animal model systems of both acute and chronic viral infections. In mice, for instance, CTL play a critical role against lymphocytic choriomeningitis virus; in this case, as well as in murine cytomegalovirus infection, it is possible to protect a naive animal from virus challenge by immunization with a recombinant vaccinia virus expressing a single viral protein and such protection is mediated by CTL (116, 117). In humans, there is convincing evidence that CTL responses play a role in the control of a number of persistent viruses, including cytomegalovirus (118). In other animal and human viral infections however, evidence is less clear-cut.

Indirect evidence for a role of CTL in the control of HIV comes from the observation that CTL responses against HIV antigens are present in persons exposed to the virus but lacking evidence of infection: these include small number of female prostitutes in Africa, sexual partners of infected persons, children born to infected mothers, and healthcare workers exposed to infectious body fluids (96). Based upon the assumption that the induction of CTL requires endogenous synthesis of viral proteins, these findings suggest that transient infection and virus clearance by CTL are indeed possible. Furthermore, studies in both HIV-infected persons (119, 120) and SIV-infected macaques (121) have shown that antiviral CTL develop rapidly after infection (day 4-7 after intravenous SIV inoculation) in both peripheral blood and lymph nodes, and that their appearance coincides with the fall in viraemia that follows the initial virus burst. CTL responses against HIV-1 can be unusually strong in comparison with other chronic viral infections. In asymptomatic HIV-1-infected adults, the frequency of circulating effector cells is sometimes high enough to allow CTL detection in freshly isolated lymphocytes, in the absence of in vitro restimulation (112). This strong response is generally maintained throughout the asymptomatic status, when is associated with increased numbers of circulating $CD8^+$ T-cells expressing activation markers such as DR , but often declines with disease progression (122). At present no satisfactory explanation exists for such decline, although both exhaustion due to prolonged high-level antigen exposure (123) and progressive loss of helper function may be advocated. Continuos activation of memory CTL, in particular, can result in low expression of Bcl-2 and CD28 that contribute to $CD8^+$ cell death (124).

Despite extensive characterisation from the moment of virus entry to full-blown disease, whether the correlation between CTL responses and disease progression is an

indication of a role for CTL in controlling HIV infection has yet to be determined. In addition, the view that persons with the most benign disease course have the highest levels of CTL activity, although widely accepted, is not well founded. In fact, results are divergent. Broad CTL responses and an inverse correlation between CTL precursor (CTLp) frequencies and cell-associated viraemia have been associated with long-term absence of progression (125-127). At the same time, either absent or very low frequencies of HIV-specific CTL have been found in long-term nonprogressors with extremely low viral load (128). Similarly, no clear relationship has been found in vertically HIV-1-infected children between CTLp frequencies and viral load or disease progression (129). However, the small number of patients examined, the heterogenous composition of the study groups, and the different methodologies for CTL assays, limit generalization of the data reported. It is also likely that qualitative factors, such as affinity of target cell-effector cells interaction, play a crucial role in determining the effectiveness of CTL response and may in part explain why the presence of high levels of CTL does not necessarily prevent development of disease. In fact, at least in some cases, high CTL frequencies may simply reflect either persistently high level of virus replication or a continuously diversifying genome.

HIV-1 has evolved several strategies to escape immune recognition (110). For instance, certain viral proteins, such as Nef, can alter MHC expression (131). Rapid evolution of viral sequences is also believed to represent a major mechanism of viral escape (132-135). In vitro, single amino acid substitutions have been shown to abrogate CTL recognition by affecting either MHC binding or TCR interaction (136). Furthermore, mutations within epitope flanking regions may reduce presentation by affecting peptide processing and transport (137). Mutant viruses which are resistant to CTL have been both generated in vitro under selective CTL pressure and detected in vivo during infection with a number of viruses including HIV-1 (138-141). Recent evidence suggests also that epitopes with mutated TCR contact sites may arise, which still interact with the TCR, but inhibit antiviral CTL responses by inducing T cell anergy, or by showing partial agonistic or antagonistic activity (142). Indeed, some Gag and RT variants have been found to induce antagonistic inhibition of CTL directed against wild-type peptides. Virus variation may also lead to the generation of mutated sequences that stimulate the expansion of variant-specific CTL. Under these circumstances, the emergence of new viral variants, which transiently avoid recognition, would be followed by successive waves of newly induced CTL (143).

Understanding the factors that may control virus spread is crucial to the development of effective therapies and vaccines against HIV. Because of the strong analogy with HIV infection of humans, SIV infection of macaques offers a highly valuable model for studying the role of CTL immunity in preventing infection after exposure of vaccinated

animals. In addition, this model offers the opportunity to explore crucial issues related to pathogenesis and disease progression, and may therefore help to clarify the complex interactions between primate lentiviruses and host immune system.

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Outline of this thesis

After an introduction to the main features of primate infections with immunodeficiency viruses in chapter 1, chapter 2 describes the results of two vaccine studies in macaques. Both studies were aimed at investigating the ability of vaccine-induced humoral and CTL responses to prevent infection of macaques upon intravenous challenge with either a molecular clone of SIVmac or a chimeric HIV-SIV virus (SHIV). Chapter 3 outlines how the optimal conditions for CTL expansion, quantitative analyses and phenotype characterization were established in vitro. Additionally, the chapter describes the identification of a minimally defined epitope in SIV Gag recognised by antiviral CTL of an SIV-infected macaque. Chapter 4 contains two prospective studies conducted during the chronic phase of HIV-1 and SIV infection respectively. The first study analyses the kinetics of HIV-1 and EBV-specific CTL and describes their relationship with changes in $CD4^+$ and $CD8^+$ cell counts over time. The second study investigates the evolution of SIV-specific CTL in relation to the kinetics of virus replication and virus attempts to evade immune surveillance. Finally, chapter 5 contains a summary and a discussion of the experiments presented in the framework of similar studies conducted in both humans and nonhuman primates.

Chapter 2

Role of CTL immunity in preventing primate lentivirus infection

2.1 Vaccine-induced virus-neutralizing antibodies and cytotoxic T cells do not protect macaques from experimental infection with simian immunodeficiency virus SIVmac32H(J5). *Journal of Virology (1995) 69:6289-6296.*

2.2 Immunization with envelope glycoproteins of primary human immunodeficiency virus type 1 (HIV-1) isolates does not protect macaques against homologous challenge. (*Submitted).*

Vaccine-Induced Virus-Neutralizing Antibodies and Cytotoxic T Cells Do Not Protect Macaques from Experimental Infection with Simian Immunodeficiency Virus SIVmac32H (J5)

ELLEN G. J. HULSKOTTE,[1,2] ANNA-MARIA GERETTI,[1] KEES H. J. SIEBELINK,[1] GEERT VAN AMERONGEN,[2] MARTIN P. CRANAGE,[3] ERLING W. RUD,[4] STEPHEN G. NORLEY,[5] PETRA DE VRIES,[2] AND ALBERT D. M. E. OSTERHAUS[1]*

Institute of Virology, Erasmus University Rotterdam, 3000 DR Rotterdam,[1] and Laboratory of Vaccine Development and Immune Mechanisms, National Institute of Public Health and Environmental Protection, 3720 BA Bilthoven,[2] The Netherlands; Centre for Applied Microbiology and Research Division of Pathology, Porton Down, Salisbury, United Kingdom[3]; Health Canada, Health Protection Branch, Laboratory Center for Disease Control, Bureau of HIV/AIDS, Ottawa, Ontario, Canada[4]; and Paul-Ehrlich Institut, Langen, Germany[5]

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To gain further insight into the ability of subunit vaccines to protect monkeys from experimental infection with simian immunodeficiency virus (SIV), two groups of cynomolgus macaques were immunized with either recombinant SIVmac32H-derived envelope glycoproteins (Env) incorporated into immune-stimulating complexes (iscoms) (group A) or with these SIV Env iscoms in combination with p27^{gag} iscoms and three Nef lipopeptides (group B). Four monkeys immunized with recombinant feline immunodeficiency virus Env iscoms served as controls (group C). Animals were immunized intramuscularly at weeks 0, 4, 10, and 16. Two weeks after the last immunization, monkeys were challenged intravenously with 50 monkey 50% infectious doses of virus derived from the J5 molecular clone of SIVmac32H propagated in monkey peripheral blood mononuclear cells. High titers of SIV-neutralizing antibodies were induced in the monkeys of groups A and B. In addition, p27^{gag}-specific antibodies were detected in the monkeys of group B. Vaccine-induced cytotoxic-T-lymphocyte precursors against Env, Gag, and Nef were detected on the day of challenge in the monkeys of group B. Env-specific cytotoxic-T-lymphocyte precursors were detected in one monkey from group A. In spite of the observed antibody and T-cell responses, none of the monkeys was protected from experimental infection. In addition, longitudinal determination of cell-associated virus loads at weeks 2, 4, 6, 9, and 12 postchallenge revealed no significant differences between vaccinated and control monkeys. These findings illustrate the need to clarify the roles of the different arms of the immune system in conferring protection against primate lentivirus infections.

Simian immunodeficiency virus (SIV) has a genomic organization similar to that of human immunodeficiency virus (HIV) and can induce a disease in certain macaques similar to human AIDS. Therefore, SIV infection of macaques is widely used as a model for HIV vaccine research. Previous observations have provided evidence that whole inactivated SIV vaccines can induce protective immunity in macaques (10, 13, 18, 29, 30, 40, 41), although in some of these studies the immune responses against cellular components of the vaccine proved to be at the basis of the observed protection (3, 12, 53). Immunization of rhesus macaques with live attenuated SIV has provided further evidence that the induction of protective immunity may be achieved by vaccination (16, 35). However, concerns about the overall safety of this approach may limit its potential for the development of HIV type 1 (HIV-1) vaccines.

Subunit vaccines have been proposed as a safe alternative to inactivated or live attenuated virus vaccines, with the advantage that only antigens relevant for the induction of protective immunity may be included. In this respect, much attention has been paid to the envelope glycoprotein (Env) as the major target of virus-neutralizing (VN) antibodies. Furthermore, Env has been shown to be recognized by cytotoxic T lymphocytes (CTL) in both HIV-infected humans and SIV-infected macaques (59). A role for CTL in controlling virus replication has been suggested from the observation that the clearance of viremia during primary infection is coincident with the emergence of HIV-1-specific CTL and prior to the development of detectable VN antibodies (34). Immunization of cynomolgus macaques with live vaccinia virus expressing Env and a subsequent booster injection with recombinant Env did confer protection against homologous challenge with SIVmne (25). However, similar immunization protocols did not induce protection against SIVmac infection (22, 51), although decreased virus loads were observed in some studies (1, 27).

The relatively conserved Gag protein is another candidate for inclusion in SIV subunit vaccines. Gag is known to be a major target for CTL responses in both HIV-1 and SIV infections (31, 59), in which Gag-specific CTL are believed to play a role in delaying the onset of disease (9).

Regulatory proteins expressed early in viral replication are also interesting components of candidate SIV subunit vaccines, since CTL directed against these proteins may kill infected cells before the expression of structural proteins and before the release of infectious virus particles. In this respect, Nef appears to be of particular interest, since it is abundantly expressed early in infection and is able to induce CTL responses in both humans and macaques (14, 15, 58). In HIV-1-infected individuals, several CTL epitopes have been identified in the central

* Corresponding author. Mailing address: Institute of Virology, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. Phone: 31-10-4088066. Fax: 31-10-4365145.

region of Nef and shown to be recognized in the context of a broad range of major histocompatibility complex (MHC) haplotypes (14, 15). In SIV-infected macaques, three Nef peptides, with a sequence highly homologous to that of the central region of the HIV-1 Nef protein, have been shown to contain CTL epitopes (7).

The system used for delivery of the selected antigens may play a crucial role in the generation of adequate B- and T-cell responses. Particular attention should be given to the induction of MHC class I-restricted $CD8^+$ CTL, because it requires antigen processing via the endogenous route, which is usually not accomplished by nonreplicating antigen presentation forms (for a review, see reference 37). However, proteins incorporated into immune-stimulating complexes (iscoms), as well as peptides bound to a lipid tail, have been shown to induce $CD8^+$ MHC class I-restricted CTL in vitro and in vivo (6, 17, 36, 39, 50, 55, 57). Interestingly, previous studies have suggested that gp120-enriched SIVmac32H iscoms are effective in inducing protection against challenge with cell-associated SIV (19, 23, 42). Furthermore, HIV-2 iscoms have been shown to induce long-lasting protective immunity against challenge with cell-free HIV-2 (43).

In this study, two groups of monkeys were immunized with either recombinant SIV Env incorporated into iscoms (group A) or with SIV Env iscoms combined with $p27^{gag}$ iscoms and three Nef lipopeptides containing the CTL epitopes previously identified in the central region of Nef (amino acids 108 to 123, 155 to 169, and 164 to 178) (7) (group B). Despite the induction of VN antibody responses and CTL responses against Env, Gag, and Nef, none of the monkeys proved to be protected against intravenous challenge with the J5 molecular clone of SIVmac32H.

MATERIALS AND METHODS

Formulation of vaccine preparations. (i) Preparation of SIV Env iscoms. Two Env glycoproteins, referred to as 8672-m and 8789-m, were produced and incorporated into iscoms as previously described (26). Briefly, peripheral blood mononuclear cells (PBMC) were derived from two rhesus macaques shortly after infection either with the 32H isolate of SIVmac251 (November 1988 pool) (macaque 8789) or with the same virus after it had undergone an 11-month in vivo passage (macaque 8672) (19). Two SIV Env genes were amplified from PBMC by PCR, and the putative primary and secondary cleavage sites were mutated by site-directed mutagenesis. These constructs were cloned in plasmid pSC65 (kindly provided by B. Moss, Bethesda, Md.) under the control of a synthetic early-late promoter. Recombinant vaccinia viruses (rVVs) were made by homologous recombination with vaccinia virus (WR strain). rVVs were used to infect baby hamster kidney cells. Twenty hours after infection, cells were harvested, and the rVV was inactivated by paraformaldehyde (PFA) fixation. Subsequently, the Env glycoprotein was solubilized from the cell membrane by using Rosenbuch-Tenside (Bachem, Bubendorf, Switzerland), purified by lentil-lectin chromatography, and eventually incorporated into iscoms. The Env protein of feline immunodeficiency virus (FIV) strain AM19 (45) was produced and incorporated into iscoms following the same procedure.

(ii) SIV Gag iscoms. The C-terminal part of p17 and p27 of the SIVmac251 *gag* gene was excised from plasmid pKA27 (kindly provided by N. Almond and P. Kitchin through the Medical Research Council) and cloned into the *Eco*RI and *Xba* sites of plasmid pMALc (New England Biolabs, Inc., Beverly, Mass.). As only hydrophobic proteins incorporate efficiently into iscoms, a sequence containing 29 amino acids of the hydrophobic part of the transmembrane sequence of HIV clone 320 (2) (amino acids 683 to 711 [WAGLWNWFSITNWLWYIKI FIMIVGGLVG]) was amplified by PCR and cloned into the C terminus of the $p27^{gag}$ gene. The resulting plasmid, pMALc-Gagp27, expresses in *Escherichia coli* a fusion product of maltose-binding protein which is linked to SIV $p27^{gag}$ by a factor Xa cleavage site. The fusion protein was first released from bacteria by sonication and subsequently purified from bacterial proteins through affinity chromatography by allowing its maltose-binding protein part to bind to an amylose column. The $p27^{gag}$ protein was released from the column by incubation with 12.5 μg of factor Xa (New England Biolabs) per ml in Xa buffer (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, 2 mM $MgCl_2$) containing 0.25% MEGA-10, 10 μg of cholesterol per ml, 10 μg of phosphatidylethanolamine per ml, and 2 μg each of the of protease inhibitors aprotinin and leupeptin per ml. The $p27^{gag}$ protein was finally collected and incorporated into iscoms by a procedure similar to that described for the preparation of SIV Env iscoms. The amount of $p27^{gag}$ present in each iscom preparation was quantified by a commercial antigen capture enzyme-linked immunosorbent assay (ELISA) (Vironostika, HIV Antigen Microelisa system; Organon Teknika B.V., Boxtel, The Netherlands) according to the procedure recommended by the manufacturer. Recombinant $p27^{gag}$ (kindly provided by I. Jones, Oxford, United Kingdom) was used as a standard protein for quantification.

(iii) Nef lipopeptides. Three Nef lipopeptides were constructed according to sequences previously found to contain CTL epitopes in SIVmac-infected macaques (7). The following Nef peptide sequences were based on the J5 molecular clone of SIVmac32H (47): peptide 1 (amino acids 108 to 123), LRTMSYK LAVDMSHFI; peptide 2 (amino acids 155 to 169), DWQDYTSGPGIRYPK; peptide 3 (amino acids 164 to 178), GIRYPKTFGWLWKLV. These peptides were synthesized by standard Fmoc solid-phase methods (20), starting with Rink amide resin. The N-terminal lysine was coupled as Fmoc-Lys(Fmoc). After Fmoc deprotection, the N-terminal and the side chain amino group of lysine were palmitoylated with palmitic anhydride. Two serine residues were introduced between the N-terminal lysine and the Nef peptide sequence.

Animals and immunization protocol (see Table 1). This study included twelve colony-bred juvenile cynomolgus macaques (*Macaca fascicularis*) between 1.5 and 3.5 years old and weighing from 1.4 to 2.9 kg at the beginning of the study. All monkeys were seronegative for SIV, type D retrovirus, and simian T-lymphotropic virus. Macaques were allocated to three groups of four monkeys each, including two male and two female monkeys and different MHC haplotypes. Animals were immunized intramuscularly at weeks 0, 4, 10, and 16 with either 20 μg of SIV Env iscoms (8672-m Env and 8789-m Env at 10 μg each) (group A) or with 20 μg of SIV Env iscoms and 10 μg of $p27^{gag}$ iscoms in combination with the three Nef lipopeptides at 1 μg each (group B). Four monkeys vaccinated with recombinant FIV Env iscoms served as controls (group C).

SIV challenge. Two weeks after the fourth immunization, monkeys were infected intravenously with 50 monkey 50% infectious doses (MID_{50}) of the cell-free March 1992 challenge stock (J5M) of SIVmac32H (pJ5), a pathogenic molecular clone derived from SIVmac32H (November 1988 pool) (47). The challenge stock derived from this molecular clone had been propagated in rhesus macaque PBMC and titrated in vivo by intravenous inoculation of rhesus macaques (47). One monkey (K2 [group A]) died during recovery from anesthesia on the day of challenge.

PBMC isolation. Heparinized blood samples were collected at two-, three-, or four-week intervals. PBMC were isolated by density gradient separation with a 68:32 (vol/vol) solution composed of 6% dextran (Sigma, St. Louis, Mo.) in distilled water and 32.8% sodium metrizoate (Nycomed, Oslo, Norway). Plasma samples were cryopreserved until used. PBMC were used immediately or stored at −135°C until used.

Quantification of SIV Env-specific serum antibodies by ELISA. Ninety-six-well plates (Costar, Cambridge, Mass.) coated with concanavalin A (Pharmacia LKB, Uppsala, Sweden) were incubated with 50 μl of 100-ng/ml lentil-lectin-purified Env derived from SIVmac32H-infected C8166 cells (19) in phosphate-buffered saline (PBS) containing 1% Triton X-100. Virus-derived SIVmac32H was used as immobilized antigen to confirm that the Env-specific antibodies detected after immunization with vaccinia virus-produced Env glycoprotein recognized the native protein. Quantification of SIV Env was performed as previously described (16). Uninfected C8166 cells were used as the negative control. After 16 h of incubation at room temperature (RT), wells were blocked with PBS containing 0.1% Tween 20 and 1% bovine serum albumin (PTB) supplemented with 10% fetal calf serum (FCS) and 0.5% nonfat dry milk. Subsequently, plates were incubated for 2 h at RT with 50 μl of twofold dilutions of plasma in PTB containing 4% FCS. After being washed, bound antibody was detected by using a biotin-conjugated goat anti-human immunoglobulin preparation (Amersham) and subsequently incubated with horseradish peroxidase-conjugated streptavidin (Amersham). For substrate reactions, 3,3′,5,5′-tetramethyl-benzidine was used (5). Endpoint titers were calculated by using a cutoff value three times above the respective dilution of the preimmune serum at an optical density at 450 nm. One-way analysis of variance (Minitab, Inc., State College, Pa.) was used to compare antibody titers between groups. The level of significance was $P < 0.05$.

Quantification of SIV Gag-specific serum antibodies by inhibition ELISA. Gag-specific antibody titers were measured in inhibition ELISAs with biotinylated polyclonal antiserum from an SIVmac-infected monkey. Ninety-six-well plates (Costar) were coated with 100 μl of a 1:200 dilution in PBS of sonicated bacterial culture of pMALc-Gagp27 containing about 10 μg of $p27^{gag}$ per ml. After incubation at RT for 16 h, wells were incubated for 30 min with PTB containing 10% FCS and then for 60 min at RT with 100 μl of twofold dilutions of monkey plasma. Fifty microliters was discarded from each well before 50 μl of biotinylated polyclonal serum in PTB containing 4% FCS was added. This polyclonal serum was derived from an SIVmacBK28-infected rhesus macaque (kindly provided by J. Heeney, TNO, Rijswijk, The Netherlands) and was shown to have a high titer of Gag-specific antibodies by immunoblotting. It was diluted to give an absorbance at an optical density at 450 nm equal to 60% of the maximum absorbance in a direct SIV ELISA. After incubation for 2 h at RT, wells were washed and incubated with horseradish peroxidase-conjugated streptavidin. For substrate reactions, 3,3′,5,5′-tetramethyl-benzidine was used (5). Inhibition titers were defined as the dilutions of monkey plasma inhibiting 50% of the absorbance at an optical density at 450 nm without the addition of plasma.

SIV neutralization assay. The SIV neutralization assay used in these experiments was performed as previously described with SIVmac32H (19). Briefly, 10 μl of serial dilutions of heat-inactivated sera were incubated in four replicate wells with 10 μl of SIVmac32H diluted to give 10 infectious particles per 10 μl. After a 30-min incubation at 37°C, 200 μl of medium containing 2×10^3 C8166 cells was added to each well. Plates were incubated for 7 days at 37°C, after which the cells from each well were transferred to poly-L-lysine-coated flat-bottom microtiter plates, fixed in methanol, and examined for the presence of SIVmac-infected cells by an SIV-specific immunoperoxidase assay. Wells containing no infected cells were scored as positive, and the number of positive wells was used to determine the 50% neutralizing dose endpoint for the relative serum. Each time point was tested twice. The differences in titers among the different groups at each time point were evaluated by Student's t test. The threshold of significance was $P < 0.05$.

Determination of frequencies for CTL precursors (CTLp). (i) Preparation of antigen-presenting cells. Lymphoblastoid B-cell lines (B-LCL) were established by the incubation of PBMC with supernatant from the S594 cell line producing herpesvirus papio (44) (kindly provided by R. Bontrop, Rijswijk, The Netherlands). Autologous B-LCL were infected for 18 h with rVV expressing either Env (v8672-m and v8789-m) (26), $p55^{gag}$ (SIVmac32H) (kindly provided by A. McMichel, Oxford, United Kingdom), or Nef (SIVmac32H clone pJ5) (47a) at a multiplicity of infection of 10 and then fixed in 1.5% PFA.

(ii) Limiting-dilution microcultures. Cryopreserved PBMC collected on the day of challenge and at week 9 or 12 postchallenge were thawed and seeded in parallel in serial dilutions ranging from 4×10^4 to 1×10^3 in RPMI 1640 (Gibco, Gaithersburg, Md.) containing 100 IU of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, and 2×10^{-5} M β-mercaptoethanol (referred to as complete medium) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah) (referred to as R-10). Cultures were set up in 24 replicate wells of 96-well round-bottom plates in a final volume of 100 μl per well. Autologous irradiated (2,500 rads) feeder PBMC were added at 10^4 per well. Responder cells were stimulated on days 0 and 7 of culture with autologous rVV-infected B-LCL expressing the appropriate SIV protein at 10^4 per well. Recombinant interleukin-2 at 10 U/ml was added to microcultures on days 3, 7, and 10. On day 14, cells from each well were resuspended and two 70-μl aliquots were screened for cytotoxicity in split-well CTL assays with either rVV expressing one of the SIV proteins or control wild-type vaccinia viruses (Copenhagen strain).

The phenotype of the expanded cell population was assessed on day 15 of culture by flow cytometry (fluorescence-activated cell sorter [FACS] analysis) with fluorescein isothiocyanate-conjugated anti-CD4 (Okt4; Ortho Diagnostics, Raritan, N.J.) and phycoerythrin-conjugated anti-CD8 (Leu 2a; Becton Dickinson, Etten-Leur, The Netherlands).

(iii) Cytotoxicity assay. Cytotoxicity was measured in standard 5-h sodium chromate (^{51}Cr) release assays. Autologous B-LCL infected for 16 h at a multiplicity of infection of 10 with either rVV expressing one of the SIV proteins or control wild-type vaccinia viruses (Copenhagen strain) were used as target cells. These were labelled for 1 h with 100 μCi of ^{51}Cr at 37°C in 5% CO_2, washed three times in complete medium, resuspended in R-10 at 10^5/ml, and added to effector cells in 50 μl (5,000 cells per well) in 96-well round-bottom plates (Costar). After a 5-h incubation at 37°C in 5% CO_2, supernatants were harvested with a Skatron harvester (Skatron, Oslo, Norway), and the release of ^{51}Cr was measured in a gamma counter. Maximum ^{51}Cr release was determined by detergent (5% Triton X-100) lysis of target cells. Spontaneous release was determined by the incubation of target cells in R-10 alone. Maximum ^{51}Cr release and spontaneous release were set up in 18 replicate wells. Spontaneous release was <25% of maximum ^{51}Cr release in all assays.

(iv) Calculation of CTLp frequencies. Individual wells were considered positive when the experimental release of specific targets but control targets exceeded spontaneous release by 3 standard deviations. CTLp frequencies were estimated by the method described by Strijbosch et al. (54). Frequencies were normalized to the number of CTLp per 10^6 plated cells.

Quantification of cell-associated virus load. Serial fivefold dilutions of freshly isolated PBMC in R-10 ranging from 1×10^6 to 1.6×10^3 were seeded in duplicate wells in the presence of 3 μg of concanavalin A per ml. PBMC were cocultured with the human T-cell line C8166 at 10^5 cells per ml. Recombinant interleukin-2 was added on day 3 at 10 U/ml. Cells were cultured for 6 weeks at 37°C in a humidified 5% CO_2 incubator. Cultures were refreshed twice a week. Supernatants were assayed regularly for $p27^{gag}$ by antigen capture ELISA (Organon Teknika). The number of infected cells was calculated from the highest positive dilution and expressed as the number of infected cells per 10^6 PBMC. The differences in the mean values of virus load among the different groups at each time point were evaluated by Student's t test. The threshold of significance was $P < 0.05$.

RESULTS

Antibody responses induced by the candidate SIV subunit vaccines. (i) SIV Env-specific serum antibodies. Monkeys were immunized intramuscularly with the different vaccine preparations at weeks 0, 4, 10, and 16 as outlined in Table 1. The first immunization induced Env-specific antibodies in three monkeys from group A and two monkeys from group B (Fig. 1A). The responses in all monkeys increased after the second and third immunizations but declined rapidly after each booster injection. After the fourth immunization, antibody titers returned to approximately the same levels as those reached after the third immunization, that is, at levels similar to those found after experimental infection. Env-specific antibody titers did not differ significantly between monkeys from groups A and B. No SIV Env-specific antibodies were found in the monkeys from control group C before challenge.

TABLE 1. Immunization schedule for cynomolgus macaques

Group	Monkeys	Immunogens[a]	μg/dose
A	769, K70, K81, K2	SIV Env iscom 8672-m	10
		SIV Env iscom 8789-m	10
B	K77, K80, K83, K88	SIV Env iscom 8672-m	10
		SIV Env iscom 8789-m	10
		SIV $p27^{gag}$ iscoms	10
		Nef lipopeptide 108–123 (LRTMSYKLAVDMSH)	1.0
		Nef lipopeptide 155–169 (DWQDYTSGPGIRYPK)	1.0
		Nef lipopeptide 164–178 (GIRYPKTGWLWKLV)	1.0
C	K66, K71, K73, K79	FIV Env iscoms	10

[a] Monkeys were immunized intramuscularly at weeks 0, 4, 10, and 16 and challenged intravenously at week 18 with 50 MID_{50} of the J5 molecular clone of SIVmac32H.

(ii) SIV Gag-specific serum antibodies. $p27^{gag}$-specific antibody levels were measured in two monkeys from group B after the first immunization and in all monkeys from group B after the second immunization (Fig. 1B). Titers increased after the second and third immunizations but declined rapidly after each booster injection. After the fourth immunization, antibody titers reached levels similar to those measured after the third immunization. No $p27^{gag}$-specific serum antibodies were found before challenge in monkeys from groups A and C.

(iii) VN serum antibodies. All monkeys from groups A and B developed VN antibodies to SIVmac32H upon immunization (Fig. 1C). These VN antibody titers were similar to or exceeded those observed after experimental SIVmac infection. VN antibody titers did not differ significantly between monkeys from groups A and B. In monkeys from control group C, no VN antibodies were found before challenge.

CTLp frequencies on the day of challenge. The abilities of candidate SIV subunit vaccines to induce SIV-specific CTLp were studied by limiting-dilution analyses of PBMC collected on the day of challenge (Table 2). The CTLp frequencies of replicate experiments fell consistently within the same range, with largely overlapping 95% confidence intervals. More specifically, the coefficient of variation for 12 replicate experiments of both Env- and Gag-specific CTL responses showed a mean of 8.1%. Relatively high levels of CTLp specific for Env, Gag, and Nef were detected in PBMC from group B monkeys K80 and K83, ranging from 7 to 105/10^6 PBMC. The highest CTLp frequencies were found for Env (105 and 25/10^6 PBMC, respectively). These responses were clearly vaccine induced since no specific CTLp were detected in PBMC collected from these monkeys before immunization. The other two monkeys in group B, K77 and K88, showed CTLp against two of the three proteins (K77, Gag- and Nef-specific CTLp; macaque K88, Env- and Gag-specific CTLp), albeit at lower levels (2 to 15/10^6 PBMC). In the monkeys from group A, only macaque

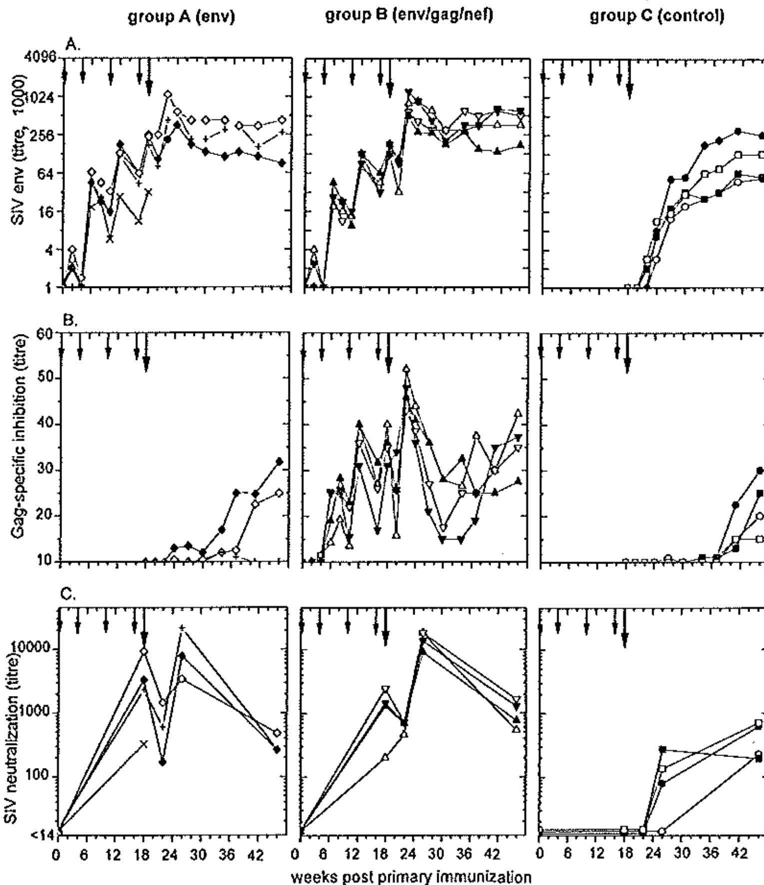

FIG. 1. Pre- and postchallenge SIV-specific antibody responses in group A, B, and C monkeys. (A) SIV Env-specific serum antibodies, as determined by indirect ELISA with SIVmac32H-derived Env as immobilized antigen. (B) SIV Gag-specific antibodies, as determined by inhibition ELISA. Bacterial $p27^{gag}$ was used as immobilized antigen. Biotinylated polyclonal serum of an SIVmac-infected monkey was used for inhibition. (C) SIVmac32H VN antibody responses. Serial dilutions of monkey plasma were incubated with SIVmac32H and added to C8166 cells. The number of negative wells was used to determine the 50% neutralizing dose endpoint titer for the relative plasma. The weeks of immunizations are indicated by small arrows. The day of challenge is indicated by a large arrow. Group A monkeys (Env immunized): ◆, 769; ◇, K70; +, K81; ×, K2. Group B monkeys (Env-Gag-Nef immunized): △, K77; ▽, K80; ▼, K83; ▲, K88. Group C monkeys (control): O, K66; ■, K71; ●, K73; □, K79. Monkey K2 (group A) died during recovery from anesthesia on the day of challenge.

769 exhibited low levels of Env-specific CTLp ($1/10^6$ PBMC). To investigate whether the CTL responses found were MHC restricted, cells expanded by Env-specific stimulation of PBMC from monkey K80 were tested against autologous and MHC class I-mismatched rVV Env-infected target cells. Lysis of the autologous target cells but not of the MHC class I-mismatched target cells was detected, indicating that the CTL responses induced were indeed predominantly MHC class I restricted (data not shown). In addition, with cells from the same monkey, FACS analysis was performed on day 15 with cultures exhibiting Env-specific CTLp activity. The majority of expanded cells proved to be of the $CD8^+$ phenotype ($CD8^+$, 69%; $CD4^+$, 17%).

SIV-specific serum antibodies after challenge. Env-specific antibody titers decreased during the first 2 weeks after challenge in the monkeys from groups A and B but showed an anamnestic response that peaked at week 4 or 6 postchallenge (Fig. 1A). Env-specific antibodies were also detected in the monkeys from control group C, starting at week 4 or 6 after infection and increasing gradually afterwards. In all three groups of monkeys, Env-specific antibody titers eventually reached a plateau at about the same level. VN antibody development in the monkeys from groups A and B also showed an anamnestic response (Fig. 1C). In the monkeys from group C, VN antibodies were induced following infection. With the exception of monkey K88, in all of the monkeys from group B,

TABLE 2. Pre- and postchallenge Env-, Gag-, and Nef-specific CTLp frequencies[a]

Group	Macaque	Frequency (CTLp/10^6 PBMC)[b] Day of challenge Env	Gag	Nef	Wk 9 or 12 postchallenge Env	Gag	Nef
A	769	1 (0–3)	ND	ND	8 (4–12)	2 (0–3)	0
	K70	0	ND	ND	3 (1–5)	1 (1–2)	0
	K81	0	ND	ND	13 (8–17)	10 (6–14)	0
B	K77	0	2 (0–5)	4 (0–10)	8 (0–16)	5 (0–10)	17 (10–24)
	K80	105 (80–130)	10 (5–15)	7 (3–11)	110 (96–151)	15 (9–20)	63 (49–76)
	K83	25 (16–34)	13 (4–22)	11 (5–17)	8 (4–12)	11 (6–16)	10 (4–16)
	K88	15 (4–26)	3 (0–8)	0	95 (74–116)	19 (7–30)	2 (0–6)

[a] Effector cells were obtained by specific stimulation with PFA-fixed autologous B-LCL infected with rVV expressing the respective SIV protein in the presence of recombinant interleukin-2. Split-well ^{51}Cr release assays were performed on day 14 of culture. Specific cytotoxicity was assayed with autologous B-LCL infected with either rVV expressing the SIV protein under investigation or wild-type control vaccinia virus. Data from limiting-dilution assays were analyzed with maximum likelihood and minimum chi-square methods.

[b] Env-, Gag-, and Nef-specific CTLp frequencies were also measured in the PBMC of K80, K83, and K88 (Env only) before immunization, and no CTLp were detected. The 95% confidence intervals are in parentheses. ND, not done.

p27^{gag}-specific plasma antibody titers declined 2 weeks after challenge but showed an anamnestic response that peaked at week 4 postchallenge (Fig. 1B). p27^{gag}-specific antibody responses were also induced after challenge in the monkeys from groups A and C.

SIV-specific CTLp frequencies after challenge. The frequencies of CTLp specific for Env, Gag, and Nef were measured at week 9 or 12 postchallenge in PBMC from group A and B monkeys (Table 2). Postchallenge CTLp measurements were carried out in parallel with those of prechallenge CTLp under identical culture and assay conditions. In monkey K80 (group B), the frequencies of Env- and Gag-specific CTLp were similar to those detected on the day of challenge, whereas the frequency of Nef-specific CTLp showed a marked increase. In monkey K83 (group B), the frequencies of Gag- and Nef-specific CTLp were similar to those measured on the day of challenge, whereas the frequency of Env-specific CTLp showed a slight decrease. Monkey K88 (group B) showed an increase in both Env- and Gag-specific CTLp after challenge and the induction of relatively low levels of Nef-specific CTLp. In monkey K77 (group B), Env-specific CTLp were induced after challenge. In the same monkey, Gag- and Nef-specific CTLp were detected, with a marked increase in the latter. Env-specific CTLp were detected in all of the monkeys from group A after challenge. In the same group, Gag-specific CTLp were detected at relatively low (monkeys 769 and K70) or high (monkey K81) frequencies. No Nef-specific CTLp were demonstrated in any monkey from group A.

Cell-associated virus loads. After challenge with 50 MID_{50} of the J5 molecular clone of SIVmac32H, all animals became infected, as illustrated by repeated isolation of SIV from their PBMC (Fig. 2). SIV was isolated from all monkeys at week 2 postchallenge. Longitudinal evaluation of cell-associated virus loads showed that the number of SIV-infected cells in circulation fluctuated over time, with a peak at 2 or 4 weeks after challenge. Furthermore, virus loads differed considerably within and among the groups. The mean value of virus load for the monkeys from control group C was consistently higher than that for the vaccinated groups, with the exception of the values at week 12. However, the differences in virus loads did not reach statistical significance.

DISCUSSION

In this paper, we have shown that SIV subunit vaccines consisting of Env glycoproteins incorporated into iscoms either

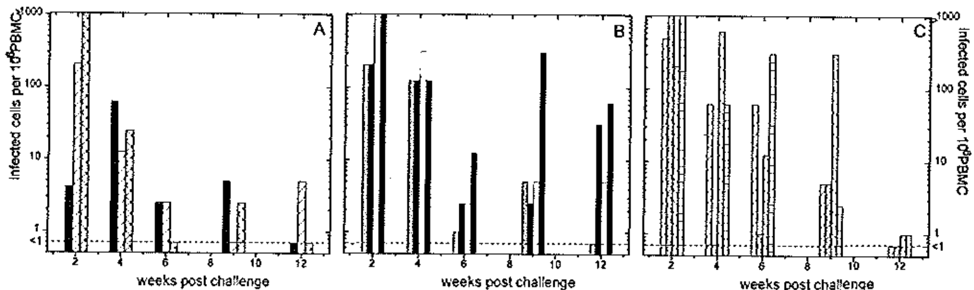

FIG. 2. Cell-associated virus loads in the PBMC of SIV-vaccinated (groups A [A] and B [B]) and control (group C [C]) monkeys during a 12-week follow-up period. Serial fivefold dilutions of PBMC ranging from 10^6 to 960 for week 2 postchallenge and from 10^6 to 1,600 for all other time points were incubated with C8166 cells for 6 weeks. The number of infected cells per 10^6 PBMC was calculated from the highest dilution that was positive in a p27^{gag} antigen capture ELISA. Group A (Env) monkeys: ▬, 769; ▭, K70; ▭, K81. Group B (Env-Gag-Nef) monkeys: ▭, K77; ▬, K80; ▭, K83; ▬, K88. Group C (control) monkeys: ▭, K66; ▭, K71; ▭, K73; ▭, K79.

alone or in combination with p27^{gag} iscoms and Nef lipopeptides failed to generate protection against intravenous SIVmac challenge, despite the induction of VN antibodies and CTL responses.

The two Env glycoproteins used for immunization, 8789-m and 8672-m, shared 98.6 and 97.0% amino acid sequence homology, respectively, with the Env protein of the J5 molecular clone of SIVmac32H used for challenge (26). Because most of the VN antibodies detected in sera from infected monkeys recognize conformational epitopes (28), it is considered important that recombinant Env proteins are presented to the immune system in a form that most closely resembles the native conformation. In fact, using a panel of 15 monoclonal antibodies recognizing both conformational and linear epitopes, we have recently shown that the antigenicity of the recombinant Env proteins used in this study is similar to that of virus-derived Env protein (26). Furthermore, we found here that the VN antibody titers induced by the two recombinant Env proteins incorporated into iscoms in group A and B monkeys were similar to or exceeded those found after experimental SIVmac infection.

VN antibodies were measured with the 32H strain of SIVmac cultured in C8166 cells. It has previously been shown that antibodies which neutralize SIVmac32H also efficiently neutralize the J5 molecular clone derived from this virus (11a). The VN antibody titers on the day of challenge did not show inverse correlations with the levels of virus load measured after challenge. This is in agreement with previous studies carried out with the SIV macaque model, which failed to demonstrate a correlation between VN antibody titers and resistance to experimental SIV infection (13, 18, 24, 40). It should be pointed out, however, that in all of these studies, VN assays were based on the neutralization of virus propagated in T-cell lines. There are clear indications that the neutralization of HIV and FIV propagated in susceptible cell lines is more easily accomplished than neutralization of virus propagated on primary lymphocyte cultures (4, 48, 52). In light of these observations, one may speculate that the biological significance of the VN antibodies measured in our vaccinated monkeys is limited. The development of VN assays with monkey PBMC and non-cell-line-adapted SIV might help to demonstrate biologically more significant antibodies.

The method we used for the determination of CTLp frequencies was established in studies carried out with HIV-1-infected individuals (21, 33, 56) as well as SIVmac-infected monkeys (20a). The frequencies of SIV Env-, Gag-, and Nef-specific CTLp were measured in parallel assays of PBMC from the day of challenge and from week 9 or 12 postchallenge. Replicate experiments showed good reproducibility of CTLp frequency estimates.

The use of both iscoms and lipopeptides proved to be effective in inducing SIV-specific CTL responses in the monkeys from group B. As can be expected for analysis of an outbred population of monkeys, the frequencies of SIV-specific CTLp differed considerably among similarly immunized monkeys. The highest CTLp frequencies were those for the Env protein and appeared to be similar to those previously observed for SIVmac251-infected macaques (58). The vaccine-induced Gag- and Nef-specific CTLp frequencies were lower but comparable to those found by Yasutomi et al. in both vaccinated and SIVmac-infected macaques (60).

To achieve in vitro expansion of SIV-specific CTLp, autologous B-LCL infected with rVV expressing the respective SIV proteins and fixed in PFA were used for specific antigen stimulation. This protocol has previously been shown to selectively expand MHC class I-restricted CD8$^+$ CTL against the Gag protein of HIV-1 (56). The same approach has now also proved to selectively expand MHC class I-restricted CD8$^+$ CTL against SIV antigens (20a). In agreement with previous findings, the Env-specific cytotoxic response of monkey K80, which showed the highest frequency after vaccination, appeared to be predominantly directed against autologous targets but not MHC class I-mismatched targets, suggesting that the killing was mediated by MHC class I-restricted CTL rather than by NK cells (46). In addition, in the same monkey the majority of cells expanded by Env-specific antigen stimulation showed a CD8$^+$ phenotype. To our knowledge, this is the first demonstration of the induction of CTL responses in primates with antigens incorporated into iscoms.

In the monkeys from group A (immunized with Env iscoms alone), only one showed low-level Env-specific CTLp responses on the day of challenge. However, the ability of these monkeys to mount a CTL response against the Env protein was demonstrated by the detection of Env-specific CTLp after challenge. Although the number of monkeys in group A is too small to allow drawing any further conclusions, it may be speculated that in the monkeys from group B, simultaneous immunization with different SIV antigens may have improved the induction of Env-specific CTL, for example, by a mechanism of increased virus-specific T-helper-cell activity (49).

After challenge, an increase in SIV-specific CTLp was observed in some cases. This variability is in line with observations that the frequencies and kinetics vary considerably among infected monkeys (20a). This observation, also made for HIV-1-infected individuals (33), may reflect various degrees of homogeneity of CTL populations induced by vaccination or infection (11, 32, 38).

Upon immunization with the three Nef lipopeptides, Nef-specific CTLp were found in three of four monkeys. These monkeys had not been selected on the basis of their MHC haplotypes. Similarly, Bourgault et al. (6) noted that 6 of 12 unselected macaques showed CTL responses after immunization with a set of peptides also derived from the central region of Nef. This indicates that this region, like the corresponding region of HIV-1 Nef (14, 15), is recognized by CTL in the context of a range of different MHC class I haplotypes, stressing its potential for vaccine development purposes.

Nef has previously been shown to be an efficient CTL target in SIV-infected macaques; 8 of 12 monkeys displayed Nef-specific CTL responses (58). In our study, a similar number (four of seven monkeys) was found to display Nef-specific CTL activity after infection. Interestingly, no Nef-specific CTLp were detected at 12 weeks postchallenge in the monkeys from group A, whereas in the monkeys from group B (which had been immunized with the Nef lipopeptides), Nef-specific CTLp frequencies were either highly increased (K77 and K80), maintained (K83), or induced (K88) following infection. This finding allows speculation of a priming effect exerted by immunization with the three Nef lipopeptides in the monkeys from group B.

In SIV-infected monkeys, the presence of SIV-specific CTL has been shown to correlate with a better clinical outcome of infection, suggesting that vaccine-induced CTL contribute to protective immunity (8, 58). In one of our previous vaccine studies, four of eight monkeys appeared to be protected from challenge with cell-associated SIVmac (19). MHC class I-associated protection was observed; all of the animals protected against cell-associated virus challenge shared the MHC class I allele Mamu-A26 with the monkey cells used for challenge (24). This suggested that virus-specific CTL directly recognized the SIV-infected donor cells in an MHC-restricted manner. In this light, it is interesting that SIV-vaccinated Mamu-A26-pos-

itive monkeys, like the monkeys in this study, were not protected from challenge with cell-free, monkey-cell-grown SIVmac. In agreement with our observations, Yasutomi et al. have recently shown that a vaccine-elicited CTL response specific for a single viral epitope does not protect macaques from SIVmne challenge (60). They proposed the presence of CTL with only one specificity and the absence of VN antibodies as an explanation for the lack of protection. In this study, however, vaccine-induced VN antibodies and CTLp specific for one regulatory and two structural proteins apparently did not protect monkeys from experimental SIVmac infection. If CTL responses do play a significant role in mediating protection against SIV infection, it may be argued that CTLp frequencies should reach very high levels to exert a protective effect. On the other hand, CTL responses may have a beneficial effect on the course of infection despite their inability to clear SIV infection. In fact, although all monkeys were still clinically healthy 1 year after challenge, preliminary data suggest that the absence of CTLp early in infection may indeed correlate with a more rapid decrease in total CD4 counts later in infection.

An evaluation of the kinetics of cell-associated virus loads showed no significant differences among the respective groups of monkeys during a 12-week follow-up period. Indeed, we found that the virus loads fluctuated considerably over time. In fact, a higher mean virus load was measured in the PBMC of control monkeys than in those of the monkeys from groups A and B, especially at week 6 postchallenge. However, the opposite was true at week 12 postchallenge. This indicates that kinetic studies rather than measurements at one time point are required when virus loads are considered as a parameter for protection.

Taken together, our results show that candidate SIV subunit vaccines based on iscoms and lipopeptides efficiently induced specific VN antibodies and CTLp. However, the presence of VN antibodies and CTLp specific for multiple SIV proteins on the day of challenge proved to be insufficient to protect monkeys from intravenous SIVmac challenge. These findings indicate that the type of immunity needed to prevent infection may be quite different from that thought to control persistent infection with primate lentiviruses.

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Immunization with envelope glycoproteins of primary human immunodeficiency virus type 1 (HIV-1) isolates does not protect macaques against homologous challenge

Ellen G. J. Hulskotte,[1] Anna-Maria Geretti,[1,2] Marlinda E. M. Dings,[1] Geert van Amerongen,[3] Francois Malet,[4] Rob A. Gruters,[1,5] and Albert D. M. E. Osterhaus[1]

Institute of Virology, Erasmus University, Rotterdam, The Netherlands[1], Department of Virology, Royal Free Hospital School of Medicine , London, UK[2], Central Animal Laboratory, National Institute of Public Health and the Environment, Bilthoven, The Netherlands[3], Ecole Normale Supérieure de Lyon, Lyon, France[4], and UMR103, CNRS-Biomérieux, Ens de Lyon, France[5].

Submitted

SUMMARY

Chimeric simian-human immunodeficiency viruses (SHIVs) used as challenge viruses in macaques provide a new tool for testing the protective potential of candidate HIV-1 vaccines. Here, four cynomolgus macaques were immunized with iscoms containing recombinant envelope glycoproteins (Env) derived from either a syncytium inducing or a non-syncytium inducing HIV-1 primary isolate from the same patient. After four immunizations, the vaccinated animals and two control monkeys were challenged intravenously with $SHIV_{320.3.1}$, carrying an HIV-1 Env also derived from this patient. On the day of challenge, HIV-1 V3-loop and CD4 binding domain specific antibodies were detected. In addition, $SHIV_{320.3.1}$ neutralizing antibodies and HIV-1 Env specific cytotoxic T lymphocyte precursors (CTLp) were demonstrated in all immunized monkeys. After $SHIV_{320.3.1}$ challenge, levels of virus replication were low, as virus could be recovered from only one of the two control monkeys during 13 weeks of follow up. Nevertheless, both control monkeys developed $SHIV_{320.3.1}$ specific antibodies and CTLp. Absence of $SHIV_{320.3.1}$ specific antibody responses and of both culturable and PCR detectable virus suggested that three of the four vaccinated macaques were protected from challenge. However, the demonstration of CTLp against multiple $SHIV_{320.3.1}$ antigens, including the non-structural proteins Rev and Tat, indicated that also in these three animals replication of the challenge virus had not been prevented. Although HIV-1 Env-based vaccination strategies may be effective in limiting virus replication, they appear to be unable to induce sterile immunity against intravenous infection.

INTRODUCTION

One of the major obstacles in the development of a vaccine against HIV-1 has been the lack of a suitable animal model for HIV-1 infection that would allow the screening of the protective potential of candidate HIV-1 vaccines. Although chimpanzees, gibbons, pig tailed macaques and human-mouse chimeric animals have all been infected successfully with HIV-1, the use of these models is hampered by major drawbacks including limited availability, ethical concerns, and limited HIV-1 pathogenicity (for review see Heeney, 1996). The construction of SHIVs, which carry HIV-1 Env and replicate in macaques, offers the unique opportunity to test the potential of HIV-1 Env-based vaccines in macaques (Li et al., 1995; Dunn et al., 1996; Joag et al., 1996; Luciw et al., 1995; Reimann et al., 1996; Igarashi et al., 1994; Ranjbar et al., 1997).

Virtually all HIV-1 Env-based vaccine formulations tested so far used envelope glycoproteins derived from T cell line adapted HIV-1 isolates (Johnston, 1996). These are known to differ in primary sequence, structure and biological properties from viruses isolated

directly from peripheral blood mononuclear cells (PBMC) (McKeating, 1996). With respect to vaccine development, the most significant difference is probably the relative resistance of primary HIV-1 isolates to antibody-mediated virus neutralization (VN) (Moore *et al.*, 1995; Poignard *et al.*, 1996). Based on their replication characteristics and cellular host range, primary HIV-1 isolates can be divided into non syncytium inducing (NSI) - usually macrophage tropic - variants, and syncytium inducing (SI) - usually T cell line tropic - variants. NSI and SI strains have been shown to differ in cellular co-receptor usage, sequences of the VN inducing variable regions V2 and V3, and the level of conservation within V3 (D'Souza & Harden, 1996; Groenink *et al.*, 1993; Bosch *et al.*, 1994; De Jong *et al.*, 1992b; Fouchier *et al.*, 1992; De Jong *et al.*, 1992a; Simmonds *et al.*, 1991; Kuiken *et al.*, 1992; Groenink *et al.*, 1991; Chesebro *et al.*, 1992). Furthermore, NSI strains have been found to predominate in the asymptomatic stage of infection, whereas a predominance of SI strains is observed in about 50% of HIV-1 infected individuals progressing to AIDS (Koot *et al.*, 1992; Zhu *et al.*, 1993; Zhang *et al.*, 1993). These progressive changes in phenotype and tropism indicate the existence of structural differences in antigenic determinants and mechanisms of escape from VN antibodies, which may have major implications for vaccine development.

In a previous study we tested the ability of SIV-Env vaccines to protect macaques against intravenous SIV_{mac} challenge (Hulskotte *et al.*, 1995a). Although no protection was observed, the view that SIV specific CTL immunity may have contributed to the rapid control of virus replication in at least some of the animals studied remains an attractive and plausible hypothesis (Hulskotte *et al.*, 1995a; A.M. Geretti 1997, unpublished). As SIV-Env differs in several structural, biological, and immunologic properties from HIV-1 Env (Burns & Desrosiers, 1994), we designed a study in which cynomolgus macaques were immunized with different HIV-1 Envs before challenge with SHIV. To investigate potential differences in the immunogenicity of SI and NSI derived envelope glycoproteins, two groups of monkeys were immunized with Envs derived from either an NSI or an SI HIV-1 biological clone, both originated from PBMC of the same seropositive individual. The antigens were presented in iscoms to allow efficient induction of both VN antibody and CTL responses (Rimmelzwaan & Osterhaus, 1995). The macaques were subsequently challenged with a molecularly cloned SHIV (Ranjbar *et al.*, 1997), carrying the envelope glycoprotein of an HIV-1 molecular clone derived from the same seropositive individual.

MATERIALS AND METHODS

Generation and characterization of HIV-1 Env iscoms. (i) Construction of recombinant vaccinia viruses (rVV). Env genes of two HIV-1 biological clones 320.2a5 and 320.2a6, isolated directly from PBMC of an asymptomatic HIV-1 infected individual (#320) from the Amsterdam cohort

of homosexual men (de Wolf *et al.*, 1987) were used as starting material (Andeweg *et al.*, 1992). The two Env display 86.1% amino acid sequence identity. 320.2a5 but not 320.2a.6 displays syncytium-inducing (SI) capacity (Groenink *et al.*, 1991; Andeweg *et al.*, 1992); they are further referred to as 320.SI and 320.NSI, respectively. To facilitate incorporation of the hydrophillic outermembrane gp120 part into iscoms (Rimmelzwaan *et al.*, 1994), amino acid substitutions were introduced into the cleavage site by site-directed mutagenesis (518K-N, 520R-T, 527R-S). RVV expressing the cleavage site mutated Env were designated v320.SI and v320.NSI respectively. Expression of full-length Env precursor was verified by western blot analysis.

(ii) Preparation of HIV-1 Env iscoms. HIV-1 320.SI-Env and 320.NSI-Env, derived from rVV v320.SI and v320.NSI respectively, and control Env of feline immunodeficiency virus (FIV) strain AM19 (Rimmelzwaan *et al.*, 1994), were produced and incorporated into iscoms using a method described previously (Hulskotte *et al.*, 1995b) with minor modifications. Briefly, 22 h after rVV infection of BHK21 cells, cells were harvested and inactivated in 1.5% paraformaldehyde (PFA) for 15 min (Hulskotte *et al.*, 1997). Env proteins were solubilized from the cell membrane using 4% n-Octyl-β-d-glycopyranoside (Sigma, Zwijndrecht, The Netherlands), purified by lentil-lectin chromatography, and incorporated into iscoms consisting of purified Quil-A components (Iscoprep 703[R]; Iscotec, Luleå, Sweden). Formation of iscom particles was confirmed by electron microscopy.

(iii) Quantification of HIV-1 Env in iscoms. Quantification of HIV-Env was carried out by an ELISA based on a previously described protocol (Hulskotte *et al.*, 1995b). Recombinant HIV-1_{IIIB} gp160 (rgp160; Cat.No. 24001 ABT, USA) was used as a standard.

(iv) Characterization of HIV-1 Env preparations by ELISA. *Antibodies and envelope glycoproteins.* The human monoclonal antibodies (HuMabs) used included: CD4bd specific HuMabs GP68 (Schutten *et al.*, 1993) and IgG1-B12 (Burton *et al.*, 1994), HIV-1 V3 specific HuMabs 257-D (Gorny *et al.*, 1993) and 391/95D (Gorny *et al.*, 1993), IAM-2G12 which recognizes a conformational sensitive gp120 epitope unrelated to the V1, V2, or V3 loop or to the CD4-binding site (Trkola *et al.*, 1996), and IAM-2F5 which is specific for a relatively conserved epitope on gp41 (ELDKWAS) (Muster *et al.*, 1993). *ELISA.* The procedures followed the protocol described above using 300 ng/ml of the HIV-1 Env under investigation or a control lysate of BHK21 cells infected with wild-type vaccinia virus in PBS containing 0.1% n-Octyl-β-d-glycopyranoside and serial two-fold dilutions of the HuMabs. The HuMab concentration giving 50% of the maximum optical density at 450 nm (OD_{450}) obtained for that HuMoab was taken as a relative measure for affinity. Differences obtained with the same monoclanal antibody (Mab) on different HIV-1 Env preparations were considered significant if more than 3 times difference in relative affinity was observed.

Animals and immunization procedure. This study included six colony-bred juvenile cynomolgus macaques *(Macaca fasicularis)* seronegative for SIV, type D retrovirus, and simian T-lymphotropic virus. Macaques were allocated randomly to three groups of two monkeys each. Animals were immunized intramuscularly at weeks 0 and 4 with 10 µg and at weeks 10 and 18 with 20 µg of HIV-1 Env iscoms (320.NSI-Env; monkeys K9 and #135, 320.SI-Env; monkeys #K84 and #144) or control FIV Env iscom preparations (monkeys #127 and #106).

$SHIV_{320.3.1}$ challenge. Two weeks after the fourth immunization, the monkeys were infected intravenously with 1500 $TCID_{50}$ of the cell-free 2/95 pool of $SHIV_{320.3.1}$ (kindly supplied by Drs. N. Almond and S. Jones, Potters Bar, United Kingdom), which is constructed and referred to as $SHIV_{w61D}$ by Ranjbar *et al.* (1997). The challenge stock had been propagated on CEMX174 cells. The HIV-1 envelope used to generate this SHIV was derived from another clone (320.3.1) of the same HIV-1 seropositive individual #320 from whom the Env used for immunization were derived (Groenink *et al.*, 1991). The $SHIV_{320.3.1}$-Env displays 81.7% and 97.5% amino acid sequence identity with the outer membrane part of the 320.NSI- and 320.SI-Env used for immunization, respectively. HIV-$1_{320.3.1}$ exhibits an SI phenotype and tropism for both macrophages and T cell lines (Groenink *et al.*, 1991).

Serology. (i) Binding of monkey sera to different HIV-1 Envs. Serum reactivity with different HIV-1-Envs was assayed using an ELISA based on an assay developed by Moore & Jarrett

(1988). Wells coated with capture antibody D7324 (Aalto BioReagents, Dublin, Ireland) were blocked for 45 min with 0.1 M Tris pH 7.5/0.1 M NaCl containing 0.1% Tween-20, 0.1% Triton X-100, 2% normal sheep serum, 2% FBS (referred to as E-buffer) supplemented with 10% sonicated lysate of monkey herpes virus papio transformed B-cell lines (4.10^7 cells/ml; HP-lysate), and subsequently incubated with 300 ng/ml of HIV-Env or a control lysate of BHK21 cells infected with wild-type vaccinia virus in E-buffer containing 4% HP-lysate for 2 h at 37°C. RVV expressing the following HIV-1 Env were used: 320.NSI and 320.SI (derived of biological clone 320.2a.6 and 320.2a.5 respectively (Andeweg *et al.*, 1992)), 168.NSI and 168.SI (kindly provided by Dr. G. Rimmelzwaan, Rotterdam, The Netherlands; derived from NSI and SI virus isolates, respectively, obtained from HIV-1 infected individual #168 (De Jong *et al.*, 1992b; Tersmette *et al.*, 1989)) and HIV-1_{IIIB} (vSC25, kind gift of B. Moss and C. Chakrabarti, Bethesda, USA). Monkey sera at a solution of 1:25 were pre-incubated in a sonicated lysate of BHK21 cells (4.10^7 cells/ml; BHK-lysate) for 1 h at 37°C. Plates were washed and incubated with serial two-fold serum dilutions starting at 1:50 in E-buffer supplemented with 2% of BHK-lysate for 1.5 h at RT, followed by an 1 h incubation with affinity isolated goat F(ab')2 anti human IgG gamma chain peroxidase conjugate (Biosource, Camarillo, USA) for 1 h at RT. The substrate reaction was carried out with 3,3',5,5'-tetramethyl-benzidine (TMB; Sigma). Endpoint titers were calculated using a cut-off value threefold above background values. The same protocol was used to determine the kinetics of $SHIV_{320.3.1}$-Env specific serum antibodies. As $SHIV_{320.3.1}$ was grown on CEMX174 cells, here BHK-lysate was replaced by CEMX174-lysate.

(ii) Serum reactivity with HIV-1 V3 loop peptides. Reactivity of monkey sera and pooled sera of HIV-1 seropositive individuals (HIV-1 pool) to V3 was measured by using an inhibition ELISA using V3-peptides as described before (2). As viral sequences within the gp120 V3 region are associated with SI or NSI viral phenotypes, different V3-mixotypes consisting of peptides based on NSI, NSI-intermediate, SI-intermediate or SI V3 consensus sequences were used (8,9,13,20,33,54): NSI, CTRPNNNTRKSI-H/P/T/N-IGPGRAFYTTG-E/D/Q-IIGDIRQAHC; NSI-intermediate, CTRPNNNTRKGIHIGPGRA-F/I-YTTG-E/N/D/Q-IIGDIRQAHC; SI-intermediate, CTRPNNNTRK-G/S-I-H/R/Y-IGPGRA-I/V/F-Y/V/H/L-TT-E/G/R-K/R-IIGDIRQAHC;SI,CTRPNNNTRKRI-H/T/R/Y-IGPGRA-F/Y-Y/V/H-TT-G/K/R-Q/K/R-IIGDIRQAHC.

(iii) Inhibition of IgG1-B12 binding. RVV expressed 320.2a.6 HIV-1 Env was adsorbed onto D7324 coated 96-well microtiter plates as described above. Subsequently, wells were incubated with 1 to 50 dilutions of monkey sera pre-incubated in BHK-lysate for 1 h at RT. Fifty µl were discarded from each well before adding 50 µl of biotinylated IgG1-B12. After incubation for 1.5 h at RT, wells were washed and incubated with HRPO-conjugated streptavidin. TMB was used for substrate reactions. Inhibition percentages were calculated on the basis of the formula: % inhibition = [(OD_{450} IgG1-B12 - OD_{450} background) - (OD_{450} test serum - OD_{450} background)] : (OD_{450} IgG1-B12 - OD_{450} background) x 100 %. Inhibition of >15% was considered significant.

(iv) Neutralization of HIV-1 infectivity. Neutralization was evaluated in an infectivity reduction assay measuring the effect of serum on the virus endpoint titer, expressed as tissue culture infectious dose ($TCID_{50}$). This method was adapted from Hogervorst *et al.* (1995) and Mascola *et al.* (1996). Phytohemagglutinin-stimulated peripheral blood lymphocytes (PHA-blasts) or CEMX174 cells (10^5) were used as target cells. One to 20 dilutions of sera and serial 3-fold dilutions of virus stock in R-10 containing 20 IU/ml recombinant interleukin-2 (rIl-2) (20 µl serum dilution and 20 µl virus) were pre-incubated in quadruplicate wells. After 30 min at 37°C, target cells in 40 µl R-10 containing 20 IU/ml rIl-2 were added to each well and incubated for 20 h at 37°C/5% CO_2. Subsequently, cells were washed 3 times and maintained in 200 µl R-10 containing 20 IU/ml rIl-2. For HIV-1_{IIIB} (MRC AIDS directed programme), at day 7 cultures were harvested and tested for the presence of p24 core antigen by an anti-p24 capture ELISA (McKeating *et al.*, 1991) using as conjugate the p24 specific Mab 14D4E11 (Janvier *et al.*, 1990). For $SHIV_{320.3.1}$, half of the supernatant was exchanged with fresh medium at day 4. At day 10 cultures were harvested and tested for the presence of HIV-1 Env using the D7324 capture ELISA described above. Background values were set by calculating the mean OD_{450} of

the negative control wells (containing cells without virus and serum) plus three times the standard deviation (SD). $TCID_{50}$ were calculated using the method of Spearman-Karber. $TCID_{50}$ reduction was defined as the ratio of the $TCID_{50}$ in the presence of normal monkey serum to the $TCID_{50}$ in the presence of test serum.

(v) Serum reactivity with SIV-Gag. A Gag specific inhibition assay was used as described previously (Hulskotte *et al.*, 1995a). Inhibition of binding of a biotinylated polyclonal serum, 1YO, of a SIV infected monkey to Gag-p27 by a tenfold dilution of the respective monkey sera was measured. Inhibition percentages were calculated on the basis of the formula: % inhibition = [(OD_{450} 1YO - OD_{450} background) - (OD_{450} test serum - OD_{450} background)] : (OD_{450} 1YO - OD_{450} background) x 100 %.

Determination of CTL precursor (CTLp) frequencies. CTLp frequencies were determined as described (Geretti *et al.*, 1997; Hulskotte *et al.*, 1995a). Briefly:

(i) Preparation of Antigen Presenting Cells (APC). Autologous B-cell lines (B-LCL) infected with rVV expressing either HIV-1 Env (v320-NSI or v320-SI as indicated), HIV-1_{IIIB}Tat (TG3196; Transgene, Strasbourg, France), HIV-1_{IIIB} Rev (TG4113; Transgene), SIVmac32H $p55^{gag}$ (kindly provided by Prof. A. McMichel, Oxford, United Kingdom), or SIVmac32H(J5) Nef (kindly provided by Drs. E.W. Rud and M. Mackett) and fixed in 1.5% PFA were used as APC.

(ii) Limiting-dilution microcultures. Freshly isolated or cryopreserved lymphocytes were seeded in R-10 in at least five dilutions of 24 replicate wells from $4x10^4$ to $4x10^3$ cells/well. Cells were cultured with autologous irradiated (2,500 rads) feeder PBMC (10^4/well, day 0), APC expressing the protein under investigation (10^4/well, day 0 and 7), and rIL2 from day 3.

(iii) Cytotoxicity assay. On day 14, aliquots from each well were screened for cytotoxicity in split-well 5 h ^{51}Cr-release assays. Autologous B-LCL infected with rVV expressing the viral protein under investigation or control wild-type vaccinia virus (vSC65, Rimmelzwaan *et al.*, 1994) or 186poly (Transgene)) were used as targets. Maximum release was determined by detergent lysis (5% Triton X-100) of target cells. Spontaneous release was determined by incubation of target cells in R-10 alone. Spontaneous release was less than 30% of maximum release.

(iv) Calculation of CTLp frequencies. Individual wells were considered positive when the experimental release exceeded spontaneous release of specific targets but control targets by three standard deviations. CTLp frequencies were estimated by the maximum likelihood method using a statistical software (Strijbosch *et al.*, 1988), that included a X^2 goodness-of fit test statistics. Frequencies were normalized to the number of CTLp per 10^6 cells. Limiting dilution assays of PBMC isolated from the monkeys before exposure to either HIV or SIV antigens showed CTLp frequency estimates consistently $<2/10^6$ PBMC.

Virus detection. (i) Virus isolation. Virus isolation was performed by cocultivation of freshly isolated PBMC, their CD8-depleted fractions, lymph node mononuclear cells (LNMC), spleen mononuclear cells (SPMC), or thymocyte,s with 10^5/ml CEMX174 cells in 3 μg/ml ConA. Recombinant Il-2 was added on day 3 at 10 IU/ml. Cultures were refreshed twice a week and kept for 6 weeks. Alternatively, virus isolation was performed by cocultivation of PBMC with mitogen stimulated PBMC using a viral load assay. A total of 2.10^6 freshly isolated PBMC were seeded at 10^5 and $3.3x10^4$ cells per well in fifteen replicates in 96-well flat-bottomed plates in the presence of 3 μg/ml ConA, 50 IU/ml rIl-2 and $2x10^4$ human PHA-blasts. At day 3, 100 μl medium was removed and 150 μl R-10 containing 50 IU/ml rIl-2 was added. At days 7, 14, 21 and 35 cultures were fed by replacing part of the old cells by fresh human PHA-blasts. Supernatants were assayed regularly by antigen capture ELISA for the presence of HIV-1 Env or SIV Gag p27 (V5-p24 antigen kit, Organon Teknika, Boxtel, The Netherlands, or Coulter SIV core antigen assay, Coulter Electronics, Mijdrecht, The Netherlands, respectively).

(ii) Nested-Gag PCR. To investigate the presence of $SHIV_{320.3.1}$ DNA, a nested PCR amplifying a 500 bp fragment of SIV Gag p27 followed by hybridization with a radioactive labelled Gag specific probe was used as described (Geretti *et al.*, 1997).

(iii) Plasma p27 antigen detection. SIV p27 core antigen in plasma was quantified by antigen-capture ELISA (Coulter Electronics, Mijdrecht, The Netherlands).

TABLE 1. Relative affinities of HuMabs for HIV-1 320.NSI and 320.SI Env

	CD4bd		V3		gp120	gp41
Envelope protein	GP68	IgG1-B12	257-D	391/95D	2G12	2F5
320.NSI	650[a]	114	25	157	118	3500
320.NSI cm[b]	700	136	12	147	175	2425
320.NSI cm PFA[c]	1000	200	32	200	262	3575
320.SI	400	342	25	60	198	3275
320.SI cm	-[d]	-	45	126	250	2450
320.SI cm PFA	-	-	70	115	85	1375

[a] Concentrations of monoclonal antibody (ng/ml) giving 50% of maximal binding.
[b] cm, after cleavage site mutation
[c] PFA, after PFA fixation
[d] OD_{450} values barely above background levels at the concentrations tested.

RESULTS

Antigenicity of recombinant HIV-1 Env preparations. The antigenicity of the 320.NSI and SI recombinant HIV-1 Env was studied by determining the relative affinities of a panel of HuMabs selected for their ability to neutralize primary HIV-1 isolates (Burton *et al.*, 1994; Schutten *et al.*, 1995; Trkola *et al.*, 1995; Schutten *et al.*, 1997) (Table 1). Both Env preparations reacted with antibodies against the CD4bd (Mabs GP68 and IgG1-B12), the V3 loop (Mabs 257-D and 391/95D), a conformational epitope on gp120 (Mab IAM-2G12), and a linear epitope on gp41 (Mab IAM-2F5). Cleavage site mutation abolished the reactivity of CD4bd specific HuMabs with the 320.SI-Env but not with the 320.NSI-Env. PFA fixation did not significantly affect the relative affinities of any of the antibodies.

Vaccine-induced HIV-1 Env specific serum antibody responses. (i) Kinetics of HIV-Env specific serum antibody response. After the second immunization, all four HIV-1 Env immunized monkeys developed serum antibodies that recognized the $SHIV_{320.3.1}$ Env (Fig. 1). Antibody titers increased after the third immunization, decreased subsequently, to increase again after the fourth immunization. The monkeys vaccinated with 320.SI-Env developed higher antibody titers and showed more pronounced booster responses than the animals vaccinated with 320.NSI-Env (Fig. 1). No HIV-1 Env specific antibodies were found in the control monkeys (not shown).

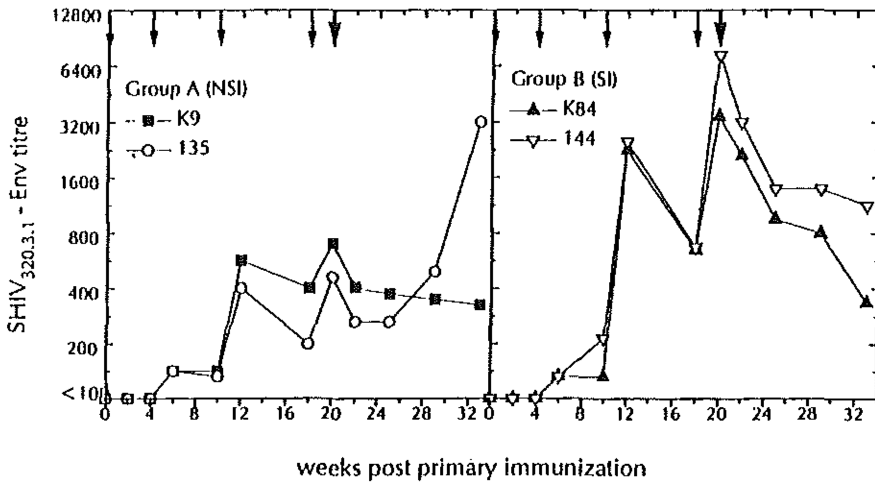

FIG. 1. Pre- and post-challnge $SHIV_{320.3.1}$-Env specific antibodies. Small arrows indicate immunisations; the large arrow indicates the day of challenge.

(ii) Reactivity of sera with different primary and T-cell line adapted HIV-1 Env preparations. Sera collected on the day of challenge were tested for their ability to bind to primary and T-cell line adapted HIV-1 Env preparations (Fig.2A). All monkeys developed the highest antibody titers against the Env used for immunization. Sera from the 320.NSI-Env immunized monkeys, but not sera from 320.SI-Env immunized monkeys, recognized the T cell line adapted $HIV\text{-}1_{IIIB}$-Env. Both groups of monkeys showed serum reactivity with 168.NSI and 168.SI Env, derived from an NSI and an SI inducing primary HIV-1 isolate respectively. The overall binding of the sera was lower than that observed with the respective #320 Env used for immunization. The sera from 320.NSI-Env immunized monkeys generally bound better to the NSI Env than to their SI counterparts. However, this was not the case for the binding of the sera from the 320.SI-Env immunized monkeys to the 168.SI protein.

(iii) Serum reactivity with the HIV-1 V3 loop. Serum reactivity with the HIV-1 V3 loop was studied on the day of challenge by a V3 peptide inhibition assay (Fig. 2B). The peptides used were V3 mixotypes representing NSI, SI and intermediate phenotype consensus sequences (De Jong *et al.*, 1992b; Fouchier *et al.*, 1992; De Jong *et al.*, 1992a; Groenink *et al.*, 1991; Simmonds *et al.*, 1991; Kuiken *et al.*, 1992). All monkeys developed V3 specific antibodies. However, neither immunization with NSI-Env nor immunization with SI-Env preferentially induced serum reactivity with the concordant consensus V3 sequences. In contrast, reactivity of pooled sera from HIV-1 seropositive individuals was preferentially inhibited by the NSI V3 consensus peptide.

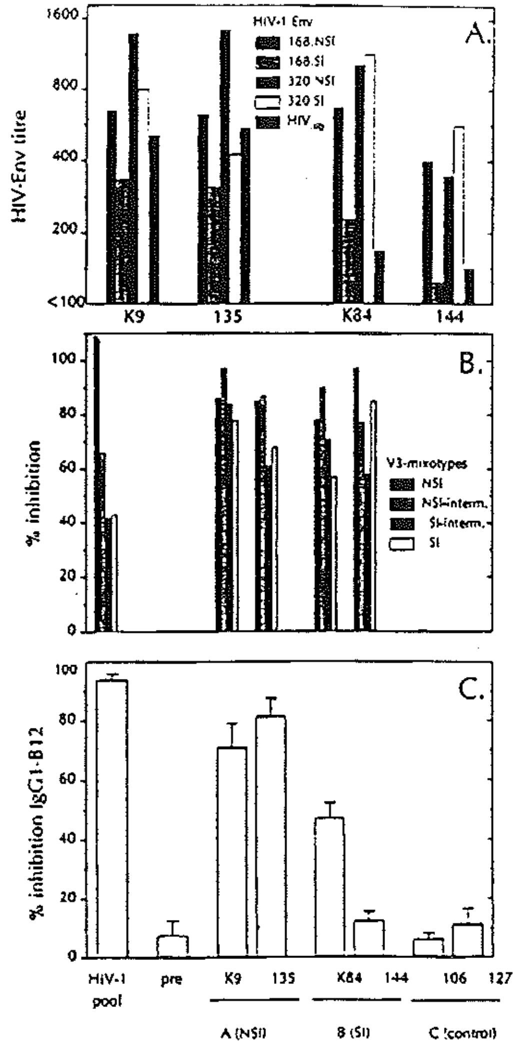

FIG. 2. Env specific antibody responses of sera collected either on the day of challenge or prior to vaccination, and of pooled sera of HIV-1 seropositive individuals (HIV-1 pool). (*A*) Env specific antibody responses against different HIV-1 Env. 320.NSI- and 320.SI- were primary isolates of seropositive individual #320 and their Env were identical to those used for immunization (Andeweg *et al.*, 1992; Groenink *et al.*, 1991); 168..NSI and 168.SI were primary isolates of seropositive individual #168 (de Jong *et al.*, 1992b; Tersmette *et al.*, 1989); HIV-1_{IIIB} is T-cell line adapted. (*B*) V3 specific antibodies, determined by inhibition ELISA. Inhibition of binding of a 1:100 serum dilution HIV-1 Env by different V3 mixotypes consisting of NSI, NSI-intermediate, SI-intermediate, or SI V3 consensus sequences is indicated. Pre-sera and serum of control monkeys were not able to bind to HIV-Env and therefore no reactivity is shown. (*C*) CD4bd specific antibodies, determined by inhibition of binding of the CD4bd specific HuMab IgG1-B12 to HIV-1 Env by 50-fold diluted serum samples. The standard error of the mean of three experiments is presented as a vertical line.

(iv) Inhibition of HIV-1 CD4bd specific HuMab binding. On the day of challenge, reactivity with the CD4bd of HIV-1 was studied by testing the serum ability to inhibit binding of HuMab IgG1-B12 to HIV-1 Env (Fig. 2C). Pre-immunization sera and sera from control monkeys did not significantly inhibit IgG1-B12 binding. Sera from the 320.NSI-Env immunized monkeys exhibited high inhibition levels, similar to those found in the pooled sera of HIV-1 seropositive individuals. In contrast, the monkeys immunized with the 320.SI-Env showed no (#144) or low (#K84) inhibition. This may have been due to the loss of CD4bd antigenicity in the 320.SI-Env used for immunization (Table 1).

(v) Virus neutralizing antibodies. The development of antibodies showing neutralizing activity towards $SHIV_{320.3.1}$ and T cell line adapted HIV-1_{IIIB} was analyzed with an HIV-1 reduction neutralization assay (Fig. 3). By the day of challenge, all HIV-1 Env vaccinated animals had developed VN antibodies to the $SHIV_{320.3.1}$ challenge virus. Neutralization levels in 320.SI-Env vaccinated monkeys exceeded those found in 320.NSI-Env immunized monkeys. In contrast, no significant neutralizing activity had developed against the T cell line adapted HIV-1_{IIIB} when measured on either primary human PHA-blasts or on a T-cell line (CEMX174). The serum pool from HIV-1 seropositive individuals neutralized both $SHIV_{320.3.1}$ and HIV-1_{IIIB}.

Vaccine-induced HIV-1 Env specific CTLp. By the day of challenge, no clear differences in CTLp induction had emerged between the 320.SI- and 320.NSI-Env immunized monkeys (Table 2). Relatively high CTLp frequencies were detected in one animal of each group (36 and $13/10^6$ PBMC, respectively). Although the other two animals showed relatively low CTLp frequencies (5 and $3/10^6$ PBMC, respectively), these responses were vaccine induced since no specific CTLp ($<2/10^6$) were detected in naive PBMC collected either from the same monkeys before immunization or from the control monkeys of group C (not shown).

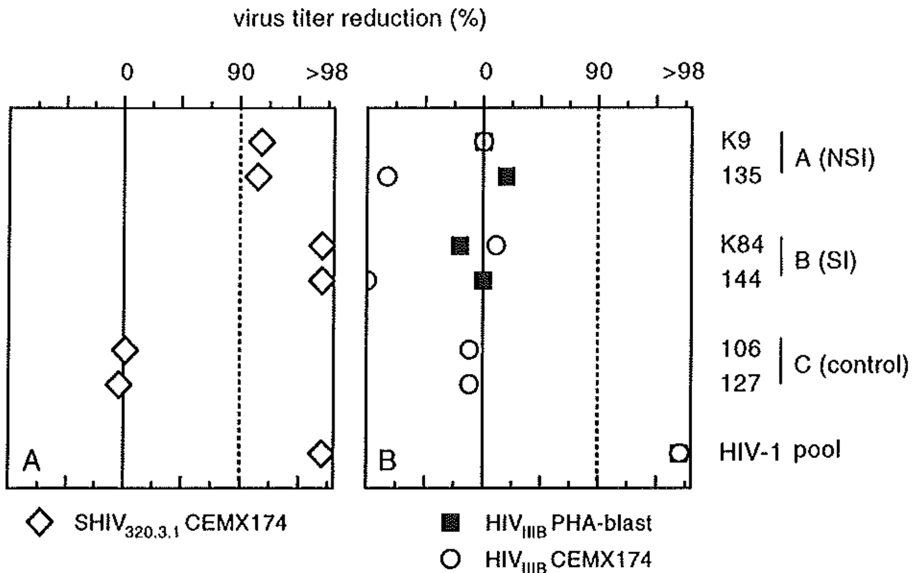

FIG. 3. VN-antibody responses against $SHIV_{320.3.1}$ *(A)* and HIV-1_{IIIB} *(B)* measured by a virus neutralization reduction assay. For HIV-1_{IIIB} reduction in virus titer on CEMX174 cells and PHA-blasts was measured. As a consequence of low-level replication in human PHA-blasts, for $SHIV_{320.3.1}$ reduction was only measured in CEMX174 cells.

TABLE 2. SHIV specific CTLp frequencies (CTLp/10^6cells) on day of challenge and week 13 postchallenge (pc)[a].

Group	Monkey	Sample	day of challenge	week 13 pc				
			HIV Env	SIV Gag	HIV Env	SIV Nef	HIV Tat	HIV Rev
A NSI	K9	PBMC	36 (19-52)	4 (1-7)	19 (12-27)			
		LN		21 (14-29)				
		spleen		39 (25-53)		20 (9-30)	15 (4-26)	10 (3-17)
	135	PBMC	5 (2-8)	5 (2-8)	32 (18-45)	0	5 (1-10)	0
		LN		3 (1-6)				
		spleen		12 (5-18)				
B SI	K84	PBMC	13 (7-20)	14 (4-24)	2 (0-4)	0	8 (3-13)	20 (11-29)*
		LN		5 (1-9)				
		spleen		8 (4-12)				
	144	PBMC	3 (1-5)	5 (2-9)	10 (3-6)	0	15 (4-26)	0
		LN		5 (1-9)				
		spleen		0				
C	127	PBMC	nd	1 (0-2)	7 (3-11)	4 (0-9)	14 (2-26)	0
		LN		5 (1-9)				
		spleen		4 (2-7)*				
	106	LN	nd	0				
		spleen		0	10 (6-15)	0	0	0

[a]Effector cells were obtained by specific stimulation with PFA fixed autologous B-LCL infected with rVV expressing the protein under investigation (in case of Env; 320.SI-Env for macaques K84, 144 and 127, 320.NSI-Env for macaques K9, 135 and 106) in the presence of rIl-2. Split-well ^{51}Cr release assays were performed on day 14 of culture. Specific cytotoxicity was assayed with autologous B-LCL infected with either rVV expressing the HIV-1 env protein under investigation or wild-type control vaccinia virus. Data from limiting-dilution assays were analyzed with the maximum likelihood method. 95% confidence intervals are given in parentheses. The cut-off value for CTL was determined at < $2/10^6$ (not shown). For all frequency calculations X^2 goodness-of-fit values were always <7, except for the two samples indicated by *, which goodness-of-fit was poor and were analyzed by the minimum chi square methond, not done.

$SHIV_{320.3.1}$ specific CTLp frequencies after challenge. We studied the development of virus specific CTL upon $SHIV_{320.3.1}$ challenge. The frequencies of CTLp specific for HIV-1 Env, Tat, Rev, and for SIV Gag and Nef were measured at week 13 post challenge in PBMC, LNMC or SPMC (Table 2). Pre- and post-challenge CTLp measurements were carried out in parallel using identical culture and assay conditions. CTLp in naive PBMC were always below $2/10^6$ cells (not shown). Consistent with the serological data, CTLp specific for multiple $SHIV_{320.3.1}$ antigens were found in both control monkeys and in the 320.NSI-Env vaccinated monkey #135 (Table 2). Surprisingly, the three other immunized monkeys also developed CTLp against multiple SHIV antigens, whihc were indicative of low-level virus replication. At week 13 post challenge, all monkeys showed CTLp (>$2/10^6$) specific for one or more SHIV proteins (Table 2). HIV-1 Env specific CTLp were induced in both control monkeys. CTL against HIV-1 Tat and Rev were detected in five out of six and two out of six animals

respectively, whereas CTL against SIV Gag and Nef were detected in five out of six and two out of six monkeys respectively. CTLp frequencies in most cases ranged between 5 and $15/10^6$. The highest CTLp frequency was $32/10^6$. After challenge, the frequencies of Env specific CTLp increased in HIV-Env vaccinated monkeys #144 and #135, but decreased in monkeys #K84 and #K9 (Tables 3 and 4).

To study the effect of $SHIV_{3203.1}$ challenge on CTLp induction in different lymphoid compartments, SIV Gag specific CTLp were determined in parallel limiting dilution analyses of PBMC, LNMC and SPMC. SIV Gag specific CTLp were detected in all three compartments in monkeys #K9, #135, and #K84. Monkey #144 showed relatively low SIV Gag specific CTLp in PBMC and LNMC but not in SPMC. Monkey #127 showed relatively low SIV Gag specific CTLp in LNMC and SPMC but not in PBMC.

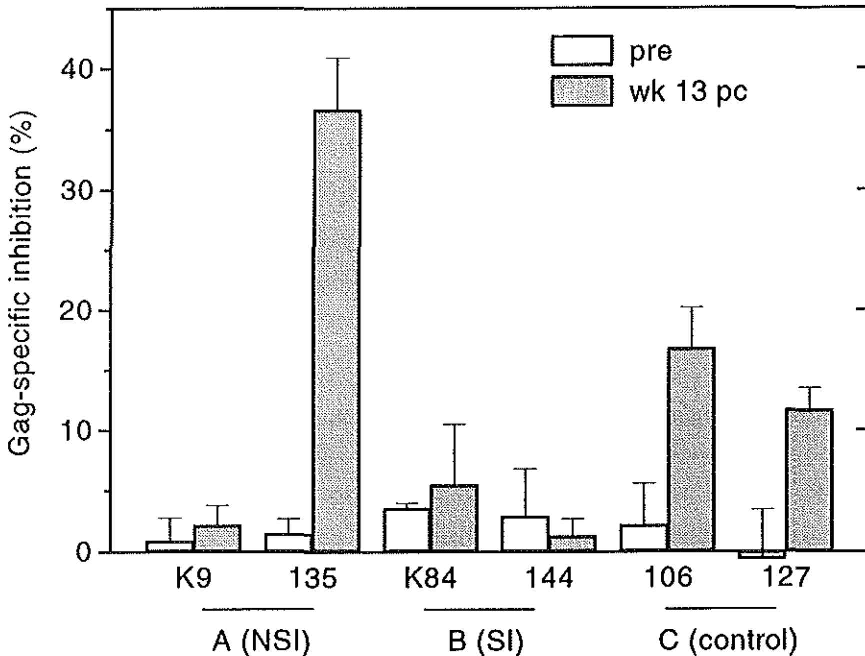

FIG. 4. Gag specific antibodies, determined by inhibition ELISA. Bacterial p27-gag was used as immobilized antigen. The percentage inhibition of a biotinylated polyclonal serum of a SIVmac-infected monkey by a tenfold dilution of monkey sera collected at 13 weeks post challenge (wk 13 pc) or prior to vaccination (pre) is shown.

TABLE 3. Longitudinal evaluation of the presence of SHIV in PBMC of HIV-Env vaccinated (groups A and B) and control monkeys (group C) in the first 13 weeks post challenge (pc)

Time pc	Macaques					
	A (NSI)		B (SI)		C (control)	
	K9	135	K84	144	127	106
day 3	- -*	- -	- -	- -	- -	- -
day 6	- -	- -	- -	- -	- -	- -
day 10	- -	- -	- -	- -	- -	- -
week 2	- -	- -	- -	- -	- -	- -
week 3	- -	+ -	- -	- -	+ -	- -
week 5	- -	- -	- -	- -	- -	- -
week 7	- -	+ nd	- nd	- nd	- nd	- nd
week 9	- nd	+ nd	- nd	- -	+ nd	- nd
week 13	- -	+ +	- -	- -	+ -	- -

*Virus detection by PCR and virus isolation respectively. The presence of $SHIV_{320.3.1}$ DNA in PBMC was assayed by a nested PCR. + indicates virus detection; - indicates lack of virus detection; nd, not done.

Virus detection after challenge. After challenge, $SHIV_{320.3.1}$ was recovere from PBMC (Table 3) and lymphoid tissues (Table 4) of the 320.NSI-Env vaccinated monkey #135 and control monkey #127. In monkey #135, proviral DNA could be detected in PBMC at weeks 3, 7, 9 and 13 after challenge. Virus was detected by virus isolation and PCR in the lymph node biopsy obtained at week 7 post challenge and in samples of lymphoid tissues (LNMC, SPMC, thymus-, bone-marrow cells) obtained at week 13 after challenge. The virus burden appeared to be less pronounced in monkey #127 : proviral DNA could repeatedly be detected in PBMC and in cells derived from the axillary and mesenteric lymph nodes obtained at weeks 7 and 13 post challenge respectively, but not in cells of the axillary and inguinal lymphnode, spleen, thymus and bone-marrow obtained at week 13 post challenge. In addition, virus could be isolated from LNMCax taken at week 7, but not from PBMC or from lymphoid tissue samples obtained at week 13 after challenge. In the other monkeys, repeated attempts failed to isolate virus from PBMC, their CD8-depleted fractions, LNMC, SPMC or thymocytes, or to detect SIV p27 core antigen in plasma (not shown). In addition, no proviral DNA could be detected at any time point in their PBMC, LNMC, SPMC, thymocytes or bone-marrow cells.

TABLE 4. Virus detection by PCR and virus isolation techniques in lymphoid tissues of HIV-Env vaccinated (groups A and B) and control (group C) monkeys at week 7 and 13 post challenge

Weeks pc	Tissue	Monkeys					
		A (NSI)		B (SI)		C (control)	
		K9	135	K84	144	127	106
7	LN_{ax}	- -*	+ +	- -	- -	+ +	- -
13	LN_{ax+in}	- nd	+ nd	- nd	- nd	- nd	- nd
13	LN_{mes}	- -	+ +	- -	- -	+ -	- -
13	spleen	- -	+ +	- -	- -	- -	- -
13	thymus	- -	+ +	- -	- -	- -	- -
13	BM	- nd	+ nd	- nd	- nd	- nd	- nd

*Virus detection by PCR and virus isolation respectively. LN: lymph node; ax: axillary , in: inguinal; mes: mesenteral; BM: bone-marrow. + indicates virus detection; - indicates lack of virus detection; nd, not done.

DISCUSSION

In the present paper, we have studied the efficacy of subunit HIV-1 vaccines based on SI or NSI envelope proteins incorporated into iscoms. Upon challenge with $SHIV_{320.3.1}$, one NSI-Env immunized monkeys became productively infected. In contrast, one other NSI- and the two SI-Env immunized monkeys were apparently protected from infection, as indicated by the absence of $SHIV_{320.3.1}$-specific antibody responses and the inability to detect $SHIV_{320.3.1}$ by virus isolation and PCR. However, the pattern of $SHIV_{320.3.1}$-specific CTLp development in the apparently protected animals indicated that also in these animals virus replication had not been prevented completely. In both control monkeys, $SHIV_{320.3.1}$ infection induced specific antibodies and CTLp. However, levels of virus replication were low, as during the 13 weeks of follow up, $SHIV_{320.3.1}$ could only be detected in one of the two control monkeys. The observation that even after challenge with such a poorly replicating virus no complete protection could be obtained, extends our previous findings in the SIV-macaque model (Hulskotte *et al.*, 1995a): in this model we showed that immunization with SIVmac Env iscoms or preparations containing SIVmac Env iscoms, Gag iscoms and Nef lipopeptides also induced VN antibodies and CTLp, but failed to induce protection against a pathogenic SIVmac challenge.

Most vaccine development strategies have used HIV-1 envelope glycoproteins of T-cell line adapted viruses, which generally induce poor VN activity against primary HIV-1 isolates (Johnston, 1996). For our study we have chosen envelope glycoproteins from primary viruses, which were selected from NSI and SI clones of one HIV-1 infected individual. The

procedures we have used to generate and purify the envelope glycoproteins have previously been shown to result in oligomeric proteins of HIV-1_{IIIB} (Earl *et al.*, 1990). Iscoms incorporating the NSI- and SI- Envs produced in this way were shown to induce neutralizing antibodies against $SHIV_{320.3.1}$, which carries an HIV-1 envelope derived from the same individual from whom also the SI and NSI Envs originated. As it may be expected from the higher degree of amino acid sequence homology between the 320.SI-Env and $SHIV_{320.3.1}$-Env(97.5%) than between the 320.NSI-Env and $SHIV_{320.3.1}$-Env (81.7%), $SHIV_{320.3.1}$-Env specific as well as VN antibody responses were higher in the monkeys immunized with the former protein. It is interesting to note that the only vaccinated animal that became productively infected had been vaccinated with the NSI-Env and had developed the lowest $SHIV_{320.3.1}$-antibody responses.

In contrast to the neutralizing activity detected against $SHIV_{320.3.1}$, no neutralizing activity against HIV-1_{IIIB} was observed in the sera of the vaccinated monkeys (Fig. 3). This may be due to the high amino acid sequence difference between the HIV-1_{IIIB}-Env and the immunizing Envs (Myers *et al.*, 1993). However, HIV-1_{IIIB} was effectively neutralized by pooled sera from seropositive individuals infected with viruses probably as distant from HIV-1_{IIIB} as the viruses of individual #320 (Fig. 3). Therefore, quantitative and qualitative differences in antibody induction upon natural infection and immunization should also be considered to explain this observation.

The sera from the immunized monkeys were also tested for their antibody reactivity against major VN inducing sites. All monkeys developed antibodies against the V3 loop. Although differences in V3 are associated with an NSI and SI phenotype (De Jong *et al.*, 1992b; Fouchier *et al.*, 1992; De Jong *et al.*, 1992a; Groenink *et al.*, 1991; Simmonds *et al.*, 1991; Kuiken *et al.*, 1992), sera of monkeys immunized with Envs containing either NSI or SI V3 loop sequences did not show a clear difference in their recognition of the consensus NSI or SI V3 loop sequences (Fig. 2B). Sera of HIV-1 infected asymptomatic individuals, which can be expected to harbour mainly NSI virus strains (Schuitemaker *et al.*, 1992), did preferentially recognize the consensus NSI V3 loop sequences. Therefore, it may be speculated that maturation of the antibody response or long term exposure is necessary to develop a more specific V3 response. CD4bd reactive antibody responses appeared to be higher in the NSI-Env immunized monkeys, which is not unexpected considering the loss of the corresponding antigenic site in the SI-Env preparation due to cleavage site mutation (Table 1).

Besides the development of virus specific antibodies, we also studied the development of specific CTL responses after vaccination and challenge. HIV-1 specific CTL have often been demonstrated in HIV-1 infected humans in the absence of *in vitro* restimulation (Cease & Berzofsky, 1994). In contrast, the frequencies of circulating CTLp in SIV-infected or vaccinated macaques is usually too low to allow detection in the absence of

in vitro expansion (Geretti *et al.*, 1997; Venet *et al.*, 1992). In previous studies we have investigated the optimal conditions for the measurement of CTL in cynomolgus macaques (Geretti *et al.*, 1997). Limiting dilution analyses of cells expanded by antigenic stimulation was shown to allow reproducible detection of SIV specific CTLp also in monkeys lacking significant responses in standard bulk CTL assays. Therefore, this approach was chosen to study the potential of the candidate HIV-1 Env iscom vaccines to induce HIV-1 Env specific CTLp in monkeys.

As expected from our previous experiments (Hulskotte *et al.*, 1995a), the use of iscoms proved to be effective in inducing HIV-1 Env specific CTLp in all the immunized cynomolgus macaques, at frequencies ranging from 3 to $36/10^6$ PBMC. Although lower than those generally found in HIV-1 infected individuals (Geretti *et al.*, 1995; Klein *et al.*, 1995), these frequencies are similar to those measured in SIVmac infected macaques (Gallimore *et al.*, 1995; Venet *et al.*, 1992; Geretti *et al.*, 1997). In particular, in our previous study of SIVmac32H(J5) infected cynomolgus macaques, CTLp measurement by the same method gave SIV Env specific CTLp frequencies of less than $30/10^6$ in 8 out of 10 monkeys tested (A.M. Geretti, unpublished results).

Although only the two control monkeys and one NSI-Env vaccinated monkey (i.e. #135) showed evidence of productive $SHIV_{320.3.1}$ infection by virus detection or antibody induction, after challenge all monkeys developed CTLp against one or more of the SHIV proteins Env, Gag, Nef, Tat, and Rev. It is unlikely that these were induced by the mere presence of antigen in the challenge material, as the stimulation protocol we used has been shown to preferentially expand major histocompatibility complex class I restricted $CD8^+$ CTL *in vitro* (Geretti *et al.*, 1997), which generally require *de novo* synthesis of viral antigen for their priming (Rock, 1996). Furthermore, most animals also developed CTL against Rev or Tat, which are not present in the virion. Taken together, these findings indicate that $SHIV_{320.3.1}$ had indeed replicated in the animals after challenge. In addition, they suggest that CTLp determination is a more sensitive technique to demonstrate virus replication than virus isolation, PCR and antibody measurements. It may be speculated that the SHIV-specific CTLp contributed to protection against productive infection. However in this small group of animals no correlation was found between either protein specificity or CTLp frequency and the outcome of challenge.

One of the major problems of using first generation SHIVs, as we did in these experiments, is their relatively poor replicative capacity *in vivo*. Although in monkey #127 $SHIV_{320.3.1}$ could be demonstrated by virus isolation and PCR, for monkey #106 only indirect proof of virus replication was obtained, by showing the induction of antibody and CTLp. Where and how long the challenge virus actively replicated in this animal, can only be speculated from our data. Most likely virus replication occurred in lymphoid tissues for a

limited period of time, since the virus could neither be demonstrated in PBMC nor in the lymphoid tissues examined at the time of euthanasia. Clearly, transfer of larger segments of HIV-1 into an SIV backbone, or additional rapid passages of SHIV in macaques, may have led to viruses with a higher replicative capacity in macaques (Li *et al.,* 1995; Luciw *et al.,* 1995; Reimann *et al.,* 1996; Joag *et al.,* 1996; Mooij *et al.*, 1997). However, a drawback of the latter approach is the possible divergence of the envelope of the challenge virus from the original HIV-1 envelope. Still, the levels of protection achieved in our study proved to be more limited than those induced by Env-based vaccination protocols followed by homologous challenge with a SHIV that had indeed been serially passaged in monkeys (Mooij et al., 1997). Differences in immunization schedules, vaccine formulation, challenge doses and animal species may all have been at the basis of the different degrees of protection achieved.

In evaluating the protective capacity of the HIV-1 Env based vaccination strategy used here, it should be born in mind that natural HIV-1 infection in humans usually occurs via the mucosal route which is not very efficient (Jones and Curran, 1994). Moreover, low virus load is associated with reduced probability of the virus being transmitted from one individual to another (Cao *et al.,* 1997). These considerations implicate that even a limited degree of protection induced by vaccination, may be important to control virus replication and thus spread of HIV-1 in humans.

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Chapter 3

Characterization of CTL responses against primate lentiviruses

3.1 Preservation of phenotype and function of positively selected virus-specific $CD8^+$ T lymphocytes following anti-Fab detachment from immunomagnetic beads. *Journal of Immunological Methods (1993)161:129-133.*

3.2 Simian immunodeficiency virus (SIV)-specific $CD8^+$ cytotoxic T lymphocyte responses of naive and vaccinated cynomolgus macaques infected with SIVmac32H-J5: quantitative analysis by in vitro antigenic stimulation. *Vaccine (1997) 15:1269-1275.*

3.2 $CD8^+$ cytotoxic T lymphocytes of a cynomolgus macaque infected with simian immunodeficiency virus (SIV)mac32H-J5 recognize a nine amino acid epitope in SIV Gag p26. *Journal of General Virology (1997) 78:821-824.*

Short communication

Preservation of phenotype and function of positively selected virus-specific CD8$^+$ T lymphocytes following anti-Fab detachment from immunomagnetic beads

A.M. Geretti, C.A.C.M. Van Els, C.A. Van Baalen, M.C.M. Poelen and A.D.M.E. Osterhaus

Laboratory of Immunobiology, National Institute of Public Health and Environmental Protection, 3720 BA Bilthoven, Netherlands

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Comparison of anti-Fab with overnight detachment of measles virus- or HIV-specific CD8$^+$ T lymphocytes, positively selected with immunomagnetic beads, showed that the anti-Fab detachment procedure is a more rapid, efficient and reproducible method, which avoids downregulation of the targeted surface marker.

Key words: CD8$^+$ T cell, human; Immunomagnetic separation; Detachment; Cellular immune response; Measles virus; HIV

Introduction

In recent years, the cell fractionation procedure with antibody-coated magnetic beads developed by Ugelstad (Ugelstad et al., 1980) has been widely applied to both positive and negative separation of T lymphocyte subsets for use in functional studies. Previous reports (Funderud et al., 1990) suggested that overnight incubation of rosetted cells is an efficient method for cell-bead detachment following positive selection. Recently, an alternative method of detachment has been proposed, based on the competitive action of a goat anti-mouse Fab antiserum on the cell-bead interaction. This method proved successful for the isolation of B lymphocytes with preservation of both phenotype and function (Rasmussen et al., 1992). We have applied the anti-Fab detachment procedure to the isolation of CD8$^+$ cytotoxic T lymphocytes (CTL) with specificity for MV or HIV. The method was evaluated for both freshly isolated and cultured T cells and proved to be a rapid, efficient, and reproducible procedure yielding highly pure and functionally intact T lymphocyte subpopulations.

Correspondence to: A.M. Geretti, Laboratory of Immunobiology, National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven, Netherlands. Tel.: 31-30-742219; Fax: 31-30-250493.

Abbreviations: MV, measles virus; HIV, human immunodeficiency virus; B-LCL, B lymphoblastoid cell lines; CTL, cytotoxic T lymphocyte; rVV, recombinant vaccinia virus; ER, experimental release; SR, spontaneous release; MR, maximal release; *E*:*T*, effector-to-target cell ratio; FMF, fluorescence mediated flow cytometry; mAb, monoclonal antibody.

Materials and methods

PBMC were obtained by Ficoll-Hypaque gradient centrifugation of blood from healthy individuals, from patient JP suffering from acute measles infection, and from five asymtomatic HIV seropositive individuals. Magnetic beads coated with anti-CD8 mAb (Dynabeads M-450, Dynal,

Oslo, Norway) were used at various target cell-to-bead ratios to fractionate lymphocytes into CD8-enriched and CD8-depleted T cell subpopulations, according to the manufacturer's instructions. For spontaneous cell-bead detachment, rosetted cells were resuspended in RPMI 1640 containing penicillin (100 U/ml), streptomycin (100 μg/ml), 10^{-5} M 2-ME and 2 mM L-glutamine (referred to as complete medium) and incubated overnight at 37°C (Funderud et al., 1990). For anti-Fab induced detachment, rosetted cells were resuspended in 200 μl of complete medium and incubated for 60 min in the presence of 10–15 μl of anti-mouse Fab polyclonal antiserum (DETACHaBEAD, Dynal). Detached cells were harvested on a magnetic separation device (MPC-6, Dynal).

Cytolytic activity was determined in standard sodium chromate (^{51}Cr) release assays. Briefly, fractionated and unfractionated effector cells were incubated with infected or control ^{51}Cr-labeled target cells at various $E:T$ ratios in duplicate wells (experimental release, ER). Spontaneous ^{51}Cr release (SR) (target cells plus medium) and maximal ^{51}Cr release (MR) (target cells plus 5% Triton X-100) were determined in quadruplicate wells. Supernatants were harvested after a 4 h incubation at 37°C and analysed in a gamma counter. The percentage of specific lysis was calculated according to the formula: (ER–SR/MR–SR)·100. SR never exeeded 25% MR.

Results and discussion

Yields and purity of T cell subsets

We fractionated PBMC from five healthy individuals with anti-CD8 coated beads using a 10:1 bead to target cell ratio. Yields by anti-Fab detachment consistently exceeded 55% of the targeted cells. In contrast to previous reports (Rasmussen et al., 1992), yields obtained after detachment by overnight incubation were more variable and less efficient, ranging from 20% to 45%. With both detachment procedures, positively selected subpopulations consisted of over 99% T cells as assessed by FMF, whereas contamination of the negative fraction by targeted cells was always below 3%. Cell viability for both enriched and depleted fractions consistently exceeded 95% as determined by dye exclusion counting.

Cell surface expression of CD3 and CD8 markers

The overnight detachment procedure led to decreased surface expression of the marker targeted for fractionation, as we observed with both positively selected CD8$^+$ (Fig. 1) and CD4$^+$ (not shown) T lymphocytes. A similar observation has been reported previously with positively selected B lymphocytes (Funderud et al., 1990; Rasmussen et al., 1992). This may be due to cross-linking and downregulation of the surface marker as a consequence of prolonged physical interaction with the immunomagnetic beads. In contrast, the surface expression of the CD8 marker was found to be unaffected on the cells detached with the anti-Fab procedure. Neither procedure affected the expression of the CD3 marker. Two representative examples are shown in Fig. 1.

Functional studies

We compared the lytic activity of MV-specific CD8$^+$ CTL detached by either the anti-Fab or the overnight incubation procedure. Bulk PBMC were obtained from patient JP 3 weeks after the onset of clinical symptoms of acute measles, and expanded in vitro by two cycles of stimulation with autologous MV infected B-LCL, as previously described (Van Binnendijk et al., 1989, 1990). As shown by FMF, the stimulated bulk PBMC consisted of predominantly CD3$^+$ T lymphocytes (87%). The percentages of the CD8$^+$CD4$^-$, CD8$^-$CD4$^-$, and CD8$^-$CD4$^+$ subsets were 56%, 26% and 5%, respectively. Cells were fractionated with anti-CD8 coated beads, detached by either the anti-Fab or the overnight incubation procedure, and tested for their lytic activity against autologous MV-infected B-LCL. As illustrated in Fig. 2*A*, the MV-specific cytotoxic activity detected in the bulk PBMC was preserved in the CD8$^+$ subset and no significant difference in activity between cells detached by the anti-Fab and or by the overnight incubation procedure was detected. Similar results were observed with the CD8$^+$ MV-specific CTL clone JP, obtained from the same donor (Van Binnendijk et al., 1989, 1990). This clone was used because of

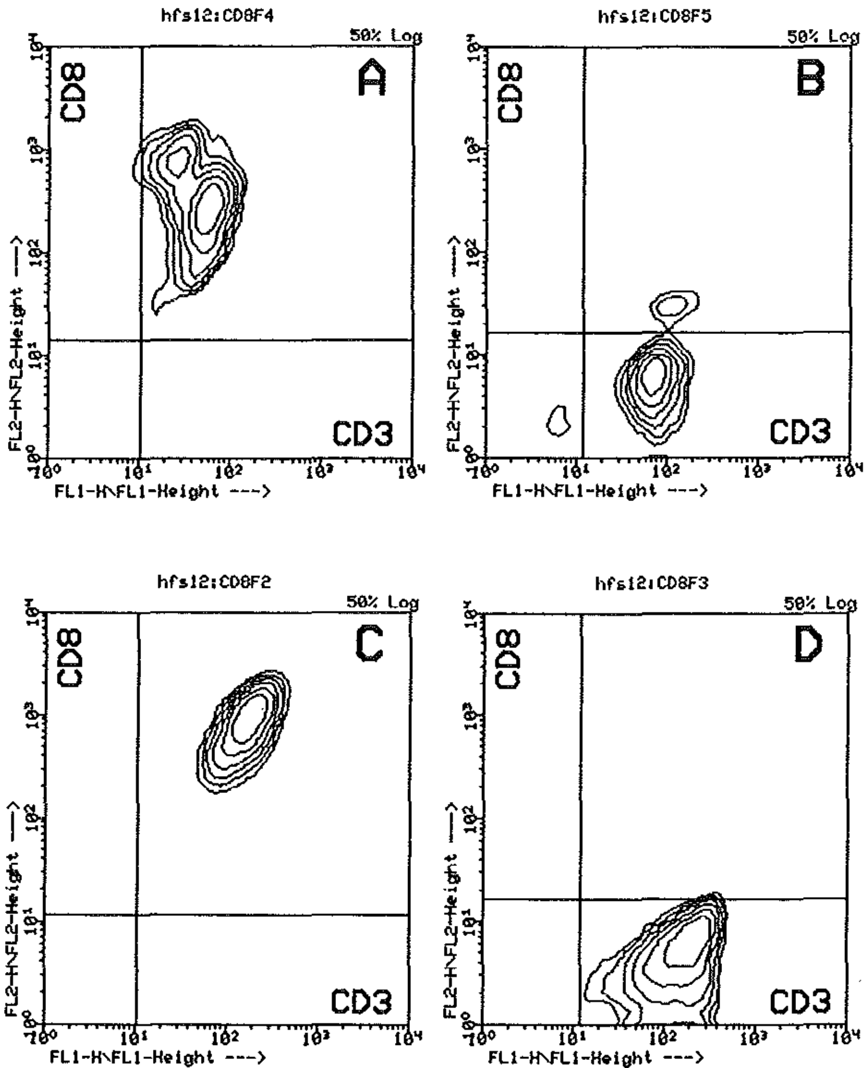

Fig. 1. Expression of surface CD3 and CD8 markers on T cells isolated with anti-CD8-coated magnetic beads and detached by either the anti-Fab or the overnight incubation procedure. Cells were stained with anti-CD3 fluorescein (FITC) conjugate and anti-CD8 phycoerythrin (PE) conjugate. Samples were analysed by two-colour cytofluorimetry shortly after isolation. *A*,*B*: cells isolated from PBMC and detached by either anti-Fab (*A*) or overnight incubation (*B*). *C*,*D*: cells isolated from a $CD8^+$ MV-specific CTL clone and detached by either anti-Fab (*C*) or overnight incubation (*D*).

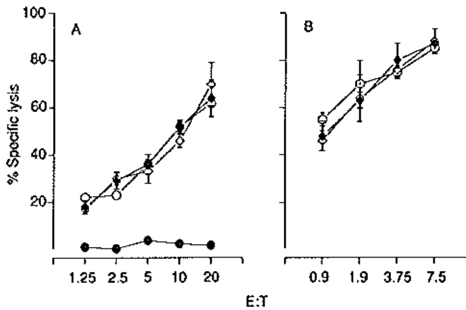

Fig. 2. *A*: cytotoxic activity of T cells isolated from bulk PBMC (patient JP), 3 weeks after the onset of symptoms of measles, stimulated with autologous MV-infected B-LCL, and fractionated with anti-CD8-coated magnetic beads. Positively selected cells were detached from the beads by either the anti-Fab or the overnight incubation procedure. Cells were tested against autologous MV-infected B-LCL. Effector to target cell ratios ($E:T$) ranged from 1.25 to 20. Legend: ◇, lysis by unfractionated PBMC; ♦, lysis by anti-Fab detached $CD8^+$ fraction; ○, lysis by overnight detached $CD8^+$ fraction; •, lysis by $CD8^-$ fraction. *B*: cytotoxic activity of a $CD8^+$ MV-specific CTL clone (JP) before and after positive selection with anti-CD8-coated magnetic beads, followed by the anti-Fab or the overnight detachment procedure. Cells were tested against autologous MV-infected B-LCL at effector-to-target cell ratios ($E:T$) ranging from 0.9 to 7.5. Results of one of two representative experiments are given. Legend: ◇, lysis by non-treated cells; ♦, lysis by anti-Fab detached cells; ○, lysis by overnight detached cells.

its well defined MV-specific MHC class I restricted lytic activity. In two independent experiments, positively selected clonal $CD8^+$ T cells showed MV-specific cytolytic activity similar to that of the untreated clone and no functional difference was detected between cells detached by either procedure (Fig. 2*B*). One explanation for the preservation of the functional activity in the cells with decreased CD8 expression is that the downregulation of the targeted surface marker is only temporary and followed by rapid recovery. On the other hand, the loss might only have been partial, with retention of sufficient residual surface molecules for the isolated cells to exert their function.

Use of the anti-Fab detachment procedure to investigate cellular immune responses against HIV

We evaluated the applicability of the immunomagnetic bead separation with anti-Fab detachment to the fractionation and analysis of HIV-specific cytolytic activity of PBMC from HIV seropositive individuals. The procedure resulted in similar yields, viability, and purity of positive and negative cell fractions as obtained with PBMC from healthy individuals (data not shown). Functional studies were performed with PBMC from HIV infected individuals, which were specifically stimulated in vitro with rVV-HIV gag infected autologous B-LCL (Van Baalen et al., submitted). Stimulated PBMC were then tested for specific cytotoxic activity against a panel of autologous and mismatched B-LCL expressing either gag or a control antigen. The unfractionated PBMC showed significant cytotoxic activity against autologous gag-expressing targets (26.7% at an $E:T$ of 45:1). Comparison of the cytolytic activity of the anti-Fab detached $CD8^+$ fraction and the $CD8^-$ fraction indicated that the gag-specific CTL activity predominantly resided in the $CD8^+$ lymphocyte subset, with a percentage of specific lysis of 38.9% compared to 3.5% of the $CD8^-$ fraction (percentages of lysis of control targets have been subtracted from those of gag-infected targets).

We conclude that the anti-Fab detachment procedure is a more rapid, efficient and reproducible method of obtaining highly pure T cell fractions than the overnight detachment procedure, and is a valuable adjunct to studying cellular immune responses to virus infections at the subset level.

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Simian immunodeficiency virus (SIV)-specific CD8$^+$ cytotoxic T lymphocyte responses of naive and vaccinated cynomolgus macaques infected with SIVmac32H(J5): quantitative analysis by *in vitro* antigenic stimulation

Anna-Maria Geretti*, †, Ellen G.J. Hulskotte*, Marlinda E.M. Dings*, Carel A. van Baalen*, Geert van Amerongen‡ and Albert D.M.E. Osterhaus*§

*Detailed analyses of simian immunodeficiency virus (SIV)-specific cytotoxic T lymphocyte (CTL) responses in vaccinated and infected macaques may help to clarify the role of CTL immunity in protection against lentiviruses. Here, the optimal conditions for the measurement of SIV Gag-specific CTL were investigated by bulk and limiting dilution assays of peripheral blood mononuclear cells (PBMC) from naive and vaccinated cynomolgus macaques (*Macaca fascicularis*) infected with SIVmac32H(J5).* In vitro *restimulation was generally required for CTL detection. Selective activation of CD8$^+$ and MHC-restricted SIV Gag-specific CTL was induced by stimulation with autologous* para-*formaldehyde-fixed B-lymphoblastoid cell lines infected with a recombinant vaccinia virus expressing SIV Gag. Applied to limiting dilution assays, antigenic stimulation reproducibly demonstrated SIV Gag-specific CTL precursors (CTLp) in PBMC of all animals studied, including those lacking significant responses in standard bulk CTL assays.* *© 1997 Elsevier Science Ltd.*

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Even though cytotoxic T lymphocytes (CTL) are believed to contribute to the control of human and animal viral infections, their role in the host defense against lentiviruses has not been firmly established[1]. Infection of macaques with several strains or clones of simian immunodeficiency virus (SIV)mac shows remarkable similarities with human immunodeficiency virus (HIV) infection of humans[2]. Following SIV inoculation, a burst of virus replication is observed, which rapidly subsides in coincidence with the development of antiviral immune responses[3]. The subsequent course of the infection varies considerably among macaques: some animals rapidly develop an AIDS-like disease and die within few weeks or months, whereas others may remain asymptomatic for > 3 years. This individual variability, combined with the opportunity to define parameters of infection such as strain, dose and route of virus inoculation, may prove valuable for clarifying the role of CTL immunity in the control of lentiviral infections.

We[4] and others[5,6] have reported recently that vaccine-induced SIV Env-, Gag-, or Nef-specific CTL failed to protect macaques upon challenge with cell-free SIVmac. In the same model, however, Gallimore *et al.*[7] found an inverse correlation between the frequency of vaccine-induced SIV Nef-specific CTL precursors (CTLp) and peak virus load measured after challenge. These findings indicate that CTL may not be able to prevent or control HIV or SIV infection, unless stringent qualitative and quantitative requirements are met. Detailed analyses of CTL responses in both vaccinated and infected macaques may help to clarify these

*Institute of Virology, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. †Present address: Department of Virology, Division of Pathology and Communicable Diseases, Rowland Hill Street, London NW3 2PF, UK. ‡Central Animal Laboratory, National Institute of Public Health and Environmental Protection, P.O.Box 1, 3720 BA Bilthoven, The Netherlands. §Author to whom all correspondence should be addressed. (Received 21 October 1996; revised version received 2 January 1997; accepted 13 Janauary 1997)

requirements, thereby facilitating the design of immunotherapeutic interventions[8]. However, initial reports have indicated that CTLp frequencies of immunized and naive rhesus[6,9] or cynomolgus[4,7,10] macaques infected with SIVmac may be relatively low by comparison with those often measured in HIV-1 infected humans[11], implying that sensitive and reproducible methods are required to evaluate CTL responses in macaques. Extending observations previously made in HIV-1 infected humans[12,13], we report here that *in vitro* expansion of effector cells under limiting dilution conditions by stimulation with SIV Gag recombinant vaccinia virus (rVV)-infected and *para*-formaldehyde (pfa)-fixed B-lymphoblastoid cell lines (B-LCL), significantly enhances CTL detection in both immunized and naive cynomolgus macaques infected with SIVmac32H(J5).

MATERIALS AND METHODS

Animals

The study included eight colony-bred juvenile (1.5–3.5 years) cynomolgus macaques seronegative for SIV, type D simian retrovirus (SRV) and simian T cell leukaemia virus-I (STLV-I). Four monkeys (designated K77*, K80*, K83* and K88*) were immunized with an SIV Env-Iscoms, Gag-Iscoms and Nef-lipopeptides subunit vaccine, as described[4]. The other four monkeys (K73, K79, K66 and K71) were naive at the time of SIV infection. Animals were inoculated intravenously with 50 monkey median infectious doses of cell-free SIVmacJ5, a pathogenic molecular clone derived from SIVmac32H and grown on rhesus peripheral blood mononuclear cells (PBMC)[14]. Infection was confirmed by virus isolation[4], antibody detection in ELISA[4] and nested-Gag PCR[15].

PBMC preparation

Blood samples were drawn from the femoral vein into tubes containing heparin. PBMC were separated by density gradient centrifugation[4], washed three times in complete RPMI 1640 medium (containing 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 10^{-5} M β-mercapto ethanol and 2 mM L-glutamine), and either used immediately or cryopreserved. Cell viability (as assessed by Trypan Blue exclusion) exceeded 95% after thawing. Cell-associated virus load was determined in serial dilutions of freshly isolated PBMC co-cultured with the human T cell line C8166; the lowest dilution contained 10^6 PBMC[4]. Herpes papio-transformed B-LCL were established by incubation of PBMC with cell-free supernatant from the Herpes papio-producing cell line S594, and maintained in complete medium containing 10% foetal calf serum (FCS; R-10). Serological MHC typing was performed by Dr R. Bontrop (BPRC, Rijswijk, The Netherlands). SIV p26 core antigen in plasma was assayed by antigen-capture ELISA (Coulter SIV Core Antigen Assay, Coulter Electronics, Mijdrecht, The Netherlands).

Immunomagnetic cell fractionation

PBMC in complete medium with 2% FCS were mixed with magnetic beads coated with anti-CD8 monoclonal antibodies (mAb) (Dynabeads M-450, Dynal, Oslo, Norway) at a 1:10 target cell-to-bead ratio[16]. After incubation for 60 min at 4°C, fractions were separated on a magnetic separation device (MPC-6, Dynal). Positively selected $CD8^+$ cells were detached from magnetic beads using a goat anti-mouse-Fab polyclonal antiserum (DETACHaBEAD, Dynal). This separation procedure yields highly pure cell populations, without interfering with either CD8 expression or cytotoxic function of effector cells[16]. By immunofluorescence analysis positively selected fractions typically consisted of >99% $CD8^+$ cells, whereas $CD8^+$ contamination of depleted fractions was consistently <2% (data not shown).

Immunofluorescence analysis

Cell samples in PBS with 0.1% BSA were incubated for 30 min at room temperature with anti-CD2 (Leu-5b-fluorescein isothiocyanate, Becton Dickinson, Mountan View, CA) and anti-CD8 (Leu-2a-phycoerythrin, Becton Dickinson) or anti-CD4 (OKT4-FITC, Ortho Diagnostic System, Raritan, NJ) mAb. Samples were washed twice with PBS, fixed in 1.5% pfa and analysed with a FACScan (Becton Dickinson).

Preparation of antigen presenting cells (APC)

Autologous B-LCL were infected overnight with rVV (10 m.o.i.) expressing either the Gag p56 gene of SIVmac32H (kindly provided by Professor A. McMichel, Institute of Molecular Medicine, Oxford, UK) or the Tat gene of SIVmac251 (TG4174, kindly provided by Dr M.P. Kieny, Transgene, Strasbourg, France), and subsequently fixed in 1.5% pfa, as described[12]. Autologous irradiated (8500 rad) SIV Gag rVV-infected B-LCL were used as APC in a limited number of experiments. Cultures stimulated with these APC showed no signs of vaccinia virus-induced cytopathic effects. Antigen expression in SIV Gag APC was confirmed by immunofluorescence analysis using a polyclonal bovine anti-vaccinia serum (RIVM, Bilthoven, The Netherlands) and the murine anti-Gag mAb CLB14 (CLB, Amsterdam, The Netherlands).

Bulk culture conditions

Bulk assays were done with either unstimulated or restimulated PBMC. For antigenic stimulation, PBMC (2.5×10^4 per well) in R-10 were cultured for 14 days in 96-well round-bottomed plates with SIV Gag or Tat APC (10^4 per well) and autologous irradiated (2500 rad) feeder PBMC (10^4 per well). Cultures were supplemented with 10 U ml^{-1} recombinant interleukin (IL)-2 (rIL-2) from day 3, and restimulated with 10^4 per well APC on day 7. During the second cycle of stimulation the culture medium was enriched with 10% supernatant from concanavalin A (ConA)-stimulated blasts. In some experiments, PBMC (10^6 ml^{-1}) were cultured for 10–14 days with autologous irradiated PHA-blasts infected with SIVmac32H(J5) (10^5 ml^{-1}), as described by Gotch *et al.*[17]. For mitogenic stimulation, PBMC (10^6 ml^{-1}) were cultured for 3 days with ConA (5 μg ml^{-1}), washed, and expanded with rIL-2 (20 U ml^{-1}) for 4–11 days. Effector cells were assayed for cytotoxicity in duplicate or triplicate wells at indicated effector-to-target cell (*E:T*) ratios.

Limiting dilution culture conditions

Limiting dilution assays were done with either unstimulated or restimulated PBMC, to estimate CTL[18] and CTLp frequencies respectively. PBMC restimulation was performed as described for bulk assays. The optimal limiting dilution assay parameters, including numbers and ranges of dilutions, and numbers of replicate wells, were predetermined for each monkey in a pilot experiment. All assays included at least four and up to eight PBMC dilutions, and each dilution included at least 24 and up to 48 replicate wells of 96-well round-bottomed plates. Three or four aliquots from each well were tested in split-well cytotoxicity assays.

Cytotoxicity assay

Cytotoxicity was measured in standard ^{51}Cr-release assays against autologous and allogenic MHC class I mismatched B-LCL infected overnight with 10 m.o.i. of SIV Gag or Tat rVV. Autologous B-LCL either infected with 186-poly rVV (containing a polycloning site without insert and kindly provided by Dr M.P. Kieny, Transgene) or incubated with medium alone served as control. Targets were labelled for 1 h with 100 μCi of ^{51}Cr, washed three times, resuspended in R-10, and added to effector cells at 5×10^3 cells per well in 96-well round-bottomed plates (Costar, Cambridge, UK). After a 5 h incubation, supernatants were harvested (Skatron Harvester, Skatron, Oslo, Norway), and the release of ^{51}Cr was measured in a gamma counter. Maximum ^{51}Cr release was determined by detergent (5% Triton X-100) lysis of target cells. Spontaneous release was determined by incubation of target cells in R-10 alone. Spontaneous release was <30% of maximum release.

Calculation of results

The percentage of lysis of specific and control targets was calculated for duplicate or triplicate wells of bulk cultures and for each well of limiting dilution cultures according to the formula: %lysis = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Individual wells of limiting dilution cultures were considered positive when lysis of specific targets exceeded by 10% that of control targets if the latter was <10%, or by 20% if the latter was >10%. In all positive wells the experimental release exceeded the spontaneous release by at least three standard deviations (S.D.). CTLp frequencies were estimated by the maximum likelihood method using the statistical software package described by Strijbosch *et al.*[19], which included a χ^2 goodness-of-fit test statistic. Frequencies were normalized to the number of CTLp per 10^6 PBMC. Rates of change/month of follow-up (slopes), and correlation coefficients (r) of virus load, CTLp frequencies and CD4$^+$ cell percentages were calculated by linear regression analysis.

RESULTS

Measurement of SIV Gag-specific CTL in bulk assays

SIV Gag-specific CTL responses were studied in eight cynomolgus macaques during the first 4 months of infection with SIVmac32H(J5). Four monkeys (indicated by an asterisk) had been immunized before infection with an SIV Env-Iscoms, Gag-Iscoms and Nef-lipopetides subunit vaccine[4], whereas the other four animals were naive at time of virus inoculation. To establish optimal conditions for CTL measurement, we first compared in bulk assays the direct SIV Gag-specific CTL responses of unstimulated PBMC with those mediated by the same PBMC following either mitogenic or antigenic *in vitro* restimulation. As shown in the examples of *Figure 1*A, no significant CTL responses against SIV Gag were mediated by unstimulated PBMC in *E:T* ratios up to 200:1. After mitogenic expansion with ConA (*Figure 1*B), effector cells specifically reactive against SIV Gag were detected in monkey K71, whereas relatively high levels of non-specific background lysis hindered reliable CTL measurement in monkey K88*. In parallel, the specific lysis of SIV Gag rVV-infected targets was markedly enhanced by two 1-week cycles of antigenic stimulation, using as APC autologous pfa-fixed B-LCL infected with SIV Gag rVV (*Figure 1*C).

Previously we[13] and others[31] have shown that after pfa-fixation, Epstein–Barr virus-transformed B-LCL retain their ability to induce CTL activation, but lose their release of helper-like soluble factors. These factors are known to stimulate the growth of both MHC restricted and unrestricted cytotoxic cells reactive against the B-LCL[13,31]. Extending these observations to Herpes papio-transformed B-LCL, we observed that stimulation of PBMC from monkey K88* with pfa-fixed SIV Gag APC elicited SIV Gag-specific

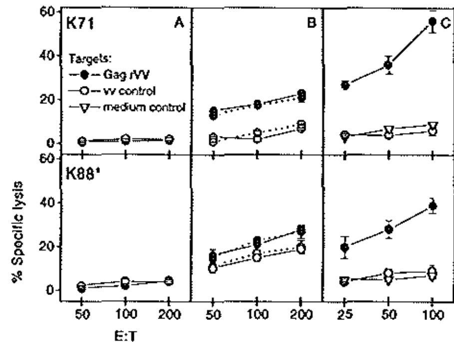

Figure 1 *In vitro* restimulation is required to detect SIV Gag-specific CTL in PBMC of immunized and non-immunized cynomolgus macaques infected with SIVmac32H(J5). Data are shown for two monkeys. Monkey K88* had been immunized with an SIV Env-Iscoms, Gag-Iscoms and Nef-lipopeptides subunit vaccine before infection. Monkey K71 was naive at the time of virus inoculation. Effector cells were: (A) unstimulated PBMC isolated either 12 weeks (monkey K71) or four months (monkey K88*) after infection; (B) the same PBMC expanded by mitogenic stimulation with ConA, and assayed twice, either on days 10 (···) and 14 (—) of culture (monkey K71), or on days 7 (···) and 10 (—) of culture (monkey K88*); (C) the same PBMC expanded by two 1-week cycles of antigenic stimulation, using as APC autologous pfa-fixed B-LCL infected with SIV Gag rVV. Targets were autologous B-LCL either infected with SIV Gag rVV or with vaccinia virus (vv) control, or uninfected (medium control). Results are expressed as mean specific lysis ±SD from triplicate (A and B) or duplicate (C) well estimations

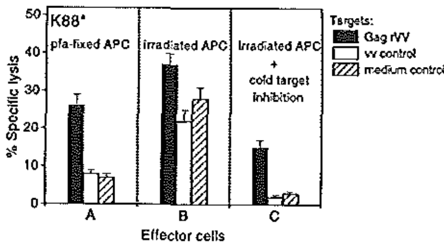

Figure 2 Stimulation efficiency of SIV Gag APC. Effector cells were generated by stimulation of PBMC from monkey K88* with either pfa-fixed (A) or irradiated (B and C) SIV Gag APC. Targets were autologous B-LCL either infected with SIV Gag rVV or with vaccinia virus (vv) control, or uninfected (medium control). For cold target inhibition (C), non-labelled uninfected B-LCL were mixed with the ^{51}Cr-labelled targets at a ratio of 5:1 before the CTL assay was initiated. Results are expressed as mean specific lysis with S.D. from duplicate well estimations at an E:T ratio of 40:1

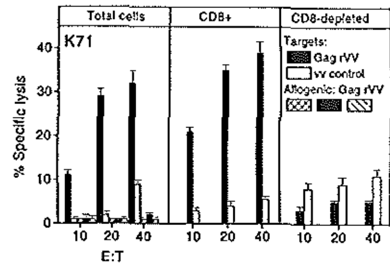

Figure 4 CD8$^+$ cell-mediated and MHC-restricted cytotoxic responses against SIV Gag. Effector cells generated by stimulation of PBMC from monkey K71 with SIV Gag APC were assayed as total cell population, positively isolated CD8$^+$ cells (>99% CD8$^+$), and CD8-depleted cell population (<2% CD8$^+$). Targets were autologous B-LCL infected with either SIV Gag rVV or with vaccinia virus (vv) control, and allogenic SIV Gag rVV-infected B-LCL from three MHC class-I mismatched monkeys

CTL without significant expansion of cytotoxic cells reactive against control targets (*Figure* 2A). In contrast, after stimulation of the same PBMC with irradiated SIV Gag APC (*Figure* 2B and C), relatively high numbers of cold (non-^{51}Cr-labelled) uninfected targets were required to inhibit non-specific background lysis and measure SIV Gag-specific CTL.

In monkey K71, effector cells generated by stimulation with either SIV Gag (*Figure 3*A) or Tat (*Figure 3*B) APC mediated significant lysis of targets infected with the inducing rVV, but not of targets infected with either the discordant rVV or with vaccinia virus control. This finding confirmed that antigenic stimulation induces selective CTL activation. Furthermore, immunofluorescence analyses indicated an expansion of CD8$^+$ cells at the end of the two cycles of SIV Gag-specific stimulation (data not shown). Depletion studies confirmed that SIV Gag-specific CTL were indeed CD8$^+$ cells: as shown in *Figure 4* for monkey K71, SIV Gag-specific CTL responses mediated by total effector cells were preserved in positively isolated CD8$^+$ cells, but were abolished by CD8-depletion. In addition, these responses were restricted to autologous targets, whereas allogenic MHC class-I mismatched targets infected with SIV Gag rVV were not recognized.

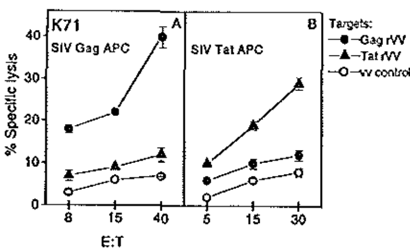

Figure 3 Selective expansion of antigen-specific CTL. Effector cells were generated by stimulation of PBMC from monkey K71 with either SIV Gag (A) or Tat (B) APC. Targets were autologous B-LCL infected with either SIV Gag or Tat rVV or with vaccinia virus (vv) control. Results are expressed as mean specific lysis with S.D. from duplicate well estimations

Measurement of SIV Gag-specific CTL and CTLp in limiting dilution assays

The frequencies of SIV Gag-specific CTL and CTLp were estimated by limiting dilution assays of unstimulated and restimulated PBMC, respectively. The optimal assay parameters (i.e. numbers and ranges of dilutions and numbers of replicate wells) yielding χ^2 goodness-of-fit test statistic <10 were predetermined in a pilot experiment for each monkeys. Single-well cytotoxic responses of restimulated PBMC from monkey K71 are shown in *Figure 5*. In agreement with the results of bulk CTL assays (*Figure 1*C), after two 1-week cycles of SIV Gag-specific stimulation, effector cells in most wells mediated killing of SIV Gag rVV-infected targets (*Figure 5*A), with negligible killing of control targets (*Figure* 5B). The relationship between the percentage of negative wells and the initial cell number was consistent with the single-hit Poisson model and indicated a CTLp frequency of 58 (95% confidence interval: 44–72; χ^2:1) per 10^6 PBMC (*Figure* 5C, filled circles). In contrast, in the absence of PBMC restimulation, SIV Gag-specific CTL were detected at a frequency of only two (0.3–3; χ^2:1) per 10^6 PBMC (*Figure* 5C, filled triangles). Similarly, 4 months after infection, the frequency of SIV Gag-specific CTL in unstimulated PBMC of monkey K88* was only 2 (0.4–4; χ^2:4) per 10^6 PBMC, whereas no CTL could be detected in unstimulated PBMC of other monkeys (data not shown). These findings confirmed that *in vitro* restimulation greatly amplifies SIV Gag-specific CTL responses.

Cell culture under limiting dilution conditions further increased the sensitivity of CTL measurement. Even after SIV Gag-specific PBMC restimulation, monkeys with low, but still measurable CTLp frequencies (<10 per 10^6 PBMC), showed no consistent CTL responses in standard bulk CTL assays, using *E:T* ratios up to 100:1. As an example, a comparison of bulk and limiting dilution assays of PBMC from monkey K66 is shown in *Figure 6*. In bulk assays (*Figure* 6A), no significant lysis of SIV Gag rVV-infected targets was mediated by PBMC that were unstimulated or restimulated with either ConA or SIV-infected blasts. Lysis increased after SIV Gag-specific stimula-

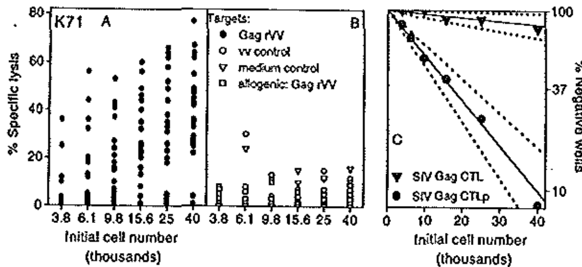

Figure 5 Limiting dilution analysis of cytotoxic responses against SIV Gag. PBMC isolated from monkey K71 at week 12 after infection were cultured in six dilutions (range: 40000 to 3815 cells per well), each including 24 replicate wells. After two 1-week cycles of SIV Gag-specific stimulation, individual wells were tested against (A) autologous B-LCL infected with SIV Gag rVV, and (B) autologous B-LCL either infected with vaccinia virus (vv) control or uninfected (medium control), and allogenic SIV Gag rVV-infected B-LCL from one MHC class-I mismatched monkey. (C) Frequency analysis of SIV Gag-specific CTL and CTLp in unstimulated and restimulated PBMC, respectively. The relationship between the percentage of negative wells and the initial cell number was consistent with the single-hit Poisson model. Frequencies were estimated by the maximum likelihood method and normalized to number of CTL or CTLp per 10^6 PBMC. Dotted lines indicate 95% confidence intervals. The frequency of SIV Gag-specific CTL was 2 (0.3–3; χ^2: 1) per 10^6 PBMC. The frequency of SIV Gag-specific CTLp was 58 (44–72; χ^2: 1) per 10^6 PBMC

tion, but it was still <10% after subtraction of non-specific background lysis. In limiting dilution assays, SIV Gag-specific CTLp were detected at a frequency of four (2–6; χ^2: 1) per 10^6 PBMC (*Figure 6B*).

The reproducibility of CTLp frequency estimates was verified in 10 replicate assays of cryopreserved PBMC, which showed a mean coefficient of variation (as 100×the S.D. of the residuals/mean CTLp frequency estimate) of 9.5% (data not shown). We also investigated the ability of SIV Gag APC to stimulate unprimed CTLp in naive PBMC isolated from the eight monkeys before exposure to either whole SIV or SIV antigens. These PBMC were assayed in parallel with those obtained 4 months after infection, using identical culture and assay conditions. The individual wells of limiting dilution assays were never or rarely positive in naive PBMC (CTLp frequencies <1 per 10^6 PBMC, data not shown), indicating that only primed CTLp were responsive to SIV Gag-specific stimulation.

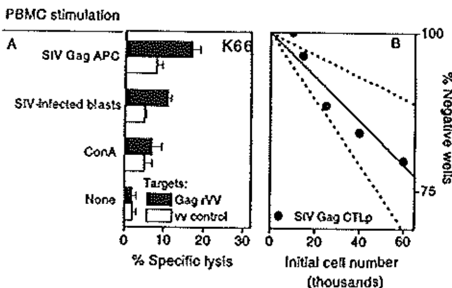

Figure 6 Bulk and limiting dilution assays of PBMC isolated from monkey K66 at week 12 after infection. In bulk assays (A), PBMC were unstimulated or restimulated with either ConA, or SIV-infected blasts, or SIV Gag APC, and assayed in duplicate at an E:T ratio of 80:1. In limiting dilution assays (B), PBMC were restimulated with SIV Gag APC. Target cells were autologous B-LCL either infected with SIV Gag rVV or with vaccinia virus (vv) control (A and B) or uninfected (medium control, B). The frequency of SIV Gag-specific CTLp was 4 (2–6; χ^2:1) per 10^6 PBMC

Kinetics of SIV Gag-specific CTLp

In kinetic studies of the four monkeys that were naive at the time of virus inoculation, SIV Gag-specific CTLp were first detected between week 1 and 4 (K71, K73, K79) or 9 (K66) after infection (*Table 1*). CTLp detection in circulation coincided therefore with the containment of PBMC-associated virus load and p26 antigenemia which followed the initial virus burst observed at week 2 or 4 after infection[4]. The four vaccinated monkeys (K77*, K80*, K83* and K88*) had developed SIV Gag-specific CTLp upon immunization, at frequencies ranging from 2 to 13 per 10^6 PBMC[4]. After infection, their CTLp frequencies were still similar to those found in the non-immunized animals (*Table 1*). In all eight monkeys, frequencies were generally maintained between week 12 and 4 months after infection, but a marked increase was observed in monkey K88*. Overall, no clear correlation emerged between CTLp frequencies and PBMC-associated virus load. In fact, 4 months after infection statistical analysis seemed to suggest a strong positive correlation between virus load and CTLp frequencies ($r = 0.89$, $P = 0.003$, not shown); however, this relationship was lost if monkey K88* was excluded from analysis. All eight monkeys remained asymptomatic during the first 4 months of infection. Six animals showed a progressive decline of CD4+ cell percentages, which was pronounced in monkeys K73 and K79 (*Table 1*).

DISCUSSION

In the present study, we have studied the CTL responses of immunized and naive cynomolgus monkeys after infection with SIVmac32H(J5). We focused our attention on responses directed against the relatively conserved Gag protein, which has been described as a major target of CTL immunity against lentiviruses[3,11,21]. Our findings demonstrate that antigenic stimulation of effector cells under limiting dilu-

Table 1 Evaluation of virological and immunological parameters in vaccinated and naive cynomolgus macaques infected with SIVmac32H(J5)[a]

Monkey	Virus load[b]		CTLp[c]			CD4+ cell slope[e]
	2–4 week (peak)	4 months	1–4 weeks[d]	6–12 weeks[d]	4 months	
K66	503	<1	0	4 (2–6; 1)	5 (3–7; 3)	-1.7 ± 1
K71	>1000	<1	27 (17–37; 4)	58 (44–72; 1)	56 (42–70; 3)	$+1 \pm 1.5$
K73	625	5	12 (8–17; 2)	9 (6–13; 1.5)	7 (5–9; 3)	-4.3 ± 0.5 ($P < 0.05$)
K79	>1000	6	14 (8–20; 1)	11 (15–26; 2.5)	12 (8–15; 4)	-3.2 ± 0.8 ($P < 0.05$)
K77*	208	25	3 (1–5; 3)	7 (4–9; 2)	9 (6–12; 4)	-0.6 ± 0.6
K80*	208	3	8 (4–12; 2)	15 (10–21; 1)	12 (8–16; 2.5)	-0.6 ± 0.3
K83*	>1000	3	12 (9–16; 1)	13 (9–17; 1)	10 (7–14; 1)	-1.3 ± 0.9
K88*	>1000	156	N.D.	18 (12–24; 1)	121 (95–147; 1)	-2.4 ± 1

[a]Data are presented for eight animals. Four monkeys (denoted by an asterisk) had been immunized with an SIV Env-Iscoms, Gag-Iscoms, Nef-lipopeptides subunit vaccine before infection, whereas the other four animals were naive at the time of virus inoculation
[b]Cell-associated virus load was measured in co-cultures of PBMC with C8166 cells and normalized to the number of infected cells per 10^6 PBMC
[c]Effector cells were generated under limiting dilution culture conditions by stimulation with autologous pfa-fixed B-LCL infected with SIV Gag rVV. Split-well ^{51}Cr-release assays were performed on day 14 of culture. Frequencies were estimated by the maximum likelihood method and normalized to the number of CTLp per 10^6 PBMC; 95% confidence intervals and χ^2 goodness-of-fit test statistics are given in parentheses
[d]Results are presented from one of at least two experiments, with coefficients of variation <10%
[e]Rates of change (slopes) in CD4+ cell percentages month^{-1} were determined by linear regression analyses of longitudinal data obtained at minimum three time points (mean = 3.75) during 4 months of observation. Positive slopes indicate increase, negative slopes correspond to a decrease

tion conditions provides a sensitive and reproducible approach to characterize CTL responses against SIV. With this approach we were able to detect SIV Gag-specific CTLp in PBMC of all animals studied, including those lacking significant CTL responses in standard bulk CTL assays (*Figure 6*).

In vitro restimulation was shown to be required to amplify SIV Gag-specific CTL responses to detectable levels (*Figure 1*). In contrast, Gag-specific CTL responses of asymptomatic HIV-1-infected adults have often been of sufficient magnitude to allow detection in primary assays, in the absence of *in vitro* restimulation[11,21]. In line with these observations, and in agreement with previous studies of SIVmac-infected rhesus[6,9] and cynomolgus[10] macaques, SIV Gag-specific CTLp were detected at frequencies generally lower than those measured in asymptomatic HIV-1-infected adults[13,21]. This discrepancy may reflect a relatively low degree of antigenic stimulation in macaques, due to rapid downregulation of SIV replication after infection. Our kinetic studies appear to support this hypothesis. Immediately after infection, we found that CTLp detection in the naive macaques coincided with the initial decline of cell-associated virus load and p26 antigenemia. Subsequently, we found no significant differences in the CTLp frequencies of immunized and naive monkeys, whereas a positive correlation seemed to develop between virus load and the frequencies of SIV Gag-specific CTLp in the monkey showing relatively poor virus containment (*Table 1*). Taken together, these observations support the hypothesis that the rapid development of CTL responses targeting certain critical viral proteins or epitopes may indeed contribute towards the initial virus containment[22,23]. However, as also suggested by Ferbas *et al.*[24], in cases of poor virus control, CTLp frequencies may become a reflection of persistently high levels of antigenic stimulation.

To stimulate the growth of SIV Gag-specific CTLp we used autologous B-LCL infected with SIV Gag rVV and fixed in pfa. With a similar approach, we have been able to demonstrate Gag-specific CTLp in PBMC from HIV-1-infected persons, even after the onset of overt disease[13]. In the macaques, *in vitro* antigenic stimulation was shown to promote the selective expansion of SIV Gag-specific CTL of the CD8+ MHC class-I restricted phenotype (*Figure 3Figure 4Figure 5*). As a result, CTL detection was enhanced compared with results obtained after mitogenic stimulation with ConA (*Figure 1*), a well-established method for CTL expansion[5,9]. Other authors have also reported the use of rVV to expand CTL against HIV[25] or SIV[26] antigens. In the study by Lubaki *et al.*[25], the use of irradiated rVV-infected B-LCL as APC caused high levels of non-specific background lysis, and cold target inhibition was required to detect HIV-specific CTL. In our study, PBMC stimulation with irradiated SIV Gag APC also allowed CTL detection in bulk assays when combined with cold target inhibition (*Figure 2*). However, this method did not allow a reproducible measurement of CTLp frequencies in limiting dilution assays (data not shown). Along the same line, Kent *et al.*[26] had to replace irradiated rVV-infected B-LCL with irradiated rVV-infected PBMC as APC to reduce the growth of non-specific cytotoxic cells and measure SIV-specific CTL. The improved ratio of specific to non-specific lysis we obtained with pfa-fixed APC (*Figure 2*), probably resulted from an adequate preservation of their stimulation efficiency, together with a reduced release of T-helper like soluble factors that may stimulate the growth of non-SIV-specific cytotoxic cells[13,20].

Interestingly, a previous study by Venet *et al.*[9] described an earlier development of SIV-induced disease in those monkeys which lacked detectable CTL responses. All our monkeys remained asymptomatic during the first 6 months of observation. Among the six animals with relatively low CTLp frequencies, five showed a progressive decrease of CD4+ cell percentages, which was pronounced in only two cases (*Table 1*). Nonetheless, a longer follow-up may clarify whether the generation of higher SIV Gag-specific CTLp frequencies may be associated with a better long-term control of the infection in some monkeys. Studies are in progress to address this hypothesis, which may contribute to the design of effective vaccina-

tion strategies for the prevention and control of lentiviral infections.

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CD8$^+$ cytotoxic T lymphocytes of a cynomolgus macaque infected with simian immunodeficiency virus (SIV) mac32H-J5 recognize a nine amino acid epitope in SIV Gag p26

Anna-Maria Geretti,[1]† Ellen G. J. Hulskotte,[1] Marlinda E. M. Dings,[1] Carel A. van Baalen,[1] Geert van Amerongen[2] and Albert D. M. E. Osterhaus[1]

[1] Institute of Virology, Erasmus University, 3000 DR Rotterdam, The Netherlands
[2] Central Animal Laboratory, National Institute of Public Health and Environmental Protection, 3720 BA, Bilthoven, The Netherlands

A detailed analysis of simian immunodeficiency virus (SIV)-specific cytotoxic T lymphocyte (CTL) responses and the identification of the proteins and epitopes they target may improve the design of immunotherapeutic interventions and provide insights into AIDS pathogenesis. Here, we identified a new CTL epitope in the SIV Gag protein, recognized by CD8$^+$ and MHC class I-restricted CTL clones from a long-term asymptomatic cynomolgus macaque (*Macaca fascicularis*) infected with SIVmac32H-J5. Using overlapping synthetic peptides, the optimal minimal epitope was characterized as a nine amino acid peptide representing amino acids 242–250 of p26 (SVDEQIQWM). CTL recognition was shown to be abolished by amino acid substitutions observed within homologous human immunodeficiency virus (HIV)-1 and HIV-2 sequences.

Infection of macaques with several strains or clones of simian immunodeficiency virus (SIV) shows remarkable similarities with human immunodeficiency virus (HIV) infection of humans and provides a valuable model for investigating the role of cytotoxic T lymphocyte (CTL) immunity in the host defence against lentiviruses (Desrosiers, 1990; Letvin *et al.*, 1994). A number of studies have shown that detailed analysis of SIV-specific CTL responses and the identification of the proteins and epitopes they target may help the design of immunotherapeutic interventions and provide insights into AIDS pathogenesis (Chen *et al.*, 1992; Yasutomi *et al.*, 1995; Hulskotte *et al.*, 1995). Extending our previous studies of HIV-1-infected humans (Van Baalen *et al.*, 1996), we present here a minimally defined CTL epitope in SIV Gag, recognized by CTL of the CD8$^+$ and MHC class I-restricted phenotype. The epitope was identified using CTL clones and bulk cultures of peripheral blood mononuclear cells (PBMC) isolated from a cynomolgus macaque (designated K71) during the first 4 months of infection with the pathogenic molecular clone SIVmac32H-J5 (Rud *et al.*, 1994). Following the initial virus burst at week 2 after infection, the monkey showed effective virus containment (Hulskotte *et al.*, 1995), and remained asymptomatic with stable CD4$^+$ cell counts during the subsequent 22 months of observation (not shown).

Author for correspondence: Albert D. M. E. Osterhaus.
Fax +31 10 4365145. e-mail osterhaus@viro.fgg.eur.nl

† Department of Virology, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, UK

Effector cells were generated from PBMC under limiting dilution conditions. Cells in R-10 medium (RPMI 1640 containing 10% FCS) were cultured with autologous paraformaldehyde-fixed B-lymphoblastoid cell lines (B-LCL) (10^4 per well) infected with a recombinant vaccinia virus (rVV) expressing Gag p55 of SIVmac32H, and with autologous irradiated (2500 rad) feeder PBMC (10^4 per well), as described (Geretti *et al.*, 1996). Cultures received recombinant interleukin-2 (rIL-2, 10 U/ml) from day 3, and were restimulated on day 7. On day 14, they were tested in split-well assays against autologous ^{51}Cr-labelled B-LCL either infected with SIV Gag rVV, or infected with a vaccinia virus control (186-poly, containing a polyclonal site without insert; Transgene), or incubated with medium alone. Individual positive cultures of dilutions showing < 33% positive wells underwent a third cycle of SIV Gag-specific stimulation and were subsequently subcloned by non-specific stimulation with PHA-L (1 μg/ml), irradiated (2500 rad) allogenic PBMC (10^5 per well), irradiated (3500 rad) allogenic human B-LCL (10^4 per well) (Van de Griend, 1984) and rIL-2 (50 U/ml). These CTL are operationally referred to as lines. Eleven CTL lines were established and maintained in culture for at least 3 months by alternate (every 14–21 days) cycles of specific and non-specific stimulation. The CTL lines' surface phenotype was CD2$^+$ CD8$^+$ CD4$^-$ (not shown). Their fine specificities were determined with three sets of 20-mer synthetic peptides, overlapping by 10 amino acids, together spanning the p26 (ADP714/1-22), p17 (ADP775/1-13) and p15 (ADP776/1-14) sequences of

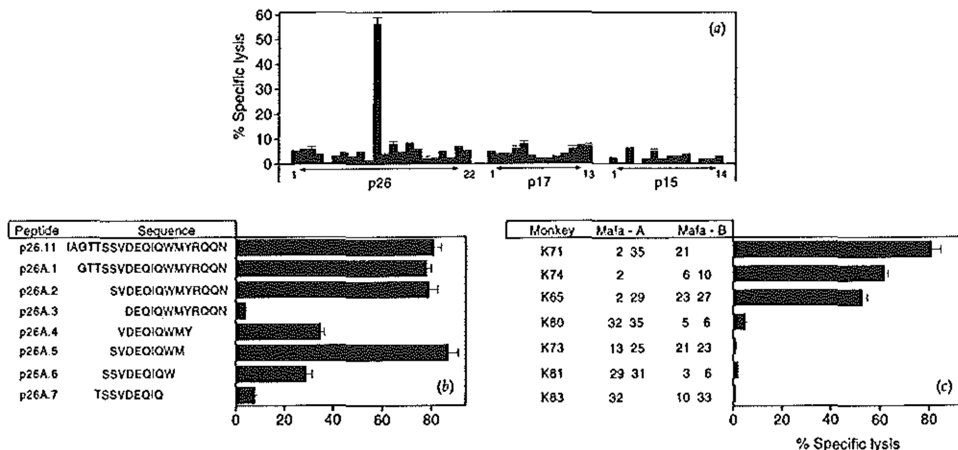

Fig. 1. Fine mapping of an MHC class I-restricted epitope in SIV Gag p26. CTL line K71/E26 was tested against: (*a*) autologous target B-LCL either pulsed with 30 µM synthetic peptides (20 residues long, with a 10 amino acid overlap) covering the p26 (peptides 1–22), p17 (peptides 1–13) and p15 (peptides 1–14) sequences of SIVmac251; (*b*) autologous target B-LCL sensitized with 30 µM synthetic peptides (9–20 residues long) spanning amino acids 236–255 of p26; (c) autologous, MHC class I mismatched and partially matched target B-LCL pulsed with 30 µM peptide p26A.5. Results are expressed as mean percentage specific lysis with standard errors from duplicate well estimations at an E:T ratio of either 5:1 (*a*) or 10:1 (*b*, *c*).

Table 1. CD8$^+$ cytotoxic T lymphocytes expanded by stimulation with peptide p26A.5

Effector cells generated by stimulation with autologous, peptide p26A.5-sensitized B-LCL were separated into positively isolated CD8$^+$ (CD8$^+$, > 99% CD8$^+$ cells) and CD8-depleted (CD8$^-$, < 2% CD8$^+$ cells) fractions immediately before testing for cytotoxicity at different E:T ratios. Target cells were autologous B-LCL, either sensitized with 30 µM of peptide p26A.5, or infected with SIV Gag rVV or vaccinia virus (vv) control, or incubated with medium alone. Results are expressed as mean percentage specific lysis from duplicate well estimations with standard error < 10%.

		% Specific lysis			
Effector cells	E:T	p26A.5	SIV Gag rVV	vv	Medium
Total	50:1	72	63	13	13
	25:1	65	55	10	11
	12:1	55	34	8	7
CD8$^+$	50:1	76	59	10	9
	25:1	61	53	8	8
	12:1	55	35	7	5
CD8$^-$	50:1	8	5	6	6
	25:1	5	4	6	4
	12:1	5	1	2	0

SIVmac251. As shown in Fig. 1(*a*) for the CTL line designated K71/E26, of the 49 peptides tested, only peptide p26.11, covering amino acids 236–255 (IAGTTSSVDEQIQWMYR) of p26, sensitized targets for lysis by the CTL lines. The two contiguous peptides p26.10 and p26.12 were not recognized, indicating that the CTL epitope was contained in the central region of peptide p26.11. To define the minimal epitope within peptide p26.11, the CTL lines were tested for recognition of synthetic peptides spanning amino acids 236–255 of p26 and varying in length from 20 to 9 residues (European Veterinary Laboratory, Woerden, The Netherlands). As shown in Fig. 1(*b*) for CTL line K71/E26, the 9-mer peptide p26A.5, representing amino acids 242–250 (SVDEQIQWM) of p26, optimally sensitized targets for lysis by the CTL lines. Two truncated peptides lacking either the N-terminal S residue (p26A.4: VDEQIQWMY) or the C-terminal M residue (p26A.6: SSVDEQIQW) were considerably less efficient. Further truncation at either terminus abolished recognition, probably by destroying MHC anchor residues. To study MHC class I restriction, the CTL lines were tested against MHC class I mismatched and partially matched B-LCL pulsed with peptide p26A.5. As shown in Fig. 1(*c*) for CTL line K71/E26, lysis was restricted to targets sharing the Mafa-A2 allele, indicating recognition in the context of this macaque MHC class I molecule. It should be noted, however, that MHC class I alleles were defined by serological techniques, and one-dimensional

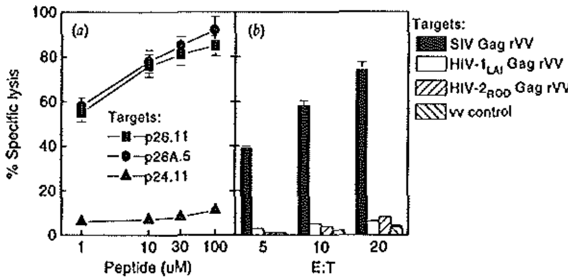

Fig. 2. Effects of sequence variation on CTL recognition. CTL line K71/E26 was tested against: (*a*) autologous target B-LCL pulsed with either peptide p26.11, or peptide p26A.5 or peptide p24.11 (GSDIAGTTSTLQEQIGWMTN, amino acids 235–254 of HIV-1_{SF2} p24), at an E:T ratio of 10:1; (*b*) autologous target B-LCL either infected with rVV expressing the Gag protein of SIVmac32H, or HIV-1_{LAI} or HIV-2_{ROD}, or infected with vaccinia virus (vv) control. Results are expressed as mean percentage specific lysis with standard errors from duplicate well estimations.

isoelectric focusing may be required to confirm the restriction element (Watkins, 1994).

In reciprocal experiments, PBMC (2×10^4 per well) were cultured for 10 days with autologous irradiated (5000 rad) B-LCL (10^4 per well) sensitized with 30 μM of peptide p26A.5, autologous irradiated feeder PBMC (10^4 per well) and rIL-2 (10 U/ml) from day 4. As a control, PBMC were stimulated with B-LCL sensitized with peptides p26.4, p26.6 or p17.6. No cytotoxic responses were mediated by these control cultures (data not shown), whereas effector cells expanded by peptide p26A.5 recognized autologous targets sensitized with the inducing peptide, as well as targets expressing endogenously processed antigen after infection with SIV Gag rVV (Table 1). The $CD8^+$ phenotype of peptide p26A.5-specific CTL was confirmed by depletion studies (Table 1), using anti-CD8 antibody-coated magnetic beads (Dynabeads M-450, Dynal) as described (Geretti *et al.*, 1993).

The amino acid region 242–250 of p26 partially overlaps with $CD4^+$ T helper epitopes previously identified in immunized cynomolgus macaques (Mills & Jones, 1994). In addition, the homologous consensus sequences of the HIV-1 A and B clades fulfil the requirements for binding the human HLA-A2.1 molecule (Brander *et al.*, 1995). The region is conserved among several strains of the HIV-2/SIV D clade, including SIVmac251, SIVmac32H, SIVmac1A11 and SIVmac239. The consensus sequence of the HIV-2/SIV D and C clades shows one amino acid substitution at position 242 (S → T), whereas the consensus sequence of the HIV-2/SIV A and B clades shows two amino acid substitutions, at positions 242 (S → T) and 244 (D → E) (Myers *et al.*, 1994). The homologous sequence is highly conserved among most HIV-1 clades (A–H). However, comparison of the SIVmac251 and HIV-1_{SF2} sequences reveals four amino acid substitutions at positions 242 (S → T), 243 (V → L), 244 (D → Q) and 248 (Q → G) (Myers *et al.*, 1994). As shown in Fig. 2(*a*), targets that were either pulsed with peptide p24.11 (ADP 788/11), covering amino acids 235–254 of HIV-1_{SF2} p24 (GSDIAGTTSTLQEQIGWMTN), or infected with an rVV expressing HIV-1_{LAI} Gag (TG1144; Transgene), were not recognized by CTL line K71/E26. These effector cells also failed to recognize targets infected with an rVV expressing HIV-2_{ROD} Gag (TG2112; Transgene), suggesting either lack of generation of the epitope, or that the two amino acid substitutions at positions 242 (S → T) and 244 (D → E) of HIV-2_{ROD} p26 were sufficient to abolish recognition.

These observations suggest that variations in the p26A.5 epitope may generate virus variants able to escape or antagonize the CTL response of monkey K71, thereby potentially affecting virus containment (Franco *et al.*, 1995). Longitudinal studies are in progress to address this hypothesis, providing an additional basis for investigating the role of CTL immunity in the control of lentiviral infections.

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Chapter 4

Evolution of CTL immunity during primate lentivirus infection

4.1 Human Immunodeficiency virus type 1 (HIV-1)- and Epstein-Barr virus-specific cytotoxic T lymphocyte precursors exhibit different kinetics in HIV-1-infected persons. *Journal of Infectious Diseases (1996) 174:35-45.*

4.2 Virus-driven evolution of simian immunodeficiency virus (SIV)-specific cytotoxic T lymphocyte responses during primary and secondary infection of cynomolgus macaques with SIVmac32H-J5. *(Submitted).*

Human Immunodeficiency Virus Type 1 (HIV-1)– and Epstein-Barr Virus–Specific Cytotoxic T Lymphocyte Precursors Exhibit Different Kinetics in HIV-1–Infected Persons

Anna M. Geretti, Marlinda E. M. Dings, Cécile A. C. M. van Els, Carel A. van Baalen, Folko J. Wijnholds, Jan C. C. Borleffs, and Albert D. M. E. Osterhaus

Institute of Virology, Erasmus University Rotterdam, Rotterdam; Laboratory of Vaccine Development and Immune Mechanisms, National Institute of Public Health and Environmental Protection, Bilthoven; and Department of Immunology and Internal Medicine, Utrecht University Hospital, Utrecht, Netherlands

The frequencies of human immunodeficiency virus type 1 (HIV-1) Gag– and Epstein-Barr virus (EBV)–specific cytotoxic T lymphocyte precursors (CTLp) were studied longitudinally in peripheral blood mononuclear cells from 9 HIV-1–infected persons. By antigen-specific stimulation, HIV-1 Gag–specific CTLp were detected in vitro throughout the course of HIV-1 infection, even after the onset of overt disease. In 4 patients, however, HIV-1 Gag–specific CTLp frequencies declined over time in the presence of maintained EBV-specific CTLp. This decline was correlated with decreasing CD4 ($r = .38$; $P < .05$) and CD8 ($r = .75$; $P < .001$) cell numbers. The maintenance of EBV-specific CTLp in patients with low CD4 cell numbers indicated that EBV-specific CTL-mediated immunity may remain longer unaffected by HIV-1–induced immune dysfunction. Consistent with this observation, the growth of EBV-specific CTL could be supported in vitro by EBV-infected lymphoblastoid B cell lines, independent of both CD4 cells and exogenous cytokines.

Human immunodeficiency virus type 1 (HIV-1)–specific cytotoxic T lymphocytes (CTL) are generated in most HIV-1–infected persons and are generally believed to play a role in the containment of virus spread [1–4]. The relatively conserved HIV-1 Gag protein is a well-described target of the CTL response against HIV-1; Gag-specific CTL of the CD8 and major histocompatibility complex (MHC) class I–restricted phenotype have been detected in most asymptomatic HIV-1–infected persons, even without in vitro restimulation [5]. In addition, limiting dilution analyses have shown that the frequencies of Gag-specific CTL precursors (CTLp), although varying among HIV-1–infected persons, can be remarkably high in peripheral blood mononuclear cells (PBMC) from some asymptomatic persons [6–9]. Even though this strong response appears eventually incapable of controlling HIV-1 infection and preventing progression to AIDS, its decline as disease advances may suggest that significant, albeit temporary, antiviral effects are provided by HIV-1–specific CTL [9–12]. The mechanisms of CTL decline, however, have not been fully elucidated and it cannot be ruled out that this decline is a consequence rather than the cause of the progression of HIV-1 infection.

Like HIV-1, Epstein-Barr virus (EBV) causes a life-long infection and induces strong and long-lasting CTL immunity [13]. Unlike HIV-1 [14], EBV establishes a state of true latency in its host, interrupted by intermittent cycles of replication [15]. Substantial evidence indicates that virus-specific CTL play a part in controlling EBV infection of B lymphocytes and in preventing the outgrowth of EBV-induced lymphoproliferative disorders [16–18]. Lymphoproliferative disorders occur with increased incidence in patients with primary or acquired immunodeficiency, including transplant recipients undergoing immunosuppressive therapy and patients with AIDS. Interestingly, EBV sequences have been detected in virtually all transplant-related but in only 25%–50% of AIDS-related lymphoproliferative disorders [19, 20]. It is also noteworthy that in HIV-1–infected persons, the majority of EBV-induced lymphoproliferative disorders occur in the central nervous system, where they may escape immunosurveillance. These observations suggest that HIV-1–infected persons may retain sufficient immunocompetence to control EBV infection in advanced stages of disease. Consistent with this hypothesis, a cross-sectional study has suggested that the frequencies of EBV-specific CTLp detectable in HIV-1–infected persons can be similar to those measurable in HIV-1–seronegative healthy persons [9]. In contrast, an earlier cross-sectional study showed that the levels of EBV-specific CTL immunity are low in HIV-1–infected patients and decline further during disease progression [21]. The occurrence of a selective dysfunction of HIV-1–specific CTL in the presence of maintained CTL activity against EBV and the possible underlying mechanisms remain a matter of debate.

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Reprints or correspondence: Dr. A. D. M. E. Osterhaus, Institute of Virology, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, Netherlands.

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Here we present the results of a longitudinal study aimed at gaining further insight into the kinetics of HIV-1 Gag– and EBV-specific CTLp, and their relationship with circulating CD4 and CD8 cells, during progression of HIV-1 infection.

Materials and Methods

Study Group

The study included 9 HIV-1–infected adults recruited from a cohort monitored at Utrecht University Hospital. HIV-1 infection was related to intravenous drug use in 1 case, to high-risk heterosexual contacts in 2 cases, and to high-risk homosexual or bisexual contacts in 6 cases. Six patients (Ew03, Fw19, Fw07, Gw21, Gw38, Gw39) remained asymptomatic throughout the study, whereas 3 patients (Gw43, Hw39, and Lw40) had clinical manifestations of AIDS (CDC stages IV-A and IV-D) at the baseline visit. Total leukocyte counts were determined at Utrecht University Hospital by standard methods. The patients' MHC phenotypes were determined according to established National Institutes of Health procedures.

PBMC Preparation

Blood samples were obtained from each patient at the baseline visit and at two or three consecutive time points. PBMC were separated from heparinized blood by density gradient centrifugation on ficoll-hypaque (Lymphoprep; Nycomed, Oslo), then washed three times in RPMI 1640 containing penicillin (100 U/mL), streptomycin (100 μg/mL), 10^{-5} M β-mercaptoethanol, and 2 mML-glutamine (complete medium), and cryopreserved in liquid nitrogen until use. Cell viability (as assessed by trypan blue exclusion) exceeded 95% after thawing.

Immunomagnetic Cell Fractionation

Magnetic beads coated with anti-CD8 or anti-CD4 monoclonal antibodies (MAbs) (Dynabeads M-450; Dynal, Oslo) were used for cell separation as described [22]. Briefly, cells were resuspended in complete medium supplemented with 2% fetal calf serum (FCS) and mixed with magnetic beads at a 10:1 target cell–to-bead ratio. After incubation for 60 min at 4°C on a Rock-n-Roller (Snijders Scientific, Tilburg, Netherlands), rosetted cells were isolated on a magnetic separation device (MPC-6; Dynal). Immediately after fractionation, positively selected cells were detached from magnetic beads using a goat anti-mouse Fab polyclonal antiserum (DETACHaBEAD; Dynal) as described [22].

Generation of B Lymphoblastoid Cell Lines

EBV-transformed B lymphoblastoid cell lines (B-LCL) were established by incubation of PBMC with cell-free supernatant from the EBV-producing marmoset cell line B95-8 (American Type Culture Collection, Rockville, MD), in the presence of 3 μg/mL cyclosporin A (Sandoz Pharma, Basel, Switzerland). B-LCL were maintained in complete medium supplemented with 10% FCS (R-10).

Preparation of HIV-1 Gag Antigen–Presenting Cells (APC)

APC expressing the p55 protein of HIV-1_{LAI} were prepared as described [23]. Briefly, autologous B-LCL were infected overnight with the recombinant vaccinia virus (rVV) TG1144 (Transgene, Strasbourg, France) [24] at an MOI of 10 and then fixed in 1.5% paraformaldehyde. Antigen expression was confirmed by immunofluorescent analysis using a polyclonal bovine anti-vaccinia serum (RIVM, Bilthoven, Netherlands) and the murine anti-p24 MAb CLB14 (CLB, Amsterdam) as described [23].

HIV-1 Gag–Specific CTL

Limiting dilution cultures. At least four PBMC dilutions in R-10 were seeded in 96-well round-bottomed plates, and each dilution was done in at least 24 replicate wells. Appropriate numbers and ranges of dilutions and numbers of replicate wells were predetermined for each patient with baseline PBMC samples. Cells were cultured with HIV-1 Gag APC (10^4/well), autologous irradiated (2500 rad) feeder PBMC (10^5/well), and recombinant interleukin-2 (rIL-2) at 10 U/mL (final concentration) from day 3. To study CTLp activation requirements, PBMC and positively isolated CD8 cells were cultured in limiting dilution with HIV-1 Gag APC (10^4/well), with or without rIL-2 (10 U/mL), as indicated. After 14 days, two 50-μL aliquots from each well were screened for cytotoxicity against autologous B-LCL infected overnight with either HIV-1 Gag rVV TG1144 or vaccinia control 186 poly (10 MOI). To analyze MHC restriction, HIV-1 Gag–infected B-LCL from MHC class I–mismatched donors were used as targets in additional experiments.

Bulk cultures. Positively selected CD8 or CD4 cells (2×10^5/well) in R-10 were seeded in 96-well round-bottomed plates, in the presence of HIV-1 Gag APC (10^4/well), with or without autologous irradiated (2000 rad) CD4 cells (2×10^4/well), and with or without rIL-2 (20 U/mL) from day 3, as indicated. After 14 days, cultures were screened for cytotoxicity against autologous B-LCL infected with either HIV-1 Gag rVV or vaccinia control, at indicated effector-to-target cell (E:T) ratios.

EBV-Specific CTL

Limiting dilution cultures. Parallel cultures were set up as described above using PBMC from the same cell sample. Autologous irradiated (5000 rad) B-LCL infected overnight with EBV were used as stimulator cells at 10^2–10^4/well (stimulator-to-effector cell ratio, <1:1), in the presence of autologous irradiated feeder PBMC (10^4/well) and rIL-2 (10 U/mL) from day 5. To study CTLp activation requirements, positively isolated CD8 cells were cultured with irradiated stimulator B-LCL alone. After 14 days, two 50-μL aliquots from each well were screened for cytotoxicity against autologous and MHC class I–mismatched EBV-infected B-LCL.

Bulk cultures. PBMC (10^5/mL) were cultured in 24-well plates with autologous irradiated stimulator B-LCL (10^5/mL), autologous irradiated feeder PBMC (10^5/mL), and rIL-2 (10 U/mL) from day 5. Alternatively, positively selected CD8 or CD4 cells (10^5/mL) were cultured with or without irradiated (2000 rad) CD4 cells (10^5/mL), and with or without rIL-2 (10 U/mL), in the presence of autologous irradiated stimulator B-LCL at indicated stimulator-to-effector cell ratios. In additional experiments, autologous B-LCL were treated with paraformaldehyde as described [23] before use as stimulator cells at 10^5/mL. After 10 days, cultures were screened for cytotoxicity against autologous and MHC class I-mismatched or partially matched B-LCL, at indicated E:T ratios. EBV-specific CTL lines were established by periodic restimulation (every 14–20 days) with irradiated stimulator B-LCL, in the presence of autologous irradiated feeder PBMC and rIL-2 (20 U/mL).

Immunofluorescence Analysis

Circulating CD3, CD4, and CD8 cells, cell fractions after immunomagnetic separation, and expanded HIV-1 Gag– and EBV-specific CTL were analyzed in double-color immunofluorescence analysis using fluorescein isothiocyanate– or phycoerythrin-conjugated anti-CD3, anti-CD4, and anti-CD8 MAbs (Becton Dickinson, Etten-Leur, Netherlands).

Cytotoxicity Assay

Cytotoxicity was measured in standard ^{51}Cr release assays. Targets were labeled for 1 h with 100 μCi of ^{51}Cr at 37°C in 7% CO_2, washed three times, resuspended in R-10 at 10^5/mL, then added to effector cells at 50 μL (5000 cells)/well in 96-well round-bottomed plates (Costar, Cambridge, UK). After a 5-h incubation at 37°C in 7% CO_2, supernatants were harvested (Skatron harvester; Skatron, Oslo), and the release of ^{51}Cr was measured in a gamma counter. Maximum ^{51}Cr release was determined by detergent (5% Triton X-100) lysis of targets. Spontaneous release was determined by incubation of target cells in R-10 alone. Spontaneous release was <25% of maximum release in all reported assays.

Calculation of Results

Percentages of lysis were calculated as follows: % lysis = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Individual wells of limiting dilution cultures were considered positive when lysis of specific targets exceeded by 10% that of control targets if the latter was <10% or by 20% if the latter was >10%. The reliability of these thresholds was tested by pilot experiments in which, after split-well CTL assays, residual cells of limiting dilution cultures were restimulated to generate short-term CTL lines (data not shown). CTLp frequencies were estimated by the maximum likelihood method using the statistical software package described by Strijbosch et al. [25]. All frequencies were normalized to the number of CTLp/10^6 PBMC. Rates of change (slopes) and correlation coefficients *(r)* were calculated by linear regression analysis.

Results

Kinetics of HIV-1 Gag– and EBV-Specific CTLp

The frequencies of HIV-1 Gag– and EBV-specific CTLp were measured longitudinally in 9 HIV-1–infected subjects. Of these, 3 (Ew03, Fw19, Gw21) remained asymptomatic and maintained CD4 cell numbers above 200/μL throughout the study, 3 (Fw07, Gw38, and Gw39) remained asymptomatic but their CD4 cell numbers declined to below 200/μL, and 3 others (Hw39, Lw40, and Gw43) had clinical manifestations of AIDS (CDC stages IV-A and IV-D; figure 1). Essentially two response patterns could be distinguished: HIV-1 Gag–specific CTLp were maintained or increased in 5 patients (figure 1A), whereas they declined in the remaining 4 patients (figure 1B). In contrast, the frequencies of EBV-specific CTLp were maintained or even increased over time in all 9 patients. Figure 2 summarizes the rates of change (slopes) of CTLp frequencies, as calculated by linear regression analysis of the longitudinal data.

Reproducibility of CTLp Frequency Estimates

Precautions were taken to reduce the influence of variations in experimental conditions on the outcome of CTLp measurements. First, to allow comparison, HIV-1 Gag– and EBV-specific CTLp were measured in parallel in the same PBMC sample. Second, to improve the goodness of fit of limiting dilution analysis lines, pilot limiting dilution assays were carried out with baseline PBMC samples, providing optimal numbers and ranges of dilutions and numbers of replicate wells for each subject [25]. Subsequently, PBMC from three or four time points were tested simultaneously using identical culture and assay conditions. The reproducibility of CTLp measurements was verified in 22 replicate experiments, which showed a mean coefficient of variation (as 100× SDs of the residuals/mean CTLp frequency estimate) of 7.4% ± 3.8% (data not shown).

Correlation of HIV-1 Gag–specific CTLp Frequencies with CD4 and CD8 Cell Numbers

The patterns of response in the 9 subjects appeared to be independent of the clinical status at baseline, as shown by the presence of symptomatic patients (i.e., Gw43, Lw40, and Hw39) in each group. A clear relationship was detected between the kinetics of HIV-1 Gag–specific CTLp and those of circulating CD8 and, to a lesser extent, CD4 cell numbers (figure 1). A cumulative analysis of this relationship is shown in figure 3. The frequencies of HIV-1 Gag–specific CTLp showed a significant correlation ($r = .38$; $P < .05$) with the absolute numbers of CD4 cells. A stronger and more significant correlation ($r = .75$; $P < .001$) was found between HIV-1 Gag–specific CTLp frequencies and the absolute numbers of CD8 cells. A significant correlation was also observed between

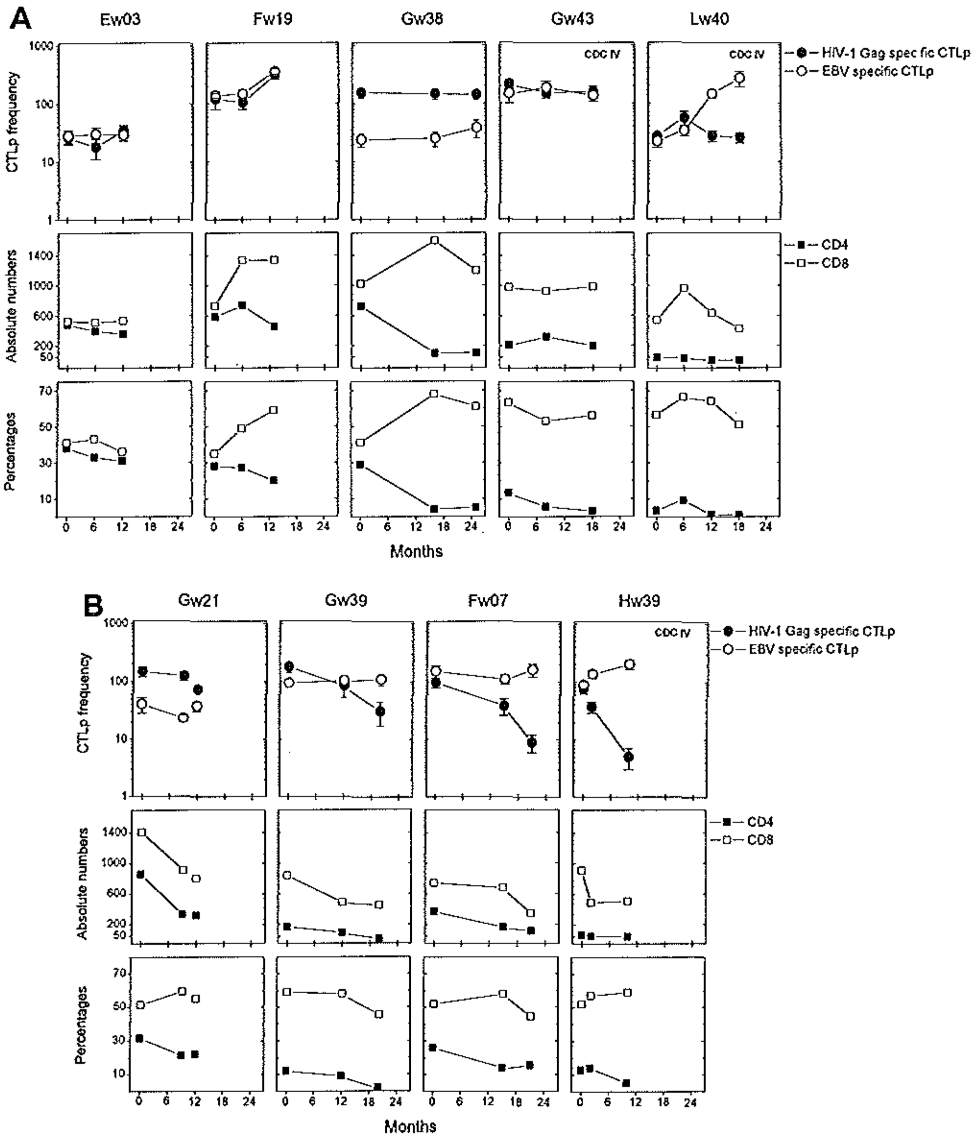

Figure 1. Kinetics of HIV-1 Gag– and EBV-specific cytotoxic T lymphocyte precursors (CTLp); their relationship to absolute nos. and % of CD4 or CD8 cells in 9 HIV-1 infected subjects with maintained or increasing (A) or decreasing HIV-1 Gag–specific CTLp (B). CTLp frequencies were determined by limiting dilution analyses of peripheral blood mononuclear cells (PBMC). Frequencies were normalized to no. of CTLp/10^6 PBMC. Error bars indicate 95% confidence intervals.

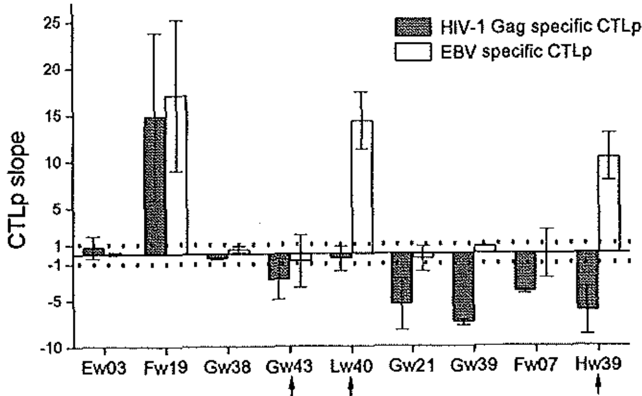

Figure 2. Rates of change (slopes) of HIV-1 Gag– and EBV-specific cytotoxic T lymphocyte precursor (CTLp) frequencies, determined from linear regression analysis of longitudinal CTLp data in figure 1. Arrows indicate symptomatic (CDC stage IV) patients. Positive slopes indicate increase, negative slopes correspond to decrease. CTLp frequencies were considered maintained if slope with SE overlaps range of −1 to +1/10⁵ PBMC/month (dotted lines).

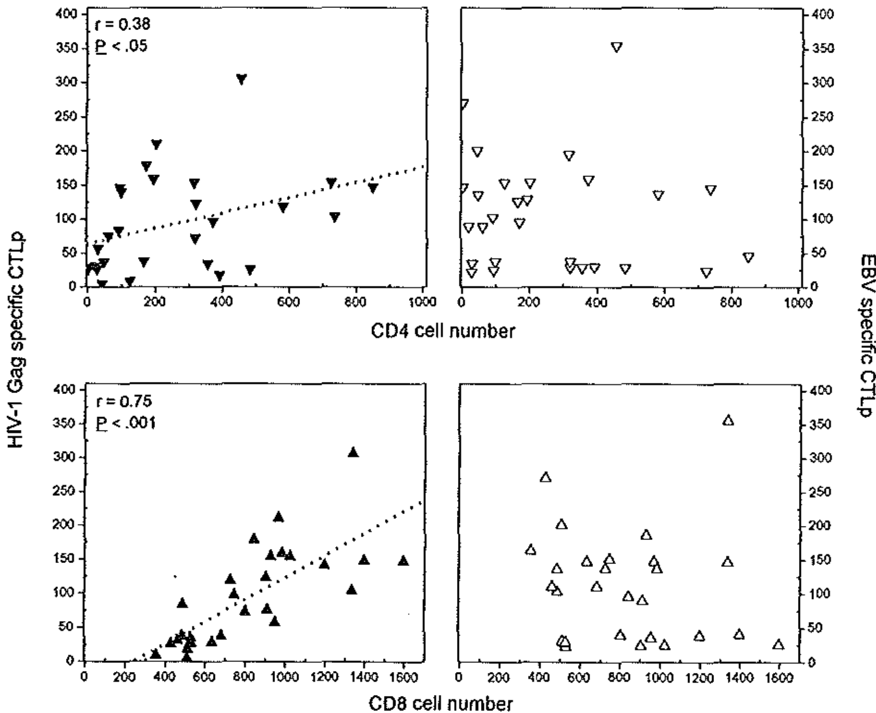

Figure 3. Frequencies of HIV-1 Gag (right)– and EBV (left)-specific cytotoxic T lymphocyte precursors (CTLp) in peripheral blood mononuclear cells (PBMC) from 9 HIV-1–infected subjects at 3 or 4 time points, plotted against patients' absolute CD4 (top) and CD8 (bottom) cell nos. Frequencies were normalized to no. of CTLp/10⁶ PBMC.

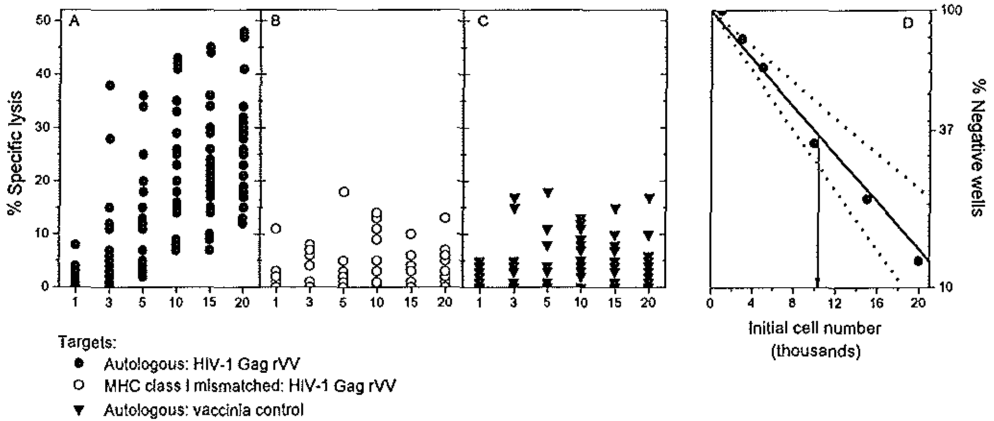

Figure 4. Single-well analysis of cytotoxic responses against HIV-1 Gag. Baseline peripheral blood mononuclear cells (PBMC) from patient Fw07 were seeded in 6 dilutions (range: 20,000–1000 cells/well), each including 24 replicate wells. After 14 days, 3 aliquots from each well were screened for cytotoxicity against autologous (A) and major histocompatibility complex (MHC) class I–mismatched (B) B lymphoblastoid cell lines (B-LCL) infected with HIV-1 Gag recombinant vaccinia virus (rVV) and against autologous B-LCL infected with vaccinia control (C). D, % of negative wells plotted against initial cell no. Linearity of relationship is consistent with single-hit Poisson model. Frequency of HIV-1 Gag-specific CTLp (1/10,100, or 99/10^6 PBMC) was estimated by maximum likelihood method (solid line interpolated at 37% negative wells). Dotted lines indicate 95% confidence intervals (74–124/10^6 PBMC).

the patients' CD4 and CD8 cell numbers (r = .43; P < .05; data not shown), whereas the frequencies of EBV-specific CTLp were not correlated with either CD4 (r = −.097; P = .62) or CD8 (r = −.057; P = .77) cell numbers.

Phenotype of HIV-1 Gag–specific CTLp

We have previously shown that in vitro stimulation of PBMC from HIV-1–infected subjects with HIV-1 Gag APC promotes the expansion of HIV-1 Gag-specific CTL of the CD8 and MHC class I–restricted phenotype [23]. In agreement with our previous observations, immunofluorescence analyses done on day 14 or 15 of limiting dilution cultures showed that cell populations in wells containing HIV-1 Gag–specific CTLp were predominantly (>85%) of the CD3CD8 phenotype and <1% were of the CD3CD4 phenotype (data not shown). Furthermore, as shown in figure 4 for patient Fw07, additional limiting dilution analyses confirmed that cytotoxic responses to HIV-1 Gag were directed predominantly against autologous rather than MHC class I–mismatched targets.

Activation Requirements of HIV-1 Gag–specific CTL

Limiting dilution assays. We studied the effects of CD4 cell depletion on the expansion of HIV-1 Gag–specific CTLp in patients Gw43 (baseline) and Hw39 (at 2 months). Limiting dilution analyses were done with PBMC and highly pure (>99%) positively isolated CD8 cells in the presence of HIV-1 Gag APC and rIL-2. As shown in table 1, the frequencies of HIV-1 Gag–specific CTLp estimated in the positively isolated CD8 cell fractions were only slightly lower than those measured in PBMC. This indicated that the absence of CD4 cells did not have adverse effects on CTLp activation and expansion. To study the requirement for exogenous rIL-2, PBMC and the positively selected CD8 cell fraction from patient Gw43 were

Table 1. Frequency of HIV-1 Gag–specific cytotoxic T lymphocyte precursors (CTLp) determined by parallel limiting dilution analyses of peripheral blood mononuclear cells (PBMC) and positively isolated CD8 cells ($CD8^+$ fraction) from 2 HIV-1–infected patients.

		HIV-1–infected patients	
Effector cells	rIL-2*	Gw43	Hw39
PBMC	+	259 (197–321)	33 (23–44)
$CD8^+$ fraction	+	213 (164–262)	21 (10–31)
PBMC	–	114 (91–138)	NT
$CD8^+$ fraction	–	31 (10–52)	NT

NOTE. NT, not tested because of limited supply of PBMC samples. To facilitate comparison, all frequencies were normalized to no. of CTLp/10^6 PBMC (95% confidence intervals).

* Recombinant interleukin-2 (rIL-2) was added to cultures on days 3, 7, and 9.

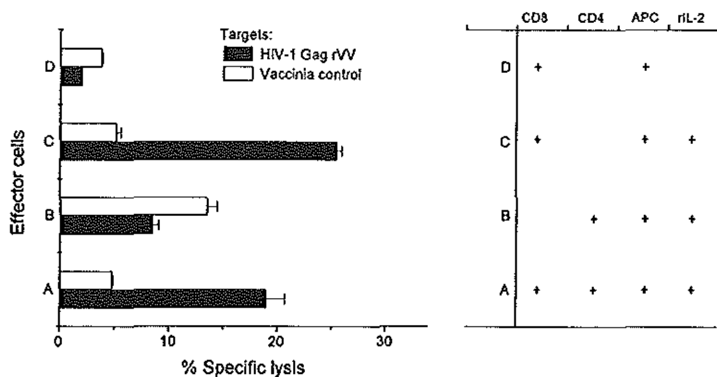

Figure 5. Activation requirements of HIV-1 Gag–specific cytotoxic T lymphocytes. Effector cells were positively selected CD8 cells from patient Gw43 cultured with HIV-1 Gag antigen-presenting cells (APC), autologous irradiated CD4 cells, and recombinant IL-2 (rIL-2) from day 3 (line A); CD4 or CD8 cells cultured with HIV-1 Gag APC and rIL-2 from day 3 (lines B, C); CD8 cells cultured with HIV-1 Gag APC alone (line D). After 14 days, cytotoxicity was measured against autologous B lymphoblastoid cells infected with either HIV-1 Gag recombinant vaccinia virus (rVV) or vaccinia control. Results are expressed as mean specific lysis with SE from duplicate well estimations at effector-to-target cell ratio of 40:1.

cultured in limiting dilution with HIV-1 Gag APC alone. The expansion of HIV-1 Gag–specific CTLp was reduced in the PBMC and, to a greater extent, in the positively isolated CD8 cell fraction (table 1). This indicated that, particularly in the absence of CD4 cells, exogenous rIL-2 was required to promote CTLp growth.

Bulk assays. The results of limiting dilution assays were confirmed in bulk assays of highly pure (>99%) positively isolated CD8 and CD4 cell fractions from patient Gw43. As shown in figure 5 (line A), HIV-1 Gag–specific CTL were expanded from CD8 cells cultured with HIV-1 Gag APC, autologous irradiated CD4 cells, and rIL-2. The positively selected CD4 cell fraction cultured in the same fashion showed a cytotoxic response mostly directed against control targets (figure 5, line B). Consistent with the results obtained in limiting dilution, HIV-1 Gag–specific CTL were expanded from CD8 cells cultured with HIV-1 Gag APC and rIL-2 in the absence of CD4 cells (figure 5, line C). Finally, no CTL were detected in CD8 cells cultured with the same APC in the absence of both CD4 cells and rIL-2.

Effects of CD4 Cell Enrichment on the Expansion of HIV-1 Gag–specific CTLp

Patient Hw39 showed a marked decrease of both HIV-1 Gag–specific CTLp and CD4 cell numbers, while maintaining stable CD8 cell numbers, between the second (2 months) and third (10 months) assessment (figure 1B). To study whether CTLp detection at 10 months could be enhanced by CD4 cell enrichment, the positively isolated CD8 cell fraction from time point 10 months (>99% CD8 cells) was reconstituted with the CD8 cell–depleted fraction from time point 2 months (31% CD4 cells, <2% CD8 cells) before stimulation in limiting dilutions. The reconstituted population contained 57% CD8 cells from time point 10 months and 13% CD4 cells from time point 2 months. In parallel, limiting dilution cultures were set up with PBMC from time points 2 months (14% CD4 cells, 57% CD8 cells) and 10 months (5% CD4 cells, 59% CD8 cells). Despite CD4 cell reconstitution, the frequency of HIV-1 Gag–specific CTLp in the reconstituted population (figure 6, bottom) was similar to that measured in PBMC from time point 10 months (figure 6, center) and still considerably lower than that measured in PBMC from time point 2 months (figure 6, top).

Phenotype of EBV-specific CTLp

As also observed with HIV-1 Gag–specific CTLp, immunofluorescence analyses done on day 14 or 15 of limiting dilution cultures showed that cell populations in wells containing EBV-specific CTLp were predominantly of the CD3CD8 phenotype (data not shown). This observation suggested that EBV-specific CTL were indeed CD8 cells and was consistent with the detection of cytotoxic responses restricted predominantly to autologous targets. However, in both limiting dilution (data not shown) and bulk cultures of positively isolated CD8 cell fractions (figure 7), the phenotype of cytotoxic cells against EBV was influenced by the ratio of stimulator to effector cells: Ratios below 1:1 were required to favor the growth of MHC class I–restricted CTL over that of unrestricted cytotoxic cells.

Characterization of EBV-specific CTL in Patient Gw43

To analyze further the phenotype of EBV-specific CTL, bulk PBMC cultures were done with stimulator B-LCL at 1:10 stimulator-to-effector cell ratios and separated into positively iso-

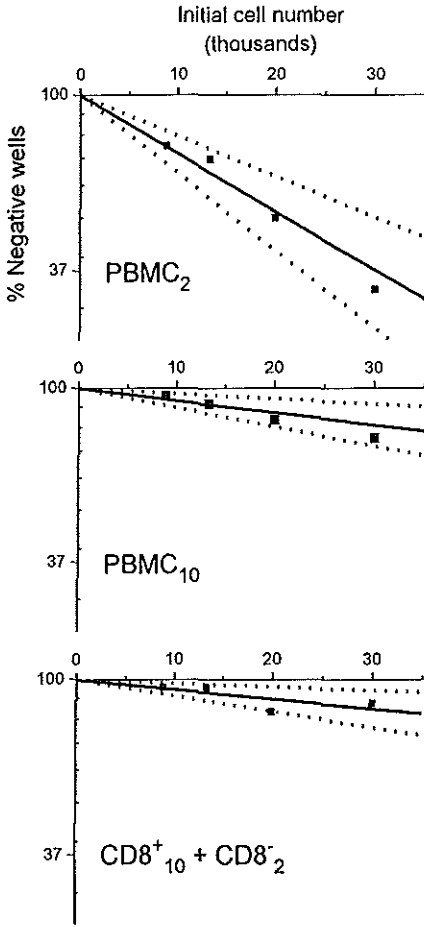

Figure 6. Frequency analysis of HIV-1 Gag-specific cytotoxic T lymphocyte precursors (CTLp) in patient Hw39. Effector cells were peripheral blood mononuclear cells (PBMC) from time points 2 and 10 months ($PBMC_2$, $PBMC_{10}$, respectively) and positively selected CD8 cells from time point 10 months ($CD8^+_{10}$) reconstituted with CD8-depleted fraction from time point 2 months ($CD8^-_2$). Parallel cultures were set up in presence of HIV-1 Gag antigen-presenting cells and recombinant interleukin-2 from day 3. After 14 days, cytotoxicity was measured against autologous B lymphoblastoid cell lines infected with either HIV-1 Gag recombinant vaccinia virus or vaccinia control. CTLp frequencies were 33 (23–44), 7 (3–11), and 6 (2–9)/ 10^5 PBMC, respectively.

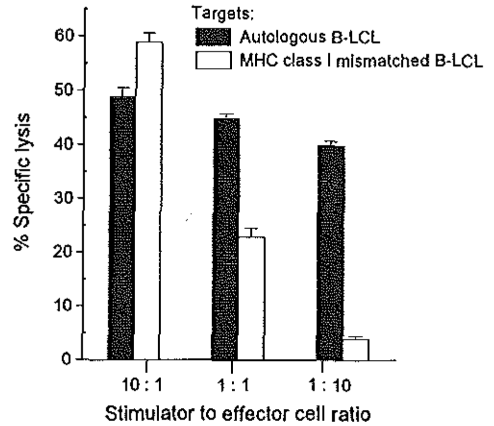

Figure 7. Effects of stimulator-to-effector cell ratios on cytotoxicity against EBV. Positively isolated CD8 cells from patient Gw43 were cultured with irradiated stimulator B lymphoblastoid cell lines (B-LCL), autologous irradiated CD4 cells, and recombinant interleukin-2 from day 5. After 10 days, cytotoxicity was measured against autologous and major histocompatibility complex (MHC) class I–mismatched B-LCL. Results are expressed as mean specific lysis with SE from triplicate well estimations at effector-to-target cell ratio of 30:1.

lated CD8 cell (>99% CD8 cells) and CD8 cell–depleted (<2% CD8 cells) fractions before use in CTL assays. As shown in figure 8 for patient Gw43, the EBV-specific CTL response detected in the total effector cell population was entirely preserved in the positively isolated CD8 cell fraction. Conversely, the response was abolished by CD8 cell depletion. In line with these observations, EBV-specific CTL lines established from PBMC of patient Gw43 all showed a CD8 and MHC class I–restricted phenotype. The restriction of CTL line Gw43/004 by MHC class I allele B62(15), determined with panels of autologous, MHC class I–mismatched and partially matched targets, is illustrated as an example in table 2.

Activation Requirements of EBV-specific CTL

Limiting dilution assays. To study the dependence of EBV-specific CTLp on CD4 cells and exogenous rIL-2, limiting dilution analyses were done with the positively isolated CD8 cell fraction from patient Gw43 (baseline), in the presence of irradiated stimulator B-LCL alone. As illustrated in figure 9, the frequency of EBV-specific CTLp estimated in the positively isolated CD8 cell fraction ($224/10^6$ cultured CD8 cells, equal to $141/10^6$ PBMC) (figure 9, bottom) was similar to that measured in parallel in the PBMC ($154/10^6$ PBMC) (figure 9, top). These results indicated that the activation and expansion of

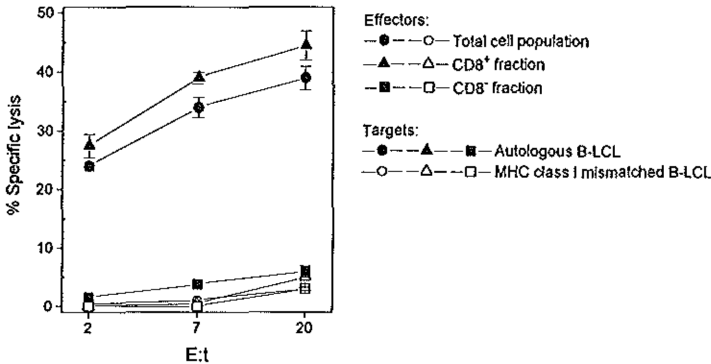

Figure 8. CD8 cell–mediated cytotoxicity against EBV. Peripheral blood mononuclear cells (PBMC) from patient Gw43 were cultured with irradiated stimulator B lymphoblastoid cell lines (B-LCL), autologous irradiated feeders, and recombinant interleukin-2 from day 5. After 10 days, cytotoxicity of total cell population and CD8-enriched ($CD8^+$) and CD8-depleted ($CD8^-$) fractions was measured against autologous (solid symbols) and major histocompatibility complex (MHC) class I–mismatched (open symbols) B-LCL. E:t, effector-to-target cell ratio.

EBV-specific CTLp was independent of both CD4 cells and exogenous rIL-2.

Bulk assays. Bulk cultures of highly pure (99%) positively isolated CD8 and CD4 cell fractions from patient Gw43 confirmed and extended the observations made in limiting dilution. As shown in figure 10, the presence of CD4 cells and rIL-2 (figure 10, line A) was not required to promote the growth of EBV-specific CTL, since this could be entirely supported by irradiated stimulator B-LCL (figure 10, line B). No CTL were detected in the CD4 cell fraction cultured in the same fashion (figure 10, line C). Similarly, no CTL were detected in the CD8 cell fraction cultured with rIL-2 alone, indicating that the presence of stimulator B-LCL was required for CTL activation (figure 10, line D).

Effects of B-LCL Fixation in Paraformaldehyde

As also shown previously by Fishwild et al. [26], the ability of stimulator B-LCL to support the growth of EBV-specific CTL was abolished by fixation with paraformaldehyde (figure 10, line E). This inhibition was not corrected by the presence of autologous irradiated CD4 cells but could be partially overcome by the addition of exogenous rIL-2 at the beginning of culture (figure 10, line F). The latter finding indicated that paraformaldehyde-treated B-LCL had retained their ability to activate EBV-specific CTL but lost their ability to promote CTL expansion, probably because of reduced release of soluble factors.

Table 2. Major histocompatibility complex (MHC) class I–restricted cytotoxicity against EBV.

Targets*	MHC class I†	% lysis
Gw43	A10, A24 (9), B35, B62 (15)	37
617	A2, A11, <u>B35</u>, <u>B62 (15)</u>	21
RV	A3, <u>A10</u> (25), B18, B51	0
233	A3, <u>A24 (9)</u>, <u>B35</u>, B60	2
067	<u>A10</u> (26), A28, B7, B57	1
Gw38	A3, A31 (19), B44 (12), B13	8

* Autologous and partially matched EBV-infected B lymphoblastoid cell lines at 15:1 effector-to-target cell ratio.
† Shared MHC class I alleles are underlined.

Discussion

We report a longitudinal study showing that HIV-1–infected persons may retain EBV-specific CTL responses while losing both their CD4 cells and their CTL responses against the Gag protein of HIV-1.

For CTLp detection we used an antigen-specific stimulation protocol that proved highly efficient in inducing selective expansion of MHC class I–restricted CD8 CTL against HIV-1 Gag [23] (figure 4). As also observed by Lubaki et al. [27], antigen-specific stimulation allowed the detection of HIV-1 Gag–specific CTL responses even in patients with advanced disease (figure 1). The frequency of HIV-1 Gag–specific CTLp differed considerably among patients, ranging from 5 to 306/10^6 PBMC. This is consistent with results obtained in cross-sectional studies of HIV-1–infected persons [6–9]: Frequencies ranging from 58 to 588/10^6 PBMC were reported by Koup et al. [7], whereas Carmichael et al. [9] measured HIV-1 Gag–specific CTLp at frequencies ranging from 0 to ~488/10^6 PBMC. Similarly, the frequency of EBV-specific CTLp differed considerably from patient to patient, ranging from 18 to 355/10^6 PBMC (figure 1), which is also in agreement with observations made by others [9, 13]. We studied extensively the reliability and reproducibility of the limiting dilution analyses in assessing changes in CTL immunity over time. Optimal assay parameters were first determined for each patient with baseline samples, and subsequently PBMC from all time points

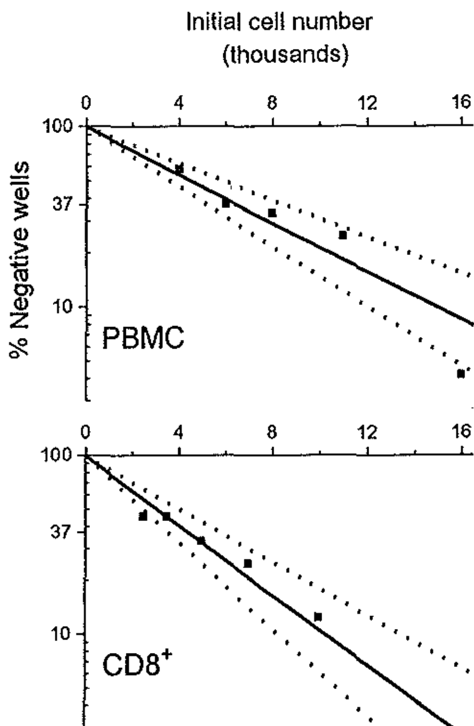

Figure 9. Expansion of EBV-specific cytotoxic T lymphocyte precursors (CTLp) in absence of CD4 cells and exogenous recombinant interleukin-2 (rIL-2). Limiting dilution cultures of peripheral blood mononuclear cells (PBMC, composed of 63% CD8 cells) from patient Gw43 were done with irradiated stimulator B lymphoblastoid cell lines (B-LCL), irradiated autologous feeder PBMC, and rIL-2 from day 5. In parallel, positively isolated CD8 cells ($CD8^+$, >99% CD8 cells) were cultured in presence of stimulator B-LCL alone. After 14 days, cytotoxicity was measured against autologous and major histocompatibility complex class I-mismatched B-LCL. EBV-specific CTLp frequencies were 154 (117–191)/10^6 PBMC and 224 (171–278)/10^6 cultured CD8 cells, respectively.

were tested simultaneously using identical culture and assay conditions. This resulted in a high reproducibility of CTLp measurements, with mean coefficient of variation among replicate experiments of 7.4%.

In two earlier cross-sectional studies, the EBV-specific CTL responses of AIDS patients were compared with those of asymptomatic HIV-1–seropositive and healthy seronegative persons: The relatively high numbers of EBV-specific CTLp detected in some HIV-1–infected persons by Carmichael et al. [9] are in agreement with our observations but seem to contradict the low levels of EBV-specific CTL immunity observed in HIV-1–infected persons by Blumberg et al. [21]. It should be pointed out, however, that the study by Blumberg et al. compared the results of bulk CTL assays rather than those of limiting dilution analyses. The latter approach may indeed increase CTL detection in HIV-1–infected persons [8]. Our longitudinal study extends these initial cross-sectional observations by providing data on individual patients followed for up to 25 months (figure 2), together with evidence of a significant relationship between the kinetics of HIV-1 Gag CTLp frequencies and those of circulating CD4 and CD8 cell numbers (figures 1, 3). Furthermore, we attempted to investigate possible mechanisms underlying EBV-specific CTLp maintenance in otherwise immunocompromised patients.

Several mechanisms have been proposed to explain HIV-1–specific CTL decline during disease progression, including impairment of APC function [28], infection of CD8 cells [29], emergence of CTL suppression [30, 31], and antigenic variation in HIV-1 Gag leading to escape from CTL surveillance [32]. The hypothesis that persistently high levels of antigenic stimulation may first lead to clonal expansion and subsequently to clonal exhaustion of virus-specific CD8 CTL has also been postulated [33, 34]. CD4 T helper cell dysfunction is likely to play an important part in the decline of HIV-1 specific CTL immunity [35–37]. In support of this view, we detected a significant correlation between the numbers of HIV-1 Gag–specific CTLp and those of circulating CD4 cells (figure 3, top left). Previous cross-sectional studies have either denied [38] or confirmed [39] this association, a discordance probably due to different methods of CTL measurement as well as different compositions of the groups of patients studied.

CTL differentiation in vivo is a complex multistep process, which obviously can only be partially explored through in vitro studies. In particular, the degree of dependence on CD4 cell–mediated help is likely to vary in relation to the stage of CTL differentiation and may become less stringent during in vitro recall. In an attempt to study this dependence, we found that the expansion of HIV-1 Gag–specific CD8 CTLp, although requiring the addition of exogenous rIL-2, was not significantly influenced by removal (table 1, figure 5) or enrichment (figure 6) of CD4 cells.

These findings imply that HIV-1–induced CD4 cell dysfunction may affect HIV-1–specific CTL responses at an early stage of CTL differentiation. As a consequence, HIV-1–specific CTL may lose their responsiveness in vitro [12] and eventually disappear [40–42]. Indeed, a progressive disappearance of HIV-1–specific CTL was suggested by the finding that the decline of HIV-1 Gag–specific CTLp was strongly correlated with the decline of circulating CD8 cells (figure 3, bottom left). This strong correlation seems to confirm the view that in the course of HIV-1 infection, the kinetics of circulating CD8 cells are mostly a reflection of the host response against HIV-1 [38, 39].

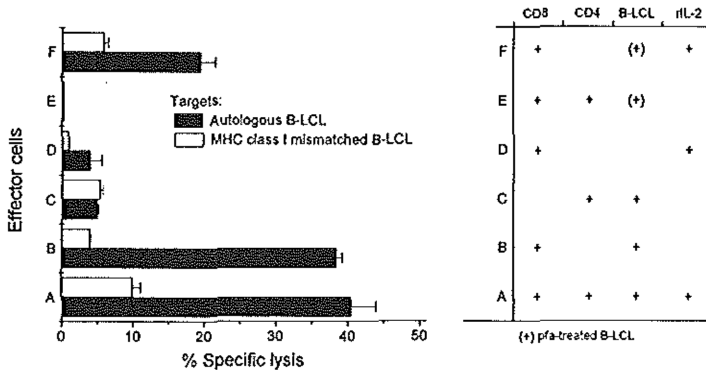

Figure 10. Activation requirements of EBV-specific cytotoxic T lymphocytes. Effector cells were positively selected CD8 cells from patient Gw43 cultured with irradiated stimulator B lymphoblastoid cell lines (B-LCL), autologous irradiated CD4 cells, and recombinant interleukin-2 (IL-2) from day 5 (line A); CD8 or CD4 cells cultured with autologous irradiated stimulator B-LCL alone (lines B, C); CD8 cells cultured with rIL-2 alone from day 1 (line D); CD8 cells cultured with autologous paraformaldehyde (pfa)-treated B-LCL and either autologous irradiated CD4 cells or rIL-2 from day 1 (lines E, F). After 10 days, cytotoxicity was measured against autologous and major histocompatibility complex (MHC) class I–mismatched B-LCL. Results are expressed as mean specific lysis with SE from triplicate well estimations at effector-to-target cell ratio of 50:1.

In contrast with the frequency of HIV-1 Gag–specific CTLp, the frequency of EBV-specific CTLp was not correlated with either CD4 or CD8 cell numbers. Indeed, the most evident differences between the kinetics of HIV-1 Gag–specific CTLp and those of EBV-specific CTLp were seen in the presence of marked CD4 cell decline (figure 1B, figure 3 [top]). This observation indicates that EBV-specific CTL immunity may remain longer unaffected by HIV-1–induced CD4 cell and cytokine dysfunction. Results of in vitro studies seem to support this view. We found that EBV-transformed B-LCL were able to finely regulate (figure 7) and support the growth of EBV-specific CD8 CTL (figure 8) in the absence of both CD4 cells and exogenous cytokines (figures 9, 10), as also reported in HIV-seronegative persons [26]. This ability of EBV-transformed B-LCL efficiently to activate EBV-specific CTL may reside in their high expression of MHC antigens and accessory molecules. Support for CTL expansion may derive from their rich production of soluble factors (figure 10) [26, 43], including IL-12, a cytokine that by itself can induce differentiation of CTLp into mature CTL [44]. Therefore, EBV infection of B lymphocytes in vivo may result in the induction of high-affinity CTL [45], displaying a low degree of dependence from CD4 cell-mediated help from the early stages of their differentiation.

In summary, our data indicate that HIV-1 Gag–specific CTLp can be detected in vitro even after the onset of symptomatic disease, although, in some persons, their numbers decline in correlation with declining CD4 and CD8 cells. The prolonged maintenance of EBV-specific CTLp in these persons may reflect the relative independence of EBV-specific CTL function from HIV-1–induced CD4 cell and cytokine dysfunction, possibly providing one explanation for the relatively low incidence of EBV-related lymphoproliferative disorders in patients with AIDS.

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Virus-driven Evolution of Simian Immunodeficiency Virus (SIV)-specific Cytotoxic T Lymphocyte Responses During Primary and Secondary Infection of Cynomolgus Macaques with SIVmac32H-J5

Anna-Maria Geretti[1,2], Ellen G. J. Hulskotte[1], Marlinda E. M. Dings[1], Carel A. van Baalen[1], Geert van Amerongen[3], Stephen G. Norley[4], Patrick Boers[1], Rob Gruters[1,5], and Albert D.M.E. Osterhaus[1]

Institute of Virology, Erasmus University, Rotterdam, The Netherlands[1],
Department of Virology, Royal Free Hospital School of Medicine, London, UK[2]
Central Animal Laboratory, National Institute of Public Health and the Environment, Bilthoven, The Netherlands[3],
Paul-Ehrlich Institute, Langen, Germany[4], and
UMR103, CNRS-Biomérieux, Ens de Lyon, France[5].

Submitted

SUMMARY

The evolution of simian immunodeficiency virus (SIV)-specific cytotoxic T lymphocyte (CTL) responses, and their relationship with the kinetics of virus replication, were studied in peripheral blood mononuclear cells (PBMC) and lymphoid organs of cynomolgus macaques infected intravenously with the molecular clone SIVmac32H-J5. In the early stage of infection, CTL precursor (CTLp) frequencies in PBMC were higher than those in lymph nodes. In a later stage, a dissociation emerged between PBMC and secondary lymphoid organs: virus load was higher in both spleen and lymph nodes than in PBMC and CTLp frequencies reflected virus distribution. During up to 22 months of observation, effective virus containment in three animals was indicated by the absence of both culturable virus and PCR-detectable provirus in PBMC. These findings were associated with a progressive decline of circulating SIV-specific CTLp, with the presence of relatively low-level virus reservoirs in lymphoid organs, and with at least partial susceptibility to homologous re-challenge. In one of these three animals, the detection of functionally immunodominant Gag-specific CTL, targeting amino acids 242-250 of p26 (SVDEQIQWM), coincided with the emergence of a variant virus carrying an aspartic acid to glutamic acid substitution at position 244. The mutated sequence was recognized poorly by CTL specific for the prototype epitope. However, CTL specific for the variant epitope were induced, which, like the variant virus itself, localized preferentially in the spleen. The observed compartmentalization of antiviral CTL to sites of virus distribution is consistent with their pivotal role in the containment of SIV infection.

INTRODUCTION

Even though the role of CTL in the host defence against lentiviruses has not as yet been firmly established, several observations indirectly support the view that CTL may indeed be effective against human immunodeficiency virus (HIV). CTL responses against HIV antigens have been detected in persons exposed to the virus but lacking evidence of infection (32). Furthermore, the detection of HIV-specific CTL in infected persons may be correlated with the length of the asymptomatic period, whereas CTL decline is usually associated with more advanced disease (6, 15, 22). A temporal association has also been described between the development of antiviral CTL and the containment of the acute phase of HIV replication (4, 24). However, studies of the relationship between CTL responses and virus burden during the subsequent course of infection have yielded conflicting results (11, 22).

The host and viral factors that facilitate virus persistence in the face of relatively strong antiviral CTL responses are still largely unknown. The high degree of variability shown by the HIV genome suggests that emergence of mutations within CTL epitopes may result in the selection of virus variants which escape recognition. In line with this hypothesis, single

amino acid substitutions have been shown to abrogate CTL recognition in vitro by affecting either MHC binding or TCR interaction (31). In addition, CTL escape variant viruses have been generated in vitro under selective CTL pressure (25). Virus variants containing mutations within CTL epitopes have also been demonstrated in vivo during infection with a number of viruses including HIV (5, 18, 23). However, the biological relevance of these variants remains somewhat controversial (8, 27). The observation that chronically infected persons generally mount a polyclonal and multi specific CTL response against several HIV antigens (6), indicates that complete evasion from immune surveillance may be a rare occurrence. Nevertheless, even reduced efficiency of CTL recognition may affect virus containment in vivo. At the same time, rapid virus evolution may favour immune evasion by causing CTL exhaustion, as a result of persistently high levels of antigenic stimulation (38).

Simian immunodeficiency virus of macaques (SIVmac) establishes a persistent infection in macaques which resembles HIV infection of humans (10). As in humans, the course of infection varies considerably: some animals rapidly develop an AIDS-like disease and die within few weeks or months, whereas others may remain asymptomatic for a few years. Infection of macaques with a molecular clone of SIVmac offers a unique opportunity to explore the contribution of host antiviral immune responses to the containment of lentiviral infections. We have previously demonstrated that vaccine-induced Env-, Gag- or Nef-specific CTL did not prevent infection of cynomolgus macaques upon intravenous challenge with the molecular clone SIVmac32H-J5, nor were they associated with significantly reduced virus load immediately after infection (20). A number of earlier studies have also addressed the contribution of CTL responses to the control of acute SIV infection (12, 21, 29). Here, we investigated the relationship between SIV replication and antiviral CTL responses in chronically infected animals. As the analysis of peripheral blood may not adequately reflect events in secondary lymphoid organs, virological and immunological data from peripheral blood were compared to those from spleen and lymph node samples.

MATERIALS AND METHODS

Animals. Eight colony-bred juvenile cynomolgus macaques were infected intravenously with either 50 (primary challenge) or 200 (re-challenge) monkey median infectious doses of cell-free SIVmac32H-J5 (33). Four monkeys (K77*, K80*, K83*, and K88*) had been previously vaccinated with an SIV Env-, Gag-, and Nef- subunit vaccine but became infected upon challenge (20). Observation lasted minimum 13 months (monkeys K73 and K79) and up to 22 months (monkey K66, K71, K77*, K80* and K88*). Monkey K83 was euthanized 18 months after infection upon development of symptomatic disease with diarrhoea and weight loss.

Preparation of peripheral blood, lymph node and splenic mononuclear cells (PBMC, LNMC and SPMC). Animals were anaesthetized with ketamine for periodic blood sampling and lymph node biopsies. Spleens were removed at the end of observation. Lymph node and spleen biopsies in

complete culture medium (RPMI 1640 with 100 U/ml penicillin, 100 µg/ml streptomycin, 10^{-5} M β-mercapto ethanol, and 2 mM L-glutamine) containing 10% FCS were teased with forceps to place cells in suspension and filtered through 112-µm-pore-size nylon mesh. Mononuclear cells were separated by density gradient centrifugation (20), and either used immediately or cryopreserved. Herpes virus papio-transformed B-LCL were established by incubation of PBMC with cell-free supernatant from cell line S594 (20).

Flow cytometry analysis. Cells in PBS with 0.1% BSA were incubated for 30' at room temperature with anti-CD2 (Leu-5b-FITC, Beckton Dickinson, Montan View, CA) and anti-CD8 (Leu-2a-PE, Beckton Dickinson) or anti-CD4 (OKT4-FITC, Ortho Diagnostic System, Raritan, NJ) monoclonal antibodies (mAb), washed twice, fixed in 1.5% paraformaldheide, and analysed with a FACscan (Beckton Dickinson).

Virus detection. *I.* Cell-associated virus load was determined in serial dilutions of freshly isolated PBMC, LNMC and SPMC, and in their CD8-depleted fractions, by co-culture with the human T cell line C8166; the lowest dilution contained 10^6 cells (20). Cell separation was performed with antibody-coated magnetic beads (Dynabeads M-450, Dynal, Oslo, Norway) as described (14). *II.* SIV p26 core antigenemia was assayed by antigen-capture ELISA (Coulter SIV Core Antigen Assay, Coulter Electronics, Mijdrecht, Netherlands). *III.* A 500-bp fragment of SIV Gag was amplified by nested PCR, modified from Chen et al. (8). Briefly, high molecular weight DNA was extracted with silica particles (3) from 10^6 freshly isolated PBMC, LNMC, SPMC, their CD4-enriched fractions, and the same cells after co-culture with C8166 cells. After heparinase treatment, 10 µl of a 100 µl solution were resuspended in a final volume of 100 µl containing 1X PCR buffer (50 mM KCl, 10mM Tris-HCl pH 9.0, 0.1% Triton X-100), $MgCl_2$ (1.5mM), dNTP (0.2mM), Taq polymerase (1 U), and primers (31 pM). Two sets of primers were used, for outer (5'- CATTCACGCAGAAGAGAAAG; 3'-GCATTTTGAATCAGCAGTG) and inner (5'-ACAAGTAGACCAACAGCACC; 3'-TGAAATGGCTCTTTTGGCC) amplification respectively. PCR conditions were: 1'10" at 94°C, 1'30" at 50°C and 1' at 72°C for 25 and 30 cycles, with a 10' final extension. Amplified DNA was analysed on 2% agarose gel. Sequence analyses were conducted both directly and after cloning in a pCR2 cloning vector with the TA cloning kit (In Vitrogen, Mountain View, CA). Automated sequencing was performed with the Dye Deoxy Terminator sequencing system (Applied Biosystems, Foster City, CA) with inner primers or T7 and SP6 primers. All clones were sequenced on both strands. Data were analysed with the Geneworks program (Intelligenetics, Mountain View, CA).

Antibody detection. Anti-Env antibodies were measured by indirect ELISA (20). Virus neutralising antibodies were measured in a yield reduction assay. Briefly, 1:50 serum dilutions (20 µl/well) were incubated for 30' at 37°C with 20 µl of serially diluted SIVmac32H in eight replicates per dilution. C8166 cells ($1.33X10^4$/ml) were added to each well in 150 µl. After 7 days, cells were transferred to poly-L-lysine coated plates, fixed in methanol and analyzed by immunoperoxidase staining (20). The yield reduction was calculated as the ratio of virus titre (TCID50/ml) in the absence of serum to the titre in the presence of the sample serum.

Recombinant vaccinia viruses. Recombinant vacinia vectors (rVV) were used to express: Env (20) and Gag p55 (provided by Prof. A. McMichael, Institute of Molecular Medicine, Oxford, UK) of SIVmac32H; Nef of SIVmac32H-J5 (20); Pol of SIVmac-BK28 (ADP263, provided by Dr. H.C. Holmes through the MRC AIDS Directed Programme (Potters Bar, South Mills, UK); and Tat of SIVmac251 (TG4174, Transgene, Strasbourg, France). The vector 186-poly, provided by Dr M. P. Kieny (Transgene) and containing a polycloning site without insert, was used as control.

Synthetic peptides. Three sets of peptides (20 residues long with a 10 amino acid overlap) covering p26 (ADP714/1-22), p17 (ADP775/1-13), and p15 (ADP776/1-14) of SIVmac251 were provided by Dr. H.C. Holmes through the MRC AIDS Directed Programme. A 9-mer peptide designated p26A.5, covering amino acids 242-250 of p26 (SVDEQIQWM), and a 9-mer variant peptide containing an aspartic acid to glutamic acid substitution (D→E) at position 244 (SVEEQIQWM), were synthesized at the European Veterinary Laboratory (Woerden, The Netherlands).

Generation of effector cells. The optimal conditions for the detection of SIV-specific CTL

and CTLp were established as described (17). Effector cells were expanded by two 1-week cycles of antigenic stimulation with 10^4/well autologous paraformaldheide-fixed B-LCL infected with rVV expressing SIV antigens. In bulk assays effector cells were cultured at $2.5X10^4$/well. In limiting dilution assays (LDA) the appropriate numbers and ranges of dilutions and numbers of replicate wells were pre-determined for each monkey in a pilot experiment; cultures included at least four and up to eight dilutions, each dilution in at least 24 and up to 48 replicate wells. Both bulk and LDA cultures also contained autologous irradiated (2500 rad) feeder PBMC (10^4/well), recombinant interleukin-2 (rIL-2, 10 U/ml) from day 3, and 10% supernatant from ConA-stimulated blasts from day 7. For peptide-specific stimulation, effector cells ($2X10^4$/well) were expanded for 10 to 14 days with autologous irradiated (5000 rad) B-LCL sensitized with synthetic peptides (10^4/well), autologous irradiated feeder PBMC (10^4/well), and rIL-2 (10 U/ml) from day 4. For mitogenic stimulation, effector cells (^{610} /ml) were cultured with ConA (5 μg/ml) for three days and further expanded with rIL-2 (20 U/ml) for 4 to 11 days. Gag-specific CTL clones were derived from PBMC and characterized as described (16).

Cytotoxicity assay. Cytotoxicity was measured in standard 5 hour ^{51}Cr-release assays (17). Autologous B-LCL incubated overnight with either SIV rVV, vaccinia virus control, synthetic peptides, or medium alone were used as targets. For anti-CD8 blocking, effector cells were incubated for 1 hour with ascitic fluid of the anti-CD8 mAb FK18 (37) at a 1:300 dilution, before adding the targets. Maximum ^{51}Cr release was determined by detergent (5% Triton X-100) lysis of target cells. Spontaneous release, determined by incubation of target cells in medium alone, was always <30% of maximum release. The percentage of lysis of specific and control targets was calculated for duplicate wells of bulk cultures and for each well of LDA according to the formula: % lysis = [(experimental release - spontaneous release)/(maximum release-spontaneous release)]×100. Individual LDA wells were considered positive when lysis of specific targets exceeded by 10% that of control targets if the latter was below 10%, or by 20% if the latter was above 10%. In all positive wells the experimental release exceeded the spontaneous release by at least three standard deviations. CTLp frequencies were estimated by the maximum likelihood (17) and normalized to the number of CTLp/10^6 mononuclear cells. Estimates were accepted when their χ^2 goodness-of-fit test statistics were <10. Rates of change (slopes) of CTLp frequencies and CD4$^+$ cell percentages were calculated by linear regression analysis.

RESULTS

Virological and clinical status of SIV-infected macaques. As described previously (20), eight cynomolgus macaques were infected intravenously with SIVmac32H-J5, a pathogenic molecular clone derived from SIVmac32H. In monkey K71, PBMC-associated virus load and p26 antigenemia showed a peak between week one and two after infection (>1000 infected cells/10^6 PBMC and 1.10 ng/ml respectively) followed by a rapid decline. Repeated attempts to isolate virus from PBMC were unsuccessful between week 12 and five months after infection (Table 1). However, during this time proviral DNA was detected in PBMC by nested Gag-PCR. Between 12 to 20 months after infection, virus isolation remained unsuccessful in both whole PBMC and their CD8-depleted fractions. In addition, repeated attempts failed to detect proviral DNA by PCR in PBMC, their CD4-enriched fractions, and the same cells after co-culture with C8166 cells. Similar results were obtained between 12 and 22 months after infection with monkeys K77* and K80*. In contrast, virus was detected in PBMC of other five monkeys (K66, K73, K79, K83*, and K88*) for the entire length of

TABLE 1. Virological status and $CD4^+$ cell slopes of SIV-infected macaques

Monkey	Virus detection[a]						$CD4^+$ cell slope[b]	
	Peak load	weeks		months				
		1-9	12	4-6	12	18-20		
K66	503	+ +	- +	- +	- +	- +	- 0.8 ± 0.2	(0.004)
K71	>1000	+	- +	- +	- -	- -	0	
K73	625	+ +	+	+	+	ND	- 1.1 ± 0.4	(0.04)
K79	>1000	+ +	+	+	+	ND	- 0.7 ± 0.3	(0.04)
K77*	208	+	- +	+	- -	- -	0	
K80*	208	+	+	+	- -	- -	0	
K83*	>1000	+	+	+	+	+	- 0.8 ± 0.2	(0.01)
K88*	>1000	+	+	+	+	(+) +	- 0.3 ± 0.1	(0.04)

[a] Virus isolation was performed in serial dilutions of PBMC and their CD8-depleted fractions. Virus load (number of infected cells/10^6 PBMC) peaked at week two or four (monkey K73) after infection. Nested-Gag-PCR was used to amplify provirus DNA from PBMC, their CD4-enriched fractions, and the same cells after co-culture with C8166 cells. + + represents virus isolation positive, PCR positive; (+) + virus isolation intermittently positive, PCR positive; - + virus isolation negative, PCR positive; - - virus isolation negative, PCR negative. ND, not done.

[b] Rate of change with standard error (and P values) in CD4 $^+$ cell percentages/month calculated from a minimum of six measurements for each monkey (mean 8.1, range 6-9).

observation (minimum 13 months) (Table 1). Seven of the eight monkeys remained asymptomatic during their respective observation periods. Monkey K83* developed symptomatic disease 18 months after infection. The percentages of circulating $CD4^+$ cells were stable over time in monkeys K71, K77* and K80*, but declined significantly in the other five monkeys (Table 1).

Frequencies of SIV-specific CTLp. The frequencies of Env-, Gag-, Pol-, Tat- and Nef-specific CTLp were determined in PBMC that were isolated from each monkey at three or four time points after infection and tested in parallel under identical culture and assay conditions (Table 2). Before infection, monkeys K77*, K80*, K83* and K88* had developed vaccine-induced Env-, Gag-, or Nef-specific CTLp at frequencies up to 105/10^6 PBMC (20). CTLp frequencies in naive PBMC isolated before exposure to either SIV or SIV antigens were always $<2/10^6$ PBMC (not shown). Multi specific CTL responses were detected between four and six months after infection: all animals had CTLp against Gag and Tat (7 of 7), and most had also CTLp against Env (6 of 7), Nef (5 of 7), or Pol (4 of 7). Frequencies were in most cases $\leq 15/10^6$ PBMC, but higher estimates (up to 121/10^6 PBMC) were obtained in monkeys K71, K80*, and K88*. Over time, frequencies appeared stable or increasing in monkeys K66, K79, and K88*, and stable or decreasing in monkeys K83*, K71, K77* and K80*. After 20

TABLE 2. Frequencies of circulating SIV-specific CTLp

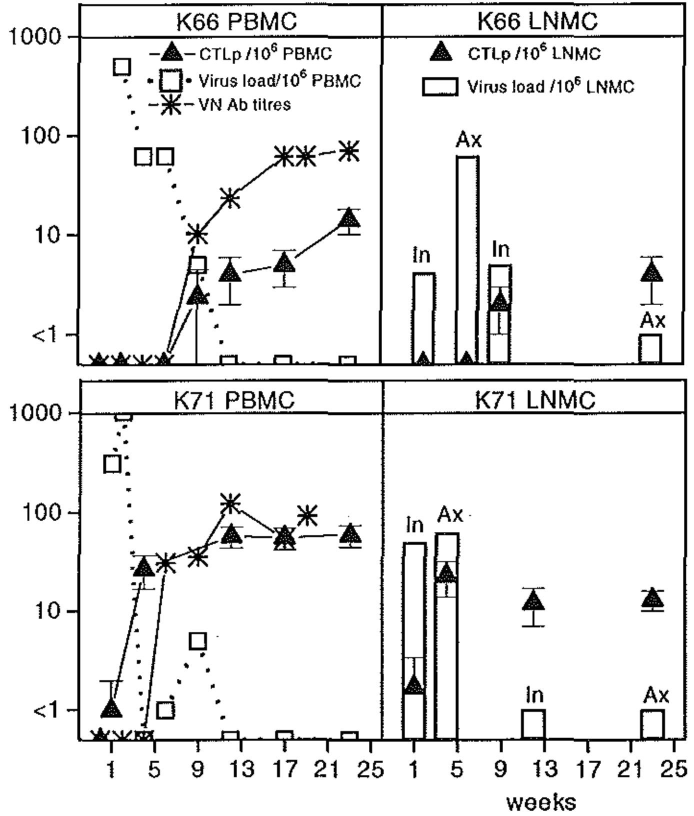
SIV protein	Month	CTLp/10^6 PBMC (with 95% confidence intervals)						
		K66	K71	K79	K77*	K80*	K83*	K88*
Env	4	0	1 (0-3)	2 (1-5)	8 (5-11)	84 (64-105)	7 (3-11)	99 (74-123)
	6	0	2 (0.3-4)	2 (0.4-4)	5 (1-9)	36 (24-48)	7 (3-10)	51 (34-68)
	12	6 (4-11)	2 (0-4)	4 (1-8)	6 (2-9)	30 (21-38)	0	46 (31-61)
	18-20	9 (4-14)	0	ND	6 (2-10)	11 (5-18)	ND	61 (46-76)
Gag	4	5 (3-7)	56 (42-70)	12 (8-15)	9 (6-12)	12 (8-16)	10 (7-14)	121 (95-174)
	6	15 (8-21)	60 (45-74)	12 (7-17)	8 (3-14)	12 (5-18)	4 (1-6)	111 (70-152)
	12	22 (13-31)	20 (13-26)	12 (6-18)	2 (0.3-3)	8 (4-12)	8 (4-13)	106 (66-146)
	18-20	26 (14-38)	3 (0.4-6)	ND	2 (0-4)	4 (1-7)	11 (6-16)	118 (85-151)
Pol	4	2 (1-4)	0	3 (1-5)	0	22 (16-29)	2 (1-4)	0
	6	7 (3-12)	1 (0-2)	4 (1-7)	2 (0-4)	17 (9-26)	2 (0.3-4)	0
	12	3 (0-6)	2 (0-4)	3 (0-6)	1 (0-3)	13 (8-19)	5 (1-10)	0
	18-20	4 (0.3-7)	0	ND	2 (0-4)	9 (4-15)	ND	1 (0-2)
Tat	4	0	23 (16-30)	9 (5-14)	5 (2-7)	6 (3-9)	10 (5-14)	111 (84-138)
	6	4 (1-8)	21 (14-29)	7 (3-11)	4 (0.5-7)	4 (1-7)	10 (5-14)	83 (50-115)
	12	11 (6-17)	10 (4-15)	11 (5-18)	1 (0-2)	0	0	94 (58-129)
	18-20	31 (20-42)	7 (4-11)	ND	3 (0-5)	0	ND	99 (72-127)
Nef	4	2 (1-3)	0	8 (4-12)	7 (4-10)	31 (22-41)	11 (6-13)	0
	6	2 (0-5)	1 (0-3)	13 (7-18)	8 (3-13)	14 (7-21)	10 (4-15)	0
	12	3 (0-7)	2 (0-4)	15 (7-22)	5 (2-8)	15 (9-20)	0	3 (0.4-5)
	18-20	2 (0-4)	0	ND	1 (0-3)	4 (1-7)	ND	3 (1-5)

months of infection, overall CTLp frequencies in the latter three monkeys waned to levels significantly ($P < 0.05$) lower than those measured at four months. Monkey K83* lost the CTL response against Env, Tat, and Nef one year after infection, but maintained the CTL response against Gag 18 months after infection, the time of onset of symptomatic disease.

Data from peripheral blood were compared with those from axillary and inguinal lymph node biopsies obtained during the first five months of infection. As shown in Fig. 1 for monkeys K66 and K71, CTLp detection in PBMC coincided with the initial decline of virus replication and the development of virus neutralizing antibodies. The kinetics of cell-associated virus load and Gag-specific CTLp in LNMC mirrored those of PBMC. However, from a quantitative perspective, CTLp frequencies in PBMC became over time higher than those measured in LNMC. The latter observation was confirmed after adjustment of CTLp frequencies to the initial input of $CD8^+$ cells in the LDA, despite the finding that $CD4^+$: $CD8^+$ cell ratios were consistently higher in LNMC (1.9 to 2.6) than in PBMC (0.5 to 0.7).

Kinetics of Gag- and p26A.5 epitope-specific CTL in monkey K71. Eleven $CD2^+$ $CD8^+$ $CD4^-$ CTL clones were derived by Gag-specific stimulation of PBMC collected from monkey K71 at three and four months after infection. The fine specificity of all clones was mapped to

FIG. 1. Kinetics of cell-associated virus load (number of infected cells/10^6 mononuclear cells), virus neutralizing antibody (VN Ab) titres (yield reduction values) and Gag-specific CTLp frequencies (number of CTLp/10^6 mononuclear cells) during the first 23 weeks of infection with SICmac32H-J5. Data from PBMC were compared to those from axillary (Ax) and inguinal (In) lymph node mononuclear cells (LNMC). Error bars indicate 95% confidence intervals.



a 9-mer peptide designated p26A.5, representing amino acids 242-250 (SVDEQIQWM) of p26 (16). Using synthetic peptides spanning the entire sequence of SIV Gag, it was shown that the same amino acid region was also the only target of Gag-specific effector cells expanded by mitogenic stimulation with ConA (Fig. 2). These findings were suggestive of a Gag-specific CTL response narrowly focused against the p26A.5 epitope. However, comparison of the frequencies of Gag- and p26A.5-specific CTLp suggested that CTL with other epitope specificities were present between 4 and 10 months after infection (Table 3). P26A.5-specific CTLp represented 77% of Gag-specific CTLp early after infection, but only 31% to 49% between four and ten months after infection. The frequencies of Gag-specific CTLp, after reaching a plateau, declined more rapidly than those of p26A.5-specific CTLp. The rates of decline from four months after infection were - 4.2 ± 0.6/month (P = 0.003) and -1.1 ± 0.3/month (P = 0.01), for Gag- and p26A.5-specific CTLp respectively. As a result, between 12 and 20 months after infection p26A.5-specific CTLp constituted 92% to 100% of Gag-specific CTLp. To ensure comparability, Gag- and p26A.5-specific CTLp frequencies were measured simultaneously in the same cultures expanded by stimulation with SIV Gag rVV.

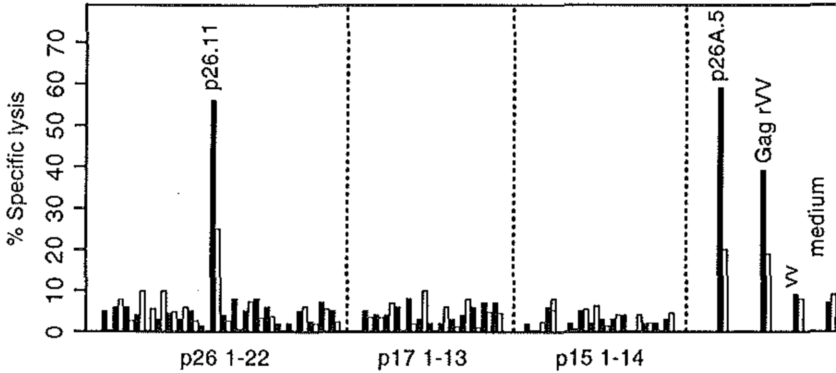


FIG. 2. Gag-specific CTL of monkey K71 recognize a nine-mer peptide in SIV p26. Effector cells derived from PBMC by stimulation with either SIV Gag rVV (solid bars) or ConA (open bars) recognized targets either pulsed with 30 μM of peptide p26A.5 (SVDEQIQWM) or with peptide p26.11 (IAGTTSSVDEQIQWMYRQQN), or expressing endogenously processed antigen after infection with SIV Gag rVV. Control targets were either infected with vaccinia virus (vv) control or incubated with medium alone. Results are expressed as mean specific lysis from duplicate well estimations at an E:T ratio of either 5:1 (solid bars) or 20:1 (open bars).

TABLE 3. Kinetics of circulating Gag- and p26A.5-specific CTLp of monkey K71[a]

Month	Gag-specific CTLp		p26A.5-specific CTLp	
	Gag-stimulation		Gag-stimulation	p26A.5-stimulation
	Expt I	Expt II		
1.4	30 (21-29)	ND	23 (16-30)	24 (16-32)
4	58 (44-73)	56 (42-70)	18 (12-25)	ND
6	62 (45-79)	60 (45-74)	21 (15-28)	ND
7	64 (48-80)	ND	25 (17-33)	23 (17-29)
10	41 (30-53)	ND	20 (13-27)	17 (11-23)
12	17 (10-23)	20 (13-26)	16 (9-22)	18 (12-24)
15	13 (9-16)	ND	12 (9-16)	ND
20	3 (1-6)	3 (0.4-6)	3 (1-6)	3 (0.4-6)

[a] After two one-week cycles of stimulation with either SIV Gag rVV or peptide p26A.5, four aliquots from each well of limiting dilution cultures were tested for cytotoxicity against autologous B-LCL either infected with SIV Gag rVV or with vaccinia virus control, sensitized with peptide p26A.5, or incubated with medium alone. Frequencies (with 95% confidence intervals) were normalized to the number of CTLp/10^6 PBMC.

CTLp frequencies were reproduced in independent replicate experiments (Table 3). In addition, the frequencies of p26A.5-specific CTLp measured after stimulation with SIV Gag rVV were reproduced in cultures expanded by stimulation with peptide p26A.5 (Table 3).Inhibition studies were conducted with anti-CD8 mAb using as effector cells i) the p26A.5-specific CTL clone designated K71E/26 (16), and ii) positively selected $CD8^+$ cells expanded by stimulation with peptide p26A.5. In bulk assays, neither type of effector cells was susceptible to anti-CD8 blockade (inhibition <10%, not shown), suggesting that the immunodominance of p26A.5-specific CTL may be related to high affinity effector cell-target cell interactions (1). Consistent with these data, anti-CD8 blocking in LDA reduced the frequency of Gag-specific CTLp but not that of p26A.5-specific CTLp (Fig. 3).

Homologous re-challenge. Twenty months after primary infection, monkeys K66 and K71 were re-challenged intravenously with SIVmac32H-J5. Two naive monkeys were challenged at the same time to serve as controls. Consistent with previous findings (Table 1), a burst of virus replication (up to 313 infected cells/10^6 PBMC) was observed in the two naive monkeys between two and five weeks after infection (not shown). In monkey K71, a transient increase in virus burden was observed at week six after re-challenge, in parallel with a transient decrease in circulating $CD4^+$ cells (Fig. 4). An increase was detected in both the numbers and percentages of circulating $CD8^+$ cells.

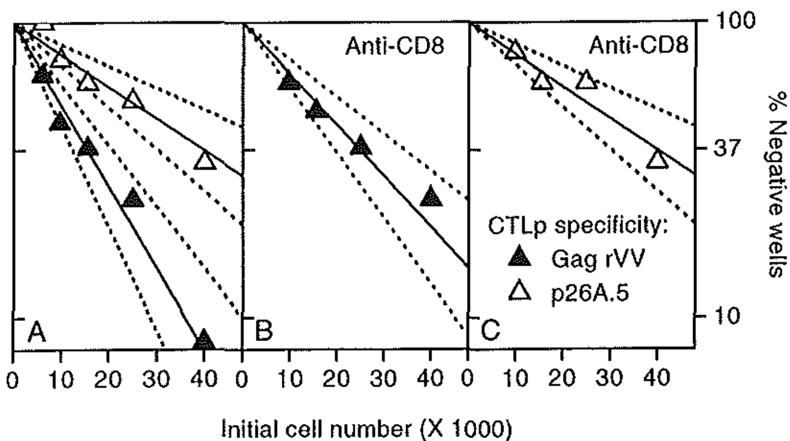
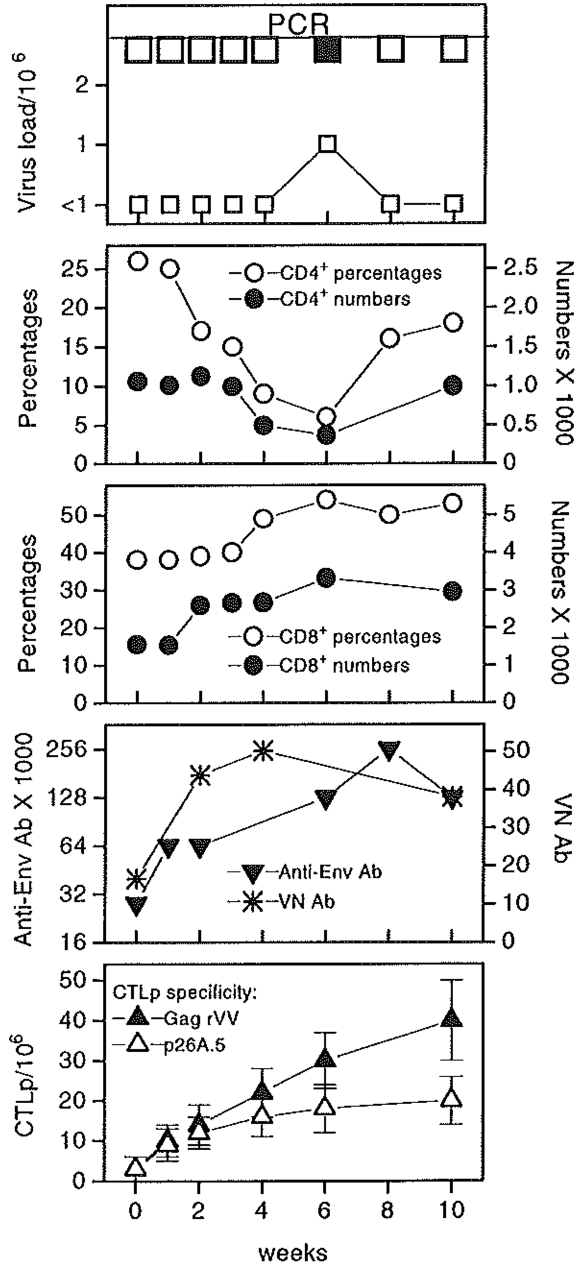


FIG. 3. P26A.5-specific CTL responses of monkey K71 are not inhibited by anti-CD8 mAb. Effector cells were generated from PBMC by Gag-specific stimulation. Autologous B-LCL either infected with SIV Gag rVV or with vaccinia virus control, sensitized with 30 µM of peptide p26A.5, or incubated with medium alone were used as targets. In the absence of anti-CD8 mAb, CTLp frequencies were 64 (range: 48-80; $\chi^2 = 1$) and 25 (17-33; $\chi^2 = 5$) for Gag- and p26A.5-specific CTLp respectively. In the presence of anti-CD8 mAb CTLp frequencies were 40 (29-51; $\chi^2 = 1$) and 25 (17-33; $\chi^2 = 1$) for Gag- and p26A.5-specific CTLp respectively. Dotted lines indicate 95% confidence intervals.

FIG. 4. Re-challenge of monkey K71. Cell-associated virus load (number of infected cells/10^6 PBMC) was measured in whole PBMC and their CD8-depleted fractions. Nested-Gag PCR was used to amplify provirus DNA from PBMC, their CD4-enriched fractions, and the same cells after co-culture with C8166 cells. Open boxes: PCR negative; solid box: PCR positive. Gag- and p26A.5-specific CTLp frequencies (number of CTLp/10^6 PBMC) were determined in cultures expanded by Gag-specific stimulation and tested against autologous B-LCL either infected with SIV Gag rVV or with vaccinia virus control, sensitized with peptide p26A.5, or incubated with medium alone. Error bars indicate 95% confidence intervals.



Parallel LDA of PBMC samples isolated during the ten weeks of observation showed a progressive increase in Gag-specific CTLp: by week four after re-challenge their frequency ($22/10^6$ PBMC; range: 15-28; $\chi^2 = 1$) was similar to that measured at week four after primary

infection (27/10^6 PBMC; range: 17-37; χ^2 = 4). In the same LDA, p26A.5-specific CTLp showed also a rapid but less pronounced increase: by week ten they represented 50% of circulating Gag-specific CTLp. These kinetics were confirmed in cultures expanded by stimulation with peptide p26A.5 (not shown). The frequencies of Tat- and Pol-specific CTLp also increased after re-challenge, whereas, as seen before re-challenge, no CTLp were detected against Env or Nef (Table 4). In monkey K66, provirus DNA was repeatedly detected by PCR in PBMC, but virus isolation was successful only at week four after re-challenge, and only from CD8-depleted cells (virus load ≤ 0.4 infected cells/10^6 PBMC). The numbers and percentages of circulating $CD4^+$ and $CD8^+$ cells remained stable, whereas virus neutralizing and anti-Env antibody titres showed a moderate increase (from 19 to 58 and from 128 to 267 respectively, not shown). CTLp frequencies in PBMC showed also a moderate increase (Table 4).

Comparison of peripheral blood and lymphoid tissues 22 months after primary infection. After 22 months of infection, virus isolation and PCR data from peripheral blood were compared to those from lymph nodes and spleen (Table 4). Overall virus burden was higher in LNMC and SPMC than in PBMC. Virus was recovered from both LNMC and SPMC of monkeys K88* and K66; the highest virus load was measured in SPMC of the latter monkey. Provirus DNA was detected by PCR in LNMC and SPMC from all five monkeys, including those lacking detectable virus in PBMC (K71, K77* and K80*). However, virus load in LNMC and SPMC of the latter three monkeys was below or barely above detection levels. CTLp frequencies were determined in parallel LDA of mononuclear cells from all three compartments, and compared after adjustment to the initial input of $CD8^+$ cells (Table 4). In the re-challenged monkey K66, and in monkeys K77* and K80*, CTLp frequencies were higher in SPMC and LNMC than in PBMC. In the re-challenged monkey K71, CTLp frequencies in LNMC were lower than those in PBMC and SPMC, whereas those in SPMC were similar to (Gag- and Tat-specific CTLp) or higher than (Pol-specific CTLp) those in PBMC. In monkey K88* CTLp frequencies in PBMC were lower than those in LNMC but similar to those in SPMC. The three compartments contained CTLp of similar protein-specificities, with the exception of Pol-specific CTLp, found in both PBMC and SPMC but not in LNMC of monkey K71, and Tat-specific CTLp, found in SPMC and LNMC, but not in PBMC of monkey K80*.

Prospective analysis of the p26.A5 epitope sequence in monkey K71. The p26A.5 epitope region of SIV Gag is conserved among several strains of the HIV-2/SIV D clade, including SIVmac251, SIVmac32H, SIVmac1A11 and SIVmac239. The homologous sequence is also highly conserved among most HIV-1 clades (A to H) (26). As amino acid substitutions in the homologous HIV-1_{SF2} and HIV-2_{ROD} epitope sequences were shown to abolish recognition by CTL clone K71/E26 (16), we tested the hypothesis that variation in the

TABLE 4. Comparison of peripheral, lymph node and splenic mononuclear cells 22 months after primary infection

Monkey	Cell type	Virus detection (with load)[a]	CTLp/10^6 mononuclear cells (with 95% confidence interval)					CD8⁺ %
			Env	Gag	Pol	Tat	Nef	
K66								
	PBMC	(+) + (≤ 0.4)	9 (6-13)	27 (19-35)	14 (10-19)	30 (21-39)	3 (1-5)	38
	LNMC	+ + (25)	17 (14-20)	35 (30-40)	16 (10-22)	35 (23-47)	3 (1-5)	36
	SPMC	+ + (>3125)	31 (20-42)	47 (34-60)	18 (13-23)	47 (31-62)	3 (1-4)	40
K71								
	PBMC	- -	0	34 (21-47)	12 (7-16)	16 (11-21)	0	50
	LNMC	- +	0	7 (3-11)	0	5 (3-7)	0	36
	SPMC	+ + (1)	2 (0.3-4)	30 (23-37)	27 (20-34)	18 (12-25)	2 (0.3-4)	47
K77*								
	PBMC	- -	0	2 (1-4)	1 (0-3)	4 (2-6)	2 (0-4)	52
	LNMC	- +	1 (0-2)	0	0	13 (6-20)	11 (7-16)	40
	SPMC	- +	0	0	2 (0.4-4)	36 (23-47)	23 (16-30)	58
K80*								
	PBMC	- -	6 (2-10)	5 (2-8)	4 (2-7)	0	4 (1-7)	48
	LNMC	+ + (1)	11 (5-18)	13 (8-18)	9 (4-15)	7 (3-10)	14 (12-17)	48
	SPMC	+ + (1)	15 (9-21)	22 (15-30)	8 (3-13)	6 (2-9)	18 (14-22)	44
K88*								
	PBMC	+ + (5)	61 (46-76)	118 (85-151)	1 (0-2)	99 (72-127)	3 (1-5)	54
	LNMC	+ + (63)	69 (51-87)	89 (73-105)	ND	75 (60-91)	3 (0.4-5)	36
	SPMC	+ + (25)	51 (40-62)	111 (99-123)	ND	89 (71-107)	4 (2-6)	49

[a]Virus isolation was performed in serial dilutions of PBMC, LNMC, SPMC and their CD8-depleted fractions. Nested-Gag-PCR was used to amplify provirus DNA from PBMC, LNMC, SPMC, their CD4-enriched fractions, and the same cells after co-culture with C8166 cells. + + represents virus isolation positive, PCR positive; (+) + virus isolation intermittently positive, PCR positive; - + virus isolation negative, PCR positive; - - virus isolation negative, PCR negative. Virus load was normalized to the number of infected cells/10^6 mononuclear cells.

HIV-2/SIV A, B, C	Consensus	TVEEQIQWM	
HIV-2/SIV D	Consensus	TVDEQIQWM	
SIVmac32H-J5	Inoculum	SVDEQIQWM	
Monkey K71			
4 months	PBMC	---------	
21 months	PBMC	---------	
	PBMC	--------I	1/10 clones
	PBMC	---------	Cultured
22 months	LNMC	---------	Superficial
	LNMC	---------	Mesenteric
	LNMC	---------	Cultured
	SPMC	--E------	
	SPMC	--E------	Cultured
Monkey K66 22 months	All sequences	---------	

FIG. 5. Genetic variation within the p26A.5 epitope. DNA samples were analysed by nested-PCR amplification of a 500 bp fragment of SIV Gag. Results of direct sequencing were confirmed with 4 to 10 independent clones from each sample. Sequencing was confirmed for both sense and antisense strands. Deduced amino acid sequences between positions 242 and 250 are shown in the single letter code. Dashes indicate amino acid identity with the SIVmac32H-J5 sequence.

p26A.5 epitope may generate CTL escape mutants. Sequence analyses of the p26A.5 epitope and its flanking regions were conducted by PCR amplification of a 500-bp fragment of SIV Gag derived from PBMC, LNMC, SPMC, their CD4-enriched fractions, and the same cells after co-culture with C8166 cells (Fig. 5). Four months after primary infection, the last time point at which provirus DNA was detected by PCR, PBMC were still harbouring the sequence of the J5 molecular clone used for challenge. Ten weeks after re-challenge, a homogenous prototype virus population was detected in both PBMC and LNMC. In contrast, a single point aspartic acid to glutamic acid (D→E) substitution at amino acid position 244 was detected in all spleen-derived sequences, including those derived from cultured samples. Outside the epitope, a second mutated region (N→S) was identified in all spleen-derived sequences at amino acid position 143 (not shown).

To study the effect of sequence variation on CTL reactivity, seven p26A.5-specific CTL clones were tested for recognition of a 9-mer variant peptide carrying the amino acid substitution D→E (SVEEQIQWM). Individual clones showed different patterns of reactivity (Fig. 6): three (e.g. K71E/26) did not recognize the variant peptide even at the highest peptide concentration tested (100 μM), two (e.g. K71E/27) recognized the variant peptide with a lower

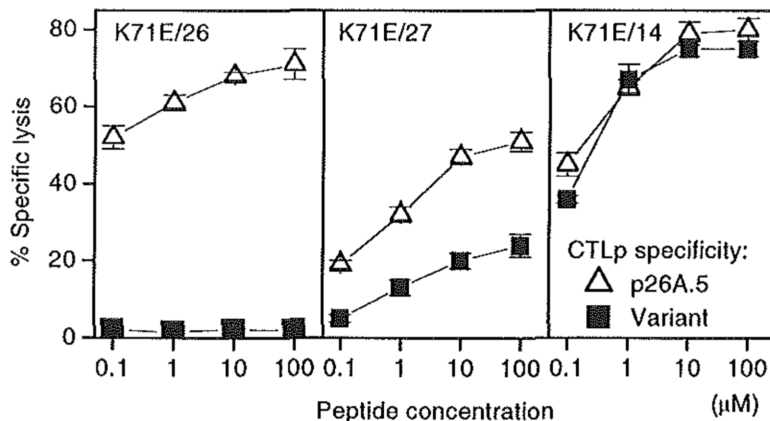


FIG. 6. Recognition of the prototype (SVDEQIQWM) and variant (SVEEQIQWM) p26A.5 epitope by CTL clones generated from PBMC of monkey K71 at three and four months after primary infection. Results are expressed as mean specific lysis from duplicate well estimations at an E:T ratio of 5:1.

TABLE 5. Recognition of prototype and variant p26A.5 epitope by CTL lines of monkey K71

Months	Peptide recognition[a]			
	Prototype only	Prototype and Variant	Prototype > Variant	Variant > Prototype
1.4	17 (65)	3 (12)	5 (19)	1 (4)
10	10 (71)	1 (7)	2 (14)	1 (7)
22	11 (69)	3 (19)	1 (6)	1 (6)

[†]Short-term CTL lines were generated by p26A.5-specific stimulation of PBMC under limiting dilution conditions and tested in split-well assays against autologous target B-LCL sensitized with 30 μM of either the prototype p25A.5 peptide (SVDEQIQWM) or a variant peptide carrying a single point mutation (SVEEQIQWM). Results are presented as numbers (and percentages) of CTL lines recognizing either the prototype peptide alone or both the prototype and the variant peptides with either similar (<10% difference in specific lysis), higher or lower (14% to 55% difference in specific lysis) efficiency.

efficiency than the prototype peptide, whereas two others (e.g. K71E/14) recognized both the variant and the prototype peptides with a similar efficiency. These observations were extended using a large panel of short-term CTL lines (Table 5). These were derived by p26A.5-specific stimulation of PBMC isolated both before and after re-challenge. The majority (65% to 71%) of CTL lines did not recognize the variant peptide, whereas the remaining recognized the

variant peptide with various degrees of efficiency.

The effect of sequence variation on CTL induction was studied by investigating the ability of the variant peptide to stimulate CTL expansion in PBMC and LNMC isolated before and after re-challenge, and in SPMC isolated at the end of observation. In bulk assays, the variant peptide was effective in inducing CTL expansion from SPMC, but was poorly immunogenic for PBMC and LNMC (Fig. 7a). Consistently, the frequencies of CTLp able to recognize the variant peptide were higher in SPMC than in PBMC or LNMC (Fig. 7b). These observations suggested the existence of three different CTL populations: one recognizing only the prototype peptide found predominantly in PBMC and LNMC; a second recognizing only the variant peptide found predominantly in SPMC; and a third smaller population recognizing both the prototype and the variant peptide found in all compartments studied.

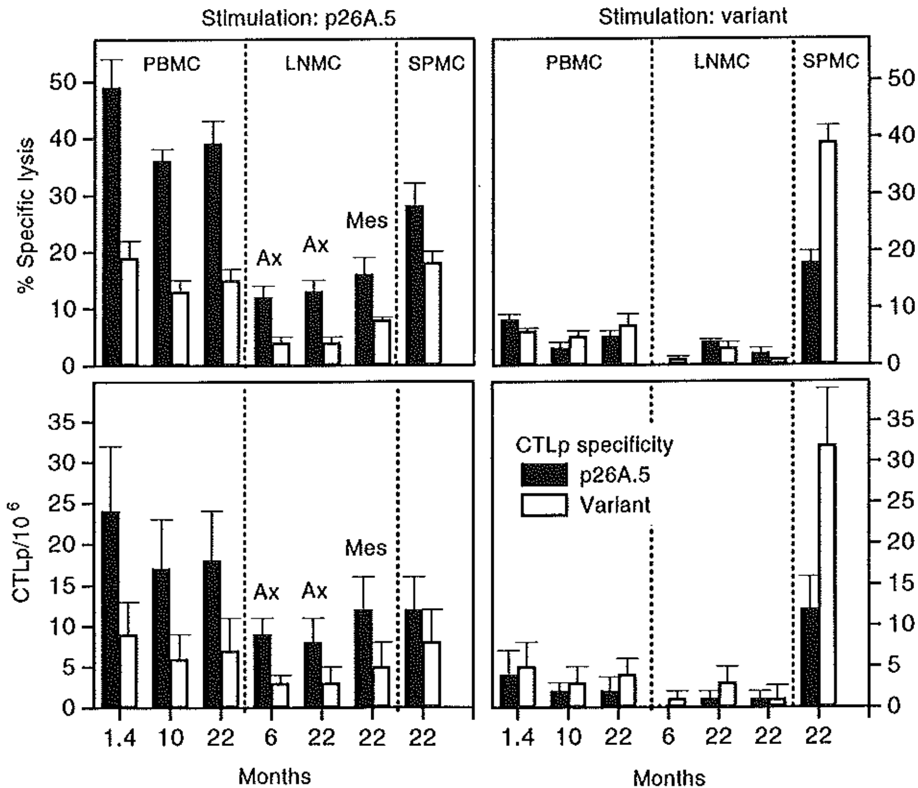


FIG. 7. Bulk (top) and limiting dilution (bottom) assays of CTL responses against prototype (SVDEQIQWM) and variant (SVEEQIQWM) p26A.5 epitope. PBMC, axillary (Ax) and mesenteric (Mes) LNMC, and SPMC were isolated from monkey K71 both before and after re-challenge. Effector cells were generated by stimulation with either the prototype (left) or the variant (right) p26A.5 peptide. Results of bulk assays are expressed as mean specific lysis from duplicate well estimations at an E:T ratio of 10:1. CTLp frequencies were normalized to the number of CTLp/10^6 mononuclear cells; error bars indicate 95% confidence intervals.

DISCUSSION

In this study we followed the evolution of SIV-specific CTL responses of macaques infected intravenously with the molecular clone SIVmac32H-J5. We showed that effective virus containment, as indicated by the absence of detectable virus in PBMC, was associated with declining CTLp frequencies in circulation and with at least partial susceptibility to re-infection. We showed also that the presence of functionally immunodominant Gag-specific CTL coincided with the emergence of a virus variant carrying a mutated epitope sequence. This was recognized poorly by CTL primed by the prototype sequence, but induced new CTL specificities. Finally, we showed that both SIV protein- and epitope-specific CTL of chronically infected animals localized preferentially in the sites of virus distribution.

We have previously reported that antigenic stimulation of effector cells under LDA conditions allows a sensitive and reproducible assessment of SIV-specific CTL immunity over time (17). With a similar approach, we have demonstrated $CD8^+$ MHC class I-restricted CTL in HIV-1 infected humans even after the onset of overt disease (15). CTLp frequencies of SIV-infected monkeys were lower than those often found in HIV-1 infected humans (Table 2), as also shown in previous studies of SIV-infected rhesus and cynomolgus macaques (12, 21). Besides the influence of host genetic factors, relatively low CTLp frequencies may reflect a low degree of antigenic stimulation in vivo, due to rapid down-regulation of SIV replication after infection. In support of this view, high frequencies of circulating CTLp were found in the monkey with the highest PBMC-associated virus load (i.e., K88*, Table 2 and reference 17). No evidence was found in the other monkeys of an inverse relationship between virus load and frequencies of circulating CTLp (17, 20).

As a consequence of rapid virus containment, CTL may eventually disappear or home to potential sites of active virus replication, such as lymph nodes (19) and spleen (9). To address this hypothesis, CTL responses and virus load were analyzed prospectively in peripheral blood and secondary lymphoid organs. Immediately after infection, CTL detection in circulation coincided with the initial down-regulation of virus load and SIV p26 antigenemia (Fig. 1). Similar observations have been made with both HIV-1-infected humans (4, 24) and SIV-infected macaques (12, 29). In contrast with previous findings (12), but in agreement with others (2), the initial virus containment also coincided with the detection of virus neutralizing antibodies (Fig. 1). During the early phase of infection, cell-associated virus load and SIV-specific CTLp frequencies showed similar kinetics in PBMC and LNMC, but, from a quantitative perspective, CTLp frequencies became rapidly higher in PBMC than in LNMC (Fig. 1). Although kinetic data obtained from different lymph nodes should be interpreted with caution, our conclusions are supported by the finding that in the early phase after intravenous SIV infection virus load and histopathological changes are similar in different (axillary and

inguinal) lymph nodes of the same animal (7). Parallel down-regulation of virus replication in both peripheral blood and lymph nodes following the initial burst of SIV replication (Fig.1) has also been reported (7, 29).

In a later stage of infection a dissociation emerged between peripheral blood and secondary lymphoid organs (Table 4). Overall virus burden was higher in both LNMC and SPMC than in PBMC, which is consistent with observations made during chronic infection with HIV-1 (9) or SIV (7). Our data extend these previous studies by demonstrating that during the chronic phase of infection SIV-specific CTLp frequencies mirrored virus distribution. These observations are consistent with CTL being the effector cells of the in vivo immune response against SIV.

Additional support for a role of CTL immunity in virus containment comes from the observation that CTLp frequencies declined before the onset of symptoms in the monkey which developed overt disease (i.e., K83*, Table 2). Longitudinal evaluation also led to the identification of three monkeys (e.i., K71, K77* and K80*) showing remarkable control of virus replication in peripheral blood (Table 1). It should be noted however, that low-level virus reservoirs were detected in their lymphoid organs (Table 4). This is an important finding, as lack of virus detection in PBMC has been regarded as an indicator of viral clearance (30).

A remarkable observation was that the lack of detectable virus in PBMC coincided with a progressive decline in the frequencies of circulating CTLp (Tables 2 and 3). As antiviral T cell memory in vivo is believed to be correlated with CTLp detected by LDA in vitro (28), our data suggest that SIV-specific memory CTL decline in the periphery under conditions of limited antigenic stimulation. This may have direct implications for the evaluation of vaccines aimed at inducing long-lasting protective CTL responses. A decline of SIV-specific CTLp over time may also be explained by a progressive state of SIV-induced immune dysfunction. However, the three animals showed no signs of disease progression during 22 months of observation (Table 1). Their T cell function, as defined by the ability to respond to allogenic cells in mixed-lymphocyte reactions, was also preserved over time (not shown). Finally, the rapid increase in CTLp frequencies observed after re-challenge in one of the animals (Fig. 4) argues against a compromized CTL function. Transient cell-associated viraemia and rising levels of SIV-specific antibodies and CTLp frequencies suggested that chronic infected animals were at least partially susceptible to re-infection (Fig. 4). Virus replication was limited in comparison with the virus burst observed upon infection of naive monkeys. This seems in line with the observation that infection with non-pathogenic HIV-2 or Nef-deleted SIVmac confers resistance to subsequent challenge with pathogenic SIV (34). Although the mechanisms underlying resistance to superinfection are still largely unclear, they are likely to include host antiviral immune responses.

In previous studies, CTL responses against a dominant T cell epitope were induced

by lower antigenic doses and lasted for a considerably longer time than CTL responses against minor epitopes (28). It was suggested that the affinity of effector cell-target cell interactions may be a determinant of T cell memory. We found that CTL responses against an immunodominant epitope in p26 designated p26A.5 (Fig. 2) developed more rapidly and lasted longer in circulation than those directed against putative minor Gag epitopes (Table 3). Resistance to anti-CD8 blocking (Fig. 3) suggested that p26A.5-specific CTLp had indeed high affinity for their targets. These CTL may require lower levels of antigenic stimulation (28), be triggered by infected cells displaying very low epitope density (35), be relatively independent of T cell help (13), and ultimately be more effective in virus containment (36).

It is conceivable that, under certain stringent circumstances, functionally immunodominant CTL may favour the negative selection of CTL escape mutants which may then replace the prototype virus, as observed in the spleen of monkey K71 (Fig. 5). It is not surprising that the mutation in the p26A.5 epitope was detected only in spleen-derived sequences, as co-existence of viral expression and CTL expansion in splenic white pulp may create a highly favourable microenvironment for immunological pressure (9). As a molecular clone was used for infection, the epitope mutation must have originated during the course of either primary or secondary infection. The absence of epitope mutations in monkey K66, which lacked p26A.5-specific CTL (not shown) and showed high virus burden in the spleen (Table 4), favours CTL pressure over sole growth advantage as the cause of virus mutation.

Overall, the variant virus was recognized poorly by CTL that had been primed by the prototype sequence (Table 5 and Figs. 5 and 6). However, the existence of multiple p26A.5-specific CTL populations, probably using different TCR gene rearrangements (39), was indicated by the recognition of the variant peptide by at least some of the CTL clones and short-term CTL lines tested. The redundancy of CTL responses targeting the same viral epitope may result in a more effective control of virus replication and represent a first-line safeguard mechanism against emerging virus variants. Lack of complete escape from CTL was also indicated by the finding that the variant virus was able to induce CTL of new specificities. These also appeared to localize preferentially in the spleen (Fig. 7). The low virus burden measured in this compartment (Table 4) suggests that the new CTL were effective in containing virus replication. However, the possibility exists that a virus variant with even a slight advantage would have eventually replaced the prototype virus in other compartments.

In conclusion, our findings add to understanding of the host-virus interactions taking place during the course of lentiviral infections, indicate a role for SIV-specific CTL in the containment of virus replication, and support the concept that the quality of antiviral CTL is as important as their quantity in determining the course of virus infections. However, the factors that ultimately lead to virus persistence and disease progression require further investigation.

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Chapter 5

Summary and discussion

Includes:

Cytotoxic T lymphocytes in AIDS pathogenesis: - lessons to be learnt from the macaque model of SIV infection. *Journal of General Virology, invited review. (Submitted).*

Increasing evidence indicates a protective role of antiviral cytotoxic T lymphocytes (CTL) in the host defence against human immunodeficiency virus (HIV). CTL reactive against HIV antigens have been detected in persons exposed to the virus but lacking evidence of infection. These include a small number of female prostitutes in Africa, sexual partners of infected persons, children born to infected mothers, and health care workers exposed to infectious body fluids (reviewed by Rowland-Jones *et al.*, 1995). Based upon the assumption that CTL induction requires endogenous synthesis of viral proteins, these findings suggest that transient HIV infection and virus clearance by CTL are indeed possible. During acute HIV infection, the detection in circulation of specific CTL coincides with the fall in viraemia that follows the initial virus burst (Borrow *et al.*, 1994; Koup *et al.*, 1994). In the subsequent stages, persistence of antiviral CTL may be associated with a prolonged asymptomatic period, whereas their decline is usually coincident with disease progression (Klein *et al.*, 1995). The relatively strong CTL response often detected during the asymptomatic stage of HIV infection is associated with increased numbers of circulating $CD8^+$ T-cells, whereas CTL decline is usually associated with marked $CD4^+$ T-cell depletion, with values well below 200/ µL (Geretti *et al.*, 1996). To explain the progressive decline of HIV-specific CTL, either exhaustion due to prolonged high-level antigenic exposure, progressive loss of helper function (Geretti *et al.* 1996), or both may be advocated. Nevertheless, it is of note that a few patients may maintain relatively strong CTL responses against HIV Gag during the late stages of the disease (Geretti *et al.*, 1996). This seems consistent with data showing that persons with the most benign disease course not necessarily have the highest levels of CTL activity.

Studies on the relationship between virus load and CTL responses in chronically infected patients have also yielded conflicting results, as both direct (Ferbas et al., 1995) and inverse (Klein et al., 1995) correlations have been reported. While further work is needed to clarify such discrepancies, it is clear that antiviral CTL responses can only be interpreted in the framework of the dynamics of virus replication, recognition and clearance of infected cells, and virus attempts to evade immune surveillance. It is also likely that qualitative factors, such as affinity of target cell-effector cells interaction, play a crucial role in determining the effectiveness of CTL response, thus explaining, at least in part, why the presence of high levels of CTL does not necessarily prevent development of disease. In fact, at least in some cases, high CTL frequencies may simply reflect either persistently high level of virus replication or a continuously diversifying genome.

Infection of macaques with simian immunodeficiency virus (SIV) offers a valuable model for studying the complex interaction between lentiviruses and host immune system. One aim of this discussion is to outline how this model has contributed to our understanding of the role of CTL in the control of lentiviral infections, and how its full potential may be exploited in the future.

General aspects of SIV infection of macaques. SIV of macaques (SIVmac) was first isolated in 1985 from animals held in captivity in the United States. Macaques, however, are not natural hosts of the virus. It is believed that cross-species transmission from sooty mangabeys, which are healthy natural carriers of SIV (SIVsm), was the source of infection. Fighting and biting among animals co-housed in outdoor corrals was the most likely route of horizontal transmission (Gardner, 1996). SIVmac shares with HIV many biological and structural features, including its tropism for the CD4 receptor and CCR5 co-receptor, a similar genomic organization and extensive genetic homology. The virus establishes persistent infection in macaques and causes an immunodeficiency syndrome closely resembling human AIDS. As in humans, prominent features of the disease include $CD4^+$ cell loss, constitutional symptoms, lymphadenopathy, skin and neurological disorders, opportunistic infections and neoplasias. Although the average time to disease is 1-2 years, the course of infection varies considerably among macaques. Even after infection with the same viral molecular clone, some animals rapidly develop disease and die within months, whereas others may remain asymptomatic for a few years, thus resembling long-term non-progressors with HIV infection.This variability, combined with the opportunity to define parameters of infection such as strain, dose, and route of virus inoculation, may prove valuable for clarifying the role of CTL immunity in lentivirus containment.

Early vaccination studies in macaques aimed primarily at preventing infection through the induction of virus neutralizing antibodies. As a result, a protective role of CTL in some of the successful vaccination strategies reported, such as those based upon live attenuated SIV vaccines (reviewed by Ruprecht *et al.*, 1996), can only be hypothesised. More recently, the prevailing view that it may be desirable for a vaccine to prevent disease if not infection, and the appreciation that SIV infection of macaques provides a valuable model for studying HIV pathogenesis, coupled with the development of reliable means of detecting SIV-specific CTL, have drawn considerable attention to antiviral cell-mediated immunity.

Detection of virus-specific CTL (i) Target cells. HIV- or SIV-specific CTL responses are currently measured against autologous B lymphoblastoid cell lines (B-LCL) immortalized either Epstein Barr virus or by Herpes virus papio respectively. After infection with recombinant vaccinia vectors (rVV) encoding HIV or SIV proteins, these cells express endogenously processed antigen in the context of MHC class I molecules. The use of rVV expressing different regions of the HIV or SIV genome has shown that antiviral CTL target both structural and regulatory viral proteins. In macaques, as also in humans, the strength and magnitude of the response vary among animals (Venet *et al.* 1992; Geretti *et al.*, 1997*a*). Alternatively, B-LCL can be sensitized for lysis by incubation with short synthetic viral peptides that bind directly to MHC class I molecules on the cell surface, thus allowing the definition of epitope specificities (Geretti *et al.*, 1997*b*).

(ii) Effector cells. In contrast with data from HIV-1-infected asymptomatic adults, in vitro restimulation has almost universally been required to amplify SIV-specific CTL responses to detectable levels. As an interesting exception, direct cytotoxicity against the envelope protein (Env), Gag, and the regulatory protein Nef has been recently observed with intestinal intraepithelial lymphocytes of two macaques infected with SIVmac$_{251}$ (Coudel *et al.*, 1997). One of the animals showed signs of advanced disease, including a marked colitis. Although a high proportion of intestinal intraepithelial lymphocytes express the CD8 marker, the exact nature and function of the activated intestinal effector cells, and their contribution to host defence or immunopathogenesis, remain to be determined. The same authors found little or no evidence of direct cytotoxicity in peripheral blood, spleen and lymph nodes. This is in line with the observation that the frequencies of circulating CTL precursors (CTLp) detected in SIV-infected or vaccinated macaques are in general lower than those measured in asymptomatic HIV-infected adults (Geretti *et al.*, 1997*a*). While the requirement for appropriate methods of CTLp restimulation may explain the apparent lack of CTL induction in some vaccine studies, it also highlights the need for standardised assays which would facilitate comparison of different experiments, often including only small numbers of animals.

One successful method for the expansion in vitro of HIV- or SIV-specific CTLp is based on stimulation with paraformaldehyde-fixed autologous B-LCL infected with rVV expressing HIV or SIV antigens (Van Baalen *et al.*, 1993; Geretti *et al.*, 1996). Compared with non-specific methods of stimulation, this approach enhances CTL detection by inducing selective CTL expansion, with reduced interference from background lysis. Cell culture under limiting dilution conditions appears to increase further the sensitivity of CTL measurement, as it allows CTLp detection, albeit at low frequencies, in macaques lacking significant responses in standard bulk CTL assays (Geretti *et al.*, 1997*a*). Autologous blasts infected with SIV have also been used successfully as stimulator cells (Gallimore *et al.*, 1996). Alternatively, synthetic peptides spanning defined regions of the HIV or SIV genome can be used to stimulate the growth of epitope-specific CTL.

Should vaccine-induced CTL prevent or limit virus replication? SIV Env-, Gag- or Nef-specific CTL have been induced in macaques through several immunization strategies, including live attenuated viruses (Cranage *et al.*, 1997), proteins either expressed by live vectors such as recombinant vaccinia virus (Gallimore *et al.*, 1995; Kent *et al.*, 1996), incorporated into iscoms (Hulskotte *et al.*, 1995) or adjuvanted by QS-21 saponin (Newman *et al.*, 1994), non-infectious virus-like particles (Klavinskis *et al.*, 1997), peptides in various formulations (Bourgault *et al.*, 1994, Huslkotte *et al.*, 1995,Yasutomi *et al.*, 1995), and DNA plasmids (Lu *et al.*, 1996). In those studies that have tested the outcome of subsequent challenge, the detection of vaccine-induced CTL has usually failed to predict complete resistance to infection. In one early report, vaccine-induced CTL against pC11, a well-defined

epitope in Gag, failed to protect macaques from intravenous challenge with SIV macaca nemestrina (SIVmne) (Yasutomi *et al.,* 1995). The induction of relatively low frequencies of CTLp of limited specificity, and the absence of antibodies, were believed to have contributed to the lack of protection. However, in a subsequent report, weshowed that immunisation with Env- and Gag-iscoms and three Nef lipopeptides induced both virus neutralizing antibodies and relatively high frequencies of Env-, Gag, or Nef-specific CTLp, but similarly failed to protect macaques from intravenous challenge with $SIVmac_{32H-J5}$ (Hulskotte *et al.,* 1995). Recently, intravenous, intramuscular and gene gun inoculations of SIV DNA plasmids have produced similar results: despite the induction of virus neutralizing antibodies and Env-specific CTL, no protection was induced against intravenous challenge with $SIVmac_{251}$ (Lu *et al.,* 1996).

Although disappointing, these findings are consistent with the view that CTL may not be able to prevent or control HIV or SIV infection, unless stringent qualitative and quantitative requirements are met. This finds indirect support in the observation that macaques immunised with whole inactivated SIVmac and protected from intravenous challenge with cell-associated $SIVmac_{32H}$, share the Mamu-A26 MHC class I allele with the donor of the infected cells (Osterhaus *et al.,* 1992; Heeney *et al.,* 1994). Mamu-A26 positive animals also behave as long time survivors (Bontrop *et al.,* 1996), suggesting that gene products of this allele may be correlated with the ability to mount protective CTL responses against SIV. In addition, certain human MHC genotypes have been found to influence the degree of susceptibility to HIV-infection. In particular, the B35 or the A1-B8-DR3 haplotypes have been associated with rapid disease progression; whereas other haplotypes such as B27-A24-DR1 have been found in long-term nonprogressors (Kaslow *et al.,* 1996). These data strengthen the case for a geneti cally determined ability of the immune system in controlling lentiviral infections.

More direct evidence of a protective role of defined CTL subpopulations is provided by a study of macaques immunised with SIV Nef rVV (Gallimore *et al.,* 1995). Although six of seven animals became infected upon intravenous challenge with $SIVmac_{32H-J5}$, the frequencies of vaccine-induced Nef-specific CTLp measured before challenge were inversely correlated with the levels of viral load measured after infection. In addition, the animal with the highest CTLp frequency was protected from infection. Recent findings also support the concept that protective CTL may limit rather than completely prevent virus replication. Vaccinated macaques lacking both detectable virus and antibody responses after either intravenous or intrarectal challenge with SIVmne showed CTL responses against SIV proteins present in the challenge virus but not in the vaccine (Kent *et al.,* 1996). Similarly, macaques immunised with Env-iscoms lacked both detectable virus and anamnestic antibody responses after intravenous challenge with a chimeric simian-human immunodeficiency virus (SHIV), but had CTLp against antigens other than Env, including the non-virionic regulatory proteins

Rev and Tat (Hulskotte *et al.*, 1997). These data indicate that CTL responses provide a highly sensitive marker of transient or low-level virus replication. It is tempting to speculate that similar silent or abortive infections may have remained undetected in some of the early vaccine studies.

What is the role of antiviral CTL in mucosal immunity? As most HIV infections are acquired by mucosal routes, current efforts are directed at inducing immune responses that may prevent or limit virus spread after mucosal exposure. The SIV model offers the opportunity to explore issues related to mucosal immunity and infection that are difficult to address in humans. Indeed, evidence that protection from sexually transmitted SIV and, by inference, HIV may be induced by effectively stimulating genital and systemic antiviral CTL is increasing. In female macaques inoculated intravaginally with $SIVmac_{251}$, at least some (1 in 2425 to 1 in 26686) of the $CD8^+$ cells recovered from the vaginal epithelium are SIV-specific CTLp directed against Env or Gag. In line with the view that progressive CTL compartmentalization occurs during persistent infection, the frequencies of genital CTLp are higher in chronically infected monkeys than in animals with recent infection (Lohman *et al.*, 1995). SIV-specific CTL have also been detected in both peripheral blood and gut-associated lymph nodes of macaques infected intravenously with live attenuated $SIVmac_{C8}$, and resistant to intrarectal challenge with either $SIVmac_{32H-J5}$ or SHIV (Cranage *et al.*, 1997). Furthermore, inoculation of macaques with SIV p27:Ty virus-like particles by either the rectal-oral and vagino-oral route, or subcutaneous immunisation targeting the iliac lymph nodes, have induced specific CTL in the rectal and cervico-vaginal mucosa, as well as in regional lymph nodes, spleen and peripheral blood (Klavinskis *et al.*, 1997). Finally, intra-vaginal inoculations with attenuated SHIV have induced at least partial resistance to intravaginal challenge with pathogenic $SIVmac_{239}$ (Miller *et al.*, 1997). Although the presence of SIV-specific CTL in the genital tract was not determined in this study, protected animals had circulating Gag-specific CTL at the time of challenge, with or without specific antibodies in genital secretions.To explain the generation of local and systemic primary immune responses upon vaccine delivery to mucosal sites, antigen up-take by resident dendritic cells is hypothesised, followed by their migration to regional lymph nodes where naive T lymphocytes are stimulated. These would then enter the circulation and migrate to the genital site, where they may be restimulated upon re-exposure to the antigen, thus providing a first line of defence against sexually transmitted viruses.

What is the relationship between virus-specific CTL and virus replication? In analogy with observations made in humans, kinetics studies have shown that the detection of Gag- or Nef-specific CTL by week 1 or 2 after intravenous SIV infection is coincident with the decrease in virus load and p26 antigenemia that follows the initial virus burst (reviewed by Letvin *et al.*, 1994). In the early phase of infection, virus load and SIV-specific CTL

responses show similar kinetics in peripheral blood and lymph nodes. In contrast, during chronic infection, virus sequestration within lymphoid organs (Chackrabarti *et al.*, 1994) is mirrored by CTL compartmentalization to the sites of infection (Geretti *et al.*, 1997*c*). These observations are consistent with CTL being the effector cells of the in vivo immune response against SIV. Further support for a protective role of antiviral CTL comes from the observation that macaques with strong CTL responses against multiple SIV antigens, including the regulatory proteins Nef, Rev and Tat, remained free of disease for at least two years after intravenous infection with $SIVmac_{251}$. In contrast either absent, transient, or weak CTL responses were observed in animals rapidly progressing to overt disease (Venet *et al.*, 1992). Preliminary findings also indicate that the detection of strong CTL responses against Rev and Tat in the early phase of infection may be associated with effective virus containment and lack of disease progression in both HIV-infected humans (Van Baalen *et al.*, 1997) and SIV-infected macaques (Geretti, unpublished).

The relatively low frequencies of SIV-specific CTLp usually detected in SIV-infected macaques seem in contrast with the strong CTL responses often detected in asymptomatic HIV-infected persons. Besides the influence of host genetic factors, or the effects of progressive immunodeficiency, relatively low CTLp frequencies may reflect a low degree of antigenic stimulation in vivo, due to rapid down-regulation of SIV replication after infection. Consistent with this view, high CTLp frequencies can be occasionally detected in animals with high viral load in their peripheral blood mononuclear cells (PBMC) (Geretti *et al.*, 1997*a*). Conversely, the frequencies of circulating SIV-specific CTLp may decline significantly over time in asymptomatic SIV-infected macaques lacking detectable virus in PBMC and showing low-level virus reservoirs in lymphoid organs (Fig. 1 and Geretti *et al.*, 1997*c*). In light of these findings, it is not surprising that the degree of protection conferred by live attenuated SIV vaccines, and the strength of the Env- and Gag-specific CTL responses they induce, are inversely correlated with the level of virus attenuation (Lohman *et al.*, 1994). These observations bear direct implications for vaccine designs aimed at inducing long-lasting protective CTL responses: a successful vaccine will need to achieve a difficult balance between safety, which is dependent upon a high degree of attenuation, and efficacy, which appears directly related to the ability of the virus to replicate.

Are virus-specific CTL deleterious to the host? It has been suggested that in a physiological attempt to eradicate persistent infection, CTL may also induce pathological changes deleterious to the host. This is supported by the finding that HIV-specific CTL are present in both the bronchoalveolar lavage of AIDS patients with lymphocytic alveolitis and in the cerebrospinal fluid (CSF) of patients with AIDS dementia complex (reviewed by Zinkernagel, 1995). The detection of SIV-specific CTL in the skin rash of SIV-infected macaques (Yamamoto *et al.*, 1992) also suggests a role for CTL in mediating tissue damage.

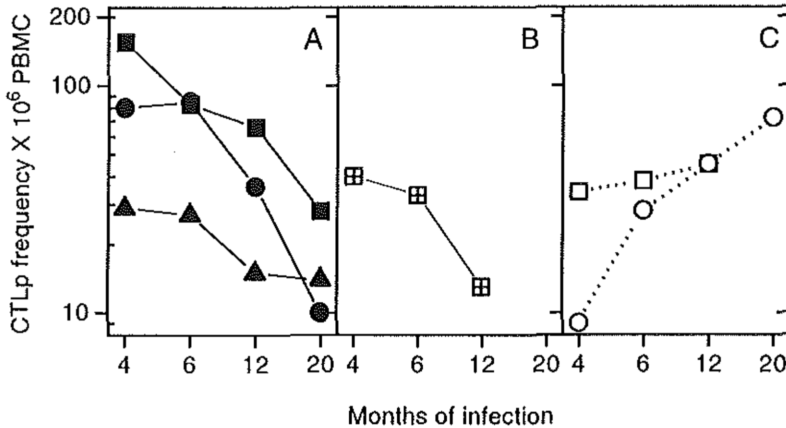

FIG. 1. Kinetics of circulating SIV-specific CTL precursors (CTLp) in macaques infected intravenously with SIVmac$_{32H\text{-}J5}$. After the initial burst of virus replication, three monkeys lost evidence of either culturable virus or PCR-detectable provirus in peripheral blood, while maintaining low-level virus reservoirs in spleen and lymph nodes; they remained asymptomatic with stable CD4$^+$ cell counts during 22 months of observation. Three other monkeys had persistent virus in peripheral blood and high viral load in lymphoid organs; two remained asymptomatic but showed a progressive CD4$^+$ cell loss, whereas the third one developed AIDS 18 months after infection. The cumulative frequencies of circulating SIV-specific CTLp, after reaching a plateau, declined significantly in the three macaques lacking detectable virus in peripheral blood (A) and in the animal which progressed to overt disease (B), but not in the two asymptomatic macaques with persistent infection in peripheral blood (C).

SIV-specific CTL have been also detected in the CSF and brain of infected macaques as early as 1 week after infection and concomitant with the detection of virus (Von Herrath *et al.*, 1995). Interestingly, different SIV proteins were targeted by CTL recovered from the brain, CSF and peripheral blood, suggesting that these can be separated compartments in SIV infection. As these promising data indicate, the SIV model provides an excellent system to explore further the role of CTL in HIV-associated neurological disorders.

Can HIV or SIV variants escape CTL recognition? Selection of mutant viruses resistant to specific CTL may be proposed as one mechanism whereby highly variable viruses escape from immune recognition. Single amino acid substitutions within CTL epitopes can abrogate recognition by affecting either MHC binding or T cell receptor (TCR) interaction (Rothbarth *et al.*, 1989), whereas mutations within epitope flanking regions may reduce presentation by affecting peptide processing and transport (Eisenlohr *et al.*, 1992). Recent studies also suggest that mutated epitopes may arise, which still interact with the TCR, but inhibit CTL function by inducing T cell anergy, or by showing partial agonistic or antagonistic activity (reviewed by Klenerman *et al.*, 1996). In this case, even virus variants that do not become the predominant viral species may affect immune surveillance, as they can block CTL

recognition of viruses that do not contain mutated epitope sequences. Finally, there is evidence that rapid virus evolution and strong antigenic stimulation may also favour immune evasion by causing CTL exhaustion (Von Boehmer, 1993).

Although mutant viruses which are resistant to CTL have been detected in persons infected with HIV (reviewed by Koup, 1994; Koenig *et al.*, 1995; Borrow *et al.*, 1997; Goulder *et al.*, 1997), and despite evidence that multiple mechanisms may be operative in HIV attempts to escape recognition (Couillin *et al.*, 1994), the biological relevance of these findings remains somewhat controversial. The fact that HIV-infected persons, or SIV-infected macaques, generally mount CTL responses against multiple viral antigens suggests that despite the loss of CTL recognition of one epitope, complete evasion from immune surveillance should be a rare occurrence. Nevertheless, qualitative aspects of antiviral CTL, such as the nature of the targeted epitope (Moskophidis *et al.*, 1995) and the affinity of effector cell-target cell interactions (Tsomides *et al.*, 1994) may be crucial determinants of effective CTL pressure. It is therefore conceivable that under stringent circumstances, strong CTL responses targeting key epitopes may promote selection of viral mutants which, at least temporarily, can evade immune recognition. The finding that pronounced HIV genetic heterogeneity is associated with strong CTL responses against Env (Wolinsky *et al.*, 1996) appears to support this view.

The effects of CTL pressure on SIV evolution have so far received limited attention. In a study of macaques infected with $SIVmac_{251}$, the presence of strong p11C-specific CTL responses was associated with the detection of amino acid mutations within the CTL epitope (Chen *et al.*, 1992). Although two of four mutated epitope sequences were recognized less efficiently than the prototype sequence, statistical analysis failed to indicate a higher rate of mutations in the epitope compared with other regions of Gag, and selection of the mutated viruses could not be demonstrated.

Infection of macaques with molecular clones of SIV may help to clarify these issues, as any mutation detected in the viral genome must have originated during the course of the infection. In a long-term non-progressor macaque infected with the molecular clone $SIVmac_{32H\text{-}J5}$, the detection of 'high affinity' immunodominant CTL targeting a nine-mer epitope in Gag designated p26A.5 (Geretti *et al.*, 1997*b*), coincided with the emergence of a variant virus carrying a mutated epitope sequence (Geretti, 1997*c*). Surprisingly, the mutated sequence was detected in the spleen, where it had uniformly replaced the challenge virus, but not in peripheral blood or lymph nodes. This probably reflects the fact that co-existence of viral expression and CTL expansion in splenic white pulp creates a highly favourable microenvironment for immunological pressure (Cheynier *et al.*, 1994). The variant virus escaped recognition by most, but not all p26A.5-specific CTL (Fig. 2). The latter observation suggests a certain redundancy of CTL responses targeting the same epitope, also described

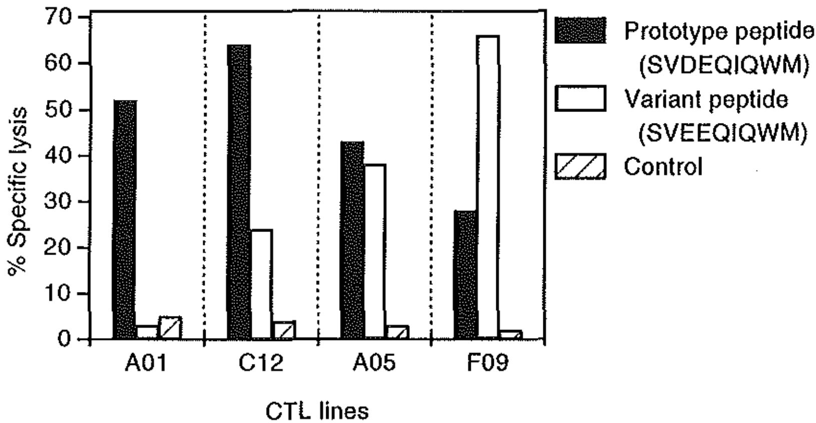

FIG. 2. Different patterns of epitope-specific CTL recognition. Short-term CTL lines were generated by stimulation of peripheral blood mononuclear cells with a nine-mer peptide representing the prototype p26A.5 epitope, and tested for their ability to lyse autologous targets pulsed with either the prototype peptide, or a variant peptide carrying an aspartic acid to glutamic acid substitution (D→E). Four patterns of reactivity were identified: most (38 of 56) CTL lines (e.g. A01) did not recognize the variant peptide even at the highest peptide concentration tested (100 μM), eight (e.g. C12) recognized the prototype peptide with higher efficiency than the variant peptide, seven (e.g. A05) recognized both the prototype and the variant peptide with a similar efficiency, and three (e.g. F09) recognised the variant peptide with higher efficiency than the prototype peptide.

for pC11-specific CTL (Shen *et al.*, 1994; Chen *et al.*, 1996), which may represent a first-line safeguard mechanism against emerging virus variants. Remarkably, CTL subpopulations able to recognise the variant sequence also localized preferentially in the spleen. The low virus load detected in this compartment suggests effective virus containment. However, the idea that a variant virus with even a slight advantage might have eventually replaced the challenge virus also in other compartments and gradually affected the control of the infection remains a valid hypothesis.

CONCLUSIONS

Over the past decade, the macaque model of SIV infection has provided significant new information on the host-virus interplay taking place during the course of lentiviral infections. Increasing evidence indicates a role for antiviral CTL in the containment of SIV replication, and supports the view that the quality of antiviral CTL is as important as their quantity in determining both the outcome of infection and the course of the disease. It is now believed that the ability to induce antiviral CTL that would limit, if not completely prevent virus spread, particularly after mucosal exposure, is an important prerequisite of an effective

HIV vaccine. The concept that the design of such a vaccine will require improved understanding of what may constitute a protective CTL response is also now widely accepted. The SIV model offers valuable opportunities to better understand the mechanisms of lentivirus-induced disease and to clarify the basis for protective immunity. Hopefully, it will fulfill its promise : - helping the development of a successful vaccination strategy against HIV.

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Curriculum vitae

I was born in Rome on the 7th of February 1962. After attending grammar school, in 1989 I received a Medical Doctorate (M.D.) cum laude from the Federico II University of Naples. Once completed my general clinical and surgical training, I obtained a specialist postgraduate diploma (cum laude) in General Oncology with a thesis on AIDS-related tumors. In 1991, I moved to London to attend an MSc course in Immunology at the Royal Postgraduate Medical School of the Hammersmith Hospital. After graduating with distinction, in 1992 I started a PhD research project at the former Laboratory of Immunobiology of the National Institute of Public Health and Environmental Protection in Bilthoven, The Netherlands. I continued my experimental work at the Institute of Virology of the Erasmus University of Rotterdam, which I left in December 1996 to take up my present post as Clinical Lecturer and Hon. Specialist Registrar at the Department of Virology of the Royal Free Hospital School of Medicine in London. Throughout my clinical and research activities, focus of my interest has been the interaction between viruses and host immune system.

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Samenvatting

AIDS werd voor het eerst als een nieuw ziektebeeld onderkend in 1981, toen een ongewone clustering van Kaposi sarcomas en Pneumocystis carinii pneumonieën werd waargenomen in jonge homosexuele mannen. Epidemiologische studies wezen in de richting van een infectieus agens dat overgebracht werd door sexuele contacten, door intraveneus druggebruik, door bloed en bloedprodukten, en ook van moeder op kind. De eerste isolatie van een virus dat later human immunodeficiency virus type 1 (HIV-1) genoemd zou worden, geschiedde in 1983. In 1985 werd een apen-equivalent van dit virus, het simian immunodeficiency virus (SIVmac), geïsoleerd uit Aziatische makaken in gevangenschap, die een ziektebeeld vertoonden dat sterk deed denken aan AIDS bij de mens. Een tweede human immunodeficiency virus (HIV-2) werd in 1986 geïsoleerd uit West Afrikaanse patiënten met een relatief milde vorm van immunodeficiëntie. Alhoewel zowel HIV-1 als HIV-2 AIDS veroorzaken, wordt bij HIV-2 geïnfecteerde individuen een relatief langere klinische latentietijd en een lagere morbiditeit waargenomen. Uit verschillende Afrikaanse apensoorten zoals groene meerkatten, sooty mangabeys, mandrillen en sykes apen, zijn later meerdere SIV stammen geïsoleerd, en in chimpansees werd het SIVcpz gevonden. Deze virussen, die natuurlijk voorkomen bij de genoemde apensoorten in Afrika, veroorzaken geen ziektesymptomen in hun natuurlijke gastheren. Opmerkelijk is dat bepaalde SIV stammen een hoge mate van sequentiehomologie vertonen met HIV-2 isolaten uit West Afrika, terwijl HIV-1 meer gelijkenis vertoont met HIVcpz. Ofschoon op dit moment de oorsprong van HIV-1 en HIV-2 nog niet geheel duidelijk is, lijkt het op grond van fylogenetische analyses waarschijnlijk dat infecties met beide virussen het gevolg zijn van zoönotische transmissies vanuit niet humane primaten reservoirs.

Het klinisch beloop van HIV-1 infecties is zeer variabel. Cohortstudies tonen aan dat ongeveer 50% van de geïnfecteerde individuen AIDS ontwikkelen na 10 jaar; sommige tonen een relatief snelle progressie (minder dan drie jaar, "snelle progressors"), terwijl andere zestien jaar of langer asymptomatisch blijven ("lange termijn non-progressors"). Inzicht in de virus-, gastheer- en omgevingsfactoren die aan deze variabele progressiesnelheid ten grondslag liggen is cruciaal voor het ontwikkelen van effectieve therapieën en vaccins tegen HIV infecties. SIVmac heeft vele biologische en structurele karakteristieken alsmede de genoomorganisatie en nucleotidesequenties gemeen met HIV. Infectie met SIVmac leidt tot een persistentie van het virus in makaken die resulteert in het syndroom dat sterke gelijkenis vertoont met AIDS. Alhoewel het gemiddeld één tot twee jaar duurt voordat ziekte wordt waargenomen, varieert het beloop van de infectie

aanzienlijk. Zelfs na infectie met dezelfde moleculaire kloon, ontwikkelen bepaalde dieren zeer snel ziektesymptomen waardoor ze binnen enkele maanden sterven, terwijl andere een aantal jaren asymptomatisch blijven. Deze variatie doet sterk denken aan de "snelle progressors" en de "lange termijn non-progressors" onder HIV geïnfecteerde mensen. Vanwege deze sterke overeenkomsten met HIV infecties van de mens, biedt SIV infectie van de makaak een zeer waardevol model om de rol van de gastheer-immuniteit bij de controle van lentivirusinfecties te bestuderen.

De studies in dit proefschrift hebben zich gericht op de bestudering van de rol die cytotoxische T lymfocyten (CTL) spelen bij het controleren van HIV en SIV infecties. Na een introductie in de belangrijkste eigenschappen van infecties met primaten lentivirussen in hoofdstuk 1, beschrijft hoofdstuk 2 de resultaten van een tweetal vaccinatiestudies in makaken. Beide studies waren gericht op de bestudering van het vermogen van vaccin-geïnduceerde humorale en CTL responsen om intraveneuze infectie van makaken te voorkomen met hetzij een moleculaire kloon van SIVmac, hetzij een HIV-SIV hybride virus (SHIV). Ondanks de inductie van virusneutraliserende antistoffen en CTL, werd in de eerste studie géén bescherming tegen infectie gevonden. Alhoewel deze bevinding teleurstellend was, was hij wel in overeenstemming met de opvatting dat CTL niet in staat zijn om een SIV of HIV infectie te controleren, tenzij aan strikte kwalitatieve en kwantitatieve voorwaarden is voldaan. De opvatting dat SIV-specifieke CTL immuniteit zou hebben bijgedragen aan het beperken van de virusreplicatie in tenminste enkele van de bestudeerde dieren blijft tegelijkertijd echter een attractieve hypothese. De resultaten van de tweede vaccinatiestudie tonen aan dat CTL responsen een zeer gevoelige marker zijn voor een tijdelijke of op een laag niveau verlopende virusreplicatie en ondersteunen de hypothese dat beschermende CTL eerder de infectie beperken dan hem compleet verhinderen. In deze studie werden in apen die geïmmuniseerd waren met Env-iscoms, geen virus en ook geen anamnostische antilichaamresponsen aantoonbaar na intraveneuze challenge-infectie met SHIV. Wel waren CTL precursor cellen tegen andere antigenen dan Env, waaronder de niet viriongebonden regulatoire eiwitten Rev en Tat aantoonbaar. Aannemend dat voor CTL inductie endogene synthese van virale eiwitten nodig is, tonen deze bevindingen aan dat een tijdelijke infectie en ook viruseradicatie door CTL inderdaad mogelijk zijn.

In hoofdstuk 3 wordt uiteengezet hoe de optimale condities voor CTL expansie, kwantitatieve analyses en fenoypische karakterisaties *in vitro* voor deze studies werden opgezet. De isolatie van T cel subsets is een belangrijke stap in de karakterisatie van CTL responsen. Resultaten in het eerste deel van het hoofdstuk tonen aan dat het mogelijk is om $CD8^+$ CTL te scheiden van andere celfracties, zonder dat hun fenotype en cytotoxische

eigenschappen worden aangetast. In het tweede deel van het hoofdstuk worden de verschillende methoden voor de *in vitro* expansie van SIV-specifieke CTL vergeleken. In tegenstelling tot gegevens verkregen met HIV-1 geïnfecteerde asymptomatische volwassen personen, bleek *in vitro* restimulatie bijna altijd nodig om in de apen SIV-specifieke CTL responsen tot detecteerbare niveaus te verhogen. Daardoor laten SIV geïnfecteerde of gevaccineerde makaken doorgaans CTL precursor (CTLp) frequenties zien die aanzienlijk lager zijn dan die in het algemeen in asymptomatische HIV geïnfecteerde volwassenen worden gevonden, hetgeen aangeeft dat de daartoe geëigende methoden van *in vitro* restimulatie nodig zijn voor hun detectie. Een succesvolle methode voor de *in vitro* expansie van antivirale CTL is gebaseerd op de stimulatie met formaldehyde gefixeerde autologe B cellijnen (B-LCL) die tevoren geïnfecteerd zijn met recombinant vacciniavirussen die SIV antigenen tot expressie brengen. Deze benaderingswijze bleek de CTL detectiegrens te verhogen door selectieve CTL expansie te bewerkstelligen. Bovendien verhoogde het kweken van de cellen onder "limiting dilution" condities de gevoeligheid van de CTL metingen en maakte het mogelijk om, zij het in lage frequenties, CTLp aan te tonen in makaken die in standaard "bulk" CTL testen geen significante CTL responsen lieten zien. Het hoofdstuk beschrijft bovendien de identificatie van een minimaal epitoop van SIV Gag dat herkend wordt door antivirale CTL van een SIV geïnfecteerde makaak. Het bepalen van virale epitopen die door specifieke CTL worden herkend kan het ontwerpen van immunotherapeutische interventies verbeteren en tevens een extra basis verschaffen voor het verkrijgen van inzicht in de pathogenese van AIDS. Inderdaad bleek dat aminozuursubstituties in de sequentie van het epitoop, zoals die werden waargenomen bij het vergelijken van de homologe regio's van HIV-1 en HIV-2, voldoende waren om de herkenning door CTL geheel teniet te doen, leidde tot de hypothese dat virusvarianten die aan de epitoop-specifieke CTL respons kunnen ontsnappen *in vivo* onder de bestaande CTL druk kunnen ontstaan. De resultaten van een longitudinale studie die erop gericht is om deze hypothese te testen worden in hoofdstuk 4 beschreven: Twee prospectieve studies die in de chronische fase van respectievelijk HIV-1 en SIV infecties werden uitgevoerd. De eerste studie analyseert de kinetiek van HIV-1 Gag en Epstein-Barr virus (EBV) specifieke CTL en beschrijft hun relatie met veranderingen in $CD4^+$ en $CD8^+$ celtellingen in de tijd. In HIV-1 geïnfecteerde volwassen individuen werd een relatief sterke CTL respons gevonden tijdens de asymptomatische fase, die geassocieerd was met verhoogde aantallen $CD8^+$ T cellen. Deze respons nam veelal af bij ziekteprogressie, tegelijk met een duidelijke $CD4^+$ T celdepletie, die tot aantallen ruim onder 200 $CD4^+$ cellen per μl leidde. Op dit moment bestaat er geen bevredigende verklaring voor deze daling, alhoewel een progressief verlies van helper functie er deel van uit kan maken. Tevens dient te worden

opgemerkt dat enkele patiënten een relatief sterke CTL respons tegen HIV-1 Gag behielden gedurende de late stadia van het ziekteverloop. Dit is in overeenstemming met de opvatting dat individuen met het meest goedaardige ziekteverloop niet noodzakelijkerwijs de hoogste niveaus van CTL activiteit hoeven te hebben. Omgekeerd hoeven zoals hierboven gesteld, hoge niveaus van CTL niet noodzakelijkerwijs de ontwikkeling van het ziekteproces te voorkomen. Een andere interessante bevinding van deze studie was, dat de frequentie van EBV-specifieke CTL niet gelijktijdig afnam met de afnemende $CD4^+$ celfrequenties, hetgeen waarschijnlijk direct samenhangt met de relatieve onafhankelijkheid van deze EBV-specifieke CTL van $CD4^+$ cel-gerelateerde T helper cel factoren.

De tweede studie die in dit hoofdstuk wordt beschreven, bestudeert de evolutie van SIV-specifieke CTL in relatie tot de kinetiek van virusreplicatie en pogingen van het virus om aan immuunsurveillance te ontsnappen. Het detecteren van virus-specifieke CTL direct na intraveneuze infectie met SIV, ging gepaard met het afnemen van de hoeveelheid virus en p26 antigeen na de initiële explosieve toename van het virus in circulatie. In deze vroege fase van de infectie, toonden virushoeveelheid en virus-specifieke CTL responsen overeenkomstige kinetieken in het perifere bloed en in de lymfeklieren. Bij de meer chronische fasen van de infectie daarentegen, werd het "wegdrukken" van het virus naar de lymfoïde organen weerspiegeld in het compartimentaliseren van de CTL op de plaatsen waar de infectie nog aanwezig was. Deze bevindingen zouden de hypothese bevestigen dat CTL de effectorcellen zijn die de *in vivo* immuunrespons tegen SIV bepalen. De relatief lage frequenties van specifieke CTLp die in de bestudeerde makaken werden gevonden schijnen niet in overeenstemming te zijn met de sterke CTL responsen die vaak in asymptomatische HIV geïnfecteerde individuen worden gevonden. Naast de invloed van door de makaak zelf bepaalde genetische factoren of de effecten van de voortschrijdende immunodeficiëntie, kunnen de lage CTLp frequenties een reflectie zijn van een lage graad van antigene stimulatie *in vivo* als gevolg van de snelle onderdrukking van SIV vermeerdering na infectie. Hiermee in overeenstemming is de opvatting dat hoge CTLp frequenties soms werden gevonden in dieren met een grote hoeveelheid virus in hun in het bloed circulerende mononucleaire cellen (PBMC). Omgekeerd namen de frequenties van circulerende SIV-specifieke CTLp significant af in de tijd in asymptomatische SIV geïnfecteerde makaken die geen aantoonbaar virus in hun PBMC hadden en ook geringe hoeveelheden virus in hun lymfoïde organen aantoonbaar aanwezig hadden. In het licht van deze bevindingen is het niet verwonderlijk dat de mate van bescherming die door levend geattenueerde SIV vaccins wordt bewerkstelligd en de sterkte van de CTL responsen die ze induceren, omgekeerd evenredig lijken te zijn met de mate van attenuatie van het virus.

Deze gegevens hebben directe gevolgen voor het ontwerpen van vaccins die zich richten op het induceren van langdurige beschermende CTL responsen en geven aan dat een succesvol vaccin een moeilijk te bereiken evenwicht dient te bewerkstelligen tussen veiligheid die afhankelijk is van een hoge graad van attenuatie en effectiviteit, die direct gerelateerd lijkt te zijn aan de mate waarin het virus zich kan vermeerderen.

Selectie van gemuteerde virussen die resistent zijn tegen specifieke CTL responsen kon worden voorgesteld als één van de mechanismen waardoor sterk variërende virussen kunnen ontsnappen aan immuunherkenning. Indien de kwaliteit van de antivirale CTL een cruciale determinant zou zijn van immunologische druk, is het voor te stellen dat bepaalde sterke CTL responsen, selectie van mutantvirussen die tenminste tijdelijk zouden kunnen ontsnappen aan herkenning, in de hand zouden werken. In een "lange termijn non-progressor" makaak geïnfecteerd met een moleculaire kloon van SIVmac, ging de detectie van "hoog affine" immunodominante CTL die het p26A.5 epitoop van Gag herkenden, gepaard met het ontstaan van een variant virus met een mutatie in de sequentie van dit epitoop. Opmerkelijk was dat de gemuteerde sequentie werd aangetoond in de milt waar het het oorspronkelijke challenge-virus geheel had verdrongen, terwijl dit niet het geval bleek in het perifere bloed of in de lymfeklieren. Het gelijktijdig aanwezig zijn van virusexpressie en CTL expansie in de witte pulpa van de milt doet waarschijnlijk een micro-omgeving ontstaan die immunologische druk in de hand werkt. Het variant virus ontsnapt daarop aan de herkenning door de meeste maar niet door alle epitoop-specifieke CTL. Interessant is dat CTL subpopulaties die de variantsequentie konden herkennen, zich ook preferentieel in de milt ophielden. De lage hoeveelheid virus die in dit compartiment werd waargenomen suggereert dat het virus er effectief onder controle werd gehouden. Echter, de speculatie ligt voor de hand dat een virus-variant die ook maar een gering selectievoordeel heeft, het challenge-virus uiteindelijk zou hebben verdrongen, ook buiten de milt, en dat dit dynamische proces geleidelijk de controle van de infectie zou ondermijnen.

Tenslotte bevat hoofdstuk 5 een samenvatting en een discussie van de experimenten uit dit onderzoek, geplaatst tegen de achtergrond van soortgelijke studies die door anderen in zowel mensen als niet-humane primaten zijn uitgevoerd. Dit laatste hoofdstuk illustreert hoe in de afgelopen tien jaar makaak-SIV modellen belangrijke nieuwe informatie hebben opgeleverd over de interactie tussen gastheer en virus gedurende het beloop van lentivirusinfecties.