HIV-1 VACCINE DEVELOPMENT:

STUDIES IN MACAQUE MODELS

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HIV-1 VACCIN ONTWIKKELING:

STUDIES IN MAKAKEN

Proefschrift

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Chapter 1

INTRODUCTION

Since its discovery about 15 years ago, human immunodeficiency virus type 1 (HIV-1) continues to spread at an alarming rate. It is estimated that by the year 2000 between 30 and 40 million people will be infected with this virus worldwide[1]. Of these, about 90 % live in developing countries. To date, more than six million people have already developed to the fatal acquired immunodeficiency syndrome (AIDS). Despite numerous research efforts no vaccination or generally accessible therapeutic approach has become available yet.

In 1981, the first case of AIDS was recognized[2]. Epidemiologic studies implicated an infectious agent and in 1983, the isolation of a previously unknown human retrovirus called lymphadenopathy-associated virus (LAV) from an AIDS patient was reported by Barré-Sinoussi *et al.*[3]. In 1984, Gallo *et al.*[4] and Levy *et al.*[5] also reported the isolation of rétroviruses from AIDS patients which they called human T-lymphotropic virus type III (HTLV-III) and AIDS-associated retrovirus (ARV), respectively. Soon thereafter, these retroviruses were recognized as members of the Lentivirinae, a subfamily of the Retroviridae, and were indeed identified as the etiological agents of AIDS. In 1986, these retroviruses were named human immunodeficiency virus (HIV). Today, two types of HIV are recognized, HIV-1 and HIV-2. Of these, HIV-1 is the primary etiologic agent of the current AIDS pandemic. The search for an HIV-1 counterpart in other primate species led to the identification of several simian immunodeficiency viruses (SIV), which may cause AIDS like syndromes in monkeys[6]. Currently, macaque lentivirus infections are considered the most appropriate animal models to study HIV pathogenesis and possible intervention strategies.

In the following section, a concise overview is given of the biology of primate lentiviruses, including the host immune response and correlates of immune-mediated protection. Aspects important for vaccine development are highlighted.

PRIMATE LENTIVIRUSES

Classification of primate lentiviruses

HIV and SIV are related members of the Lentivirinae subfamily of the Retroviridae family (see Figure 1). Lentiviruses cause lifelong infections in their respective hosts and may induce a slowly progressing and degenerative disease[7]. Lentivirus isolates from humans can be divided in two groups, designated HIV-1 and HIV-2. HIV-1 isolates have been genetically classified into the major (M) and outlier (O) group. The M group is further divided into nine subtypes (clades A through I) based on their envelope sequences[8]. Members of the same subtype differ by less than 10 % and those of different subtypes by 15 % or more. Subtype B is the most prevalent in Europe and the United States, while clade E is the most prevalent in Africa. HIV-2 isolates have been classified into five subtypes (clades A through E)[8], and are mainly found in West-

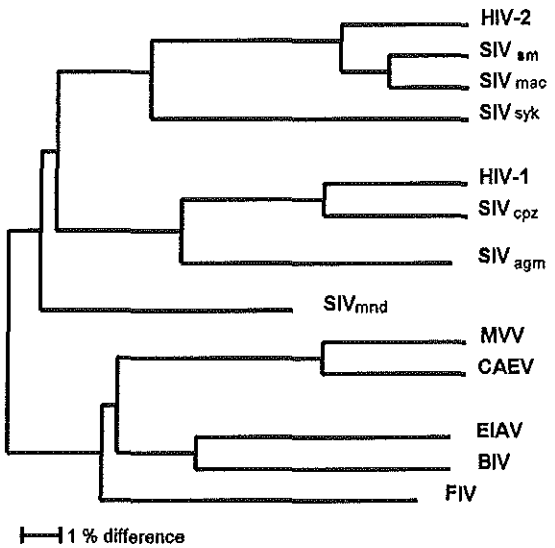

Figure 1. Phyologenetic relationships of lentiviruses. Representative lentiviruses are compared using *pol* gene nucleotide sequences for establishing phylogenetic relationships. The scale indicates percentage difference in nucleotide sequences in the *pol* gene. The braking order of the primate lentiviruses is will controversial[149,150].

Africa. This enormous viral diversification is a major concern in the development of an HIV vaccine.

In the search for an animal model for HIV-1 infection, several lentiviruses have been identified in non-human primfiate species including african green monkeys (SIV_{agm})[9], sooty mangabeys (SIV_{sm})[10], mandrills (SIV_{mnd})[11], sykes (SIV_{syk})[12] and chimpanzees (SIV_{cpz})[13] (see Figure 1). These unique strains of SIV are endemic to the respective African monkey species and appear not to induce disease in their natural host. In contrast, Asian macaque species apparently infected with SIV in captivity, including rhesus, cynomolgus, and nemestrina macaques, did show fatal AIDS-like disease. However, the viruses isolated from these animals, SIV_{mac}, SIV_{cyn}, and SIV_{mne} respectively, appear very closely related to SIV_{sm}, and are therefore thought to have originated from the accidental transmission of SIV_{sm} from naturally infected sooty mangabeys to macaques[14]. A similar cross-species transmission of a primate lentivirus is likely to be at the basis of the development of AIDS in humans infected with HIV-1, although the non-human primate reservoir for HIV-1 has not been identified definitively.

Besides HIV and SIV, five other subgenera of lentiviruses have been recognized: feline immunodeficiency virus (FIV)[15], bovine immunodeficiency virus (BIV)[16], equine infectious anaemia virus (EIAV)[17], maedi-visna virus (MVV)[18] and caprine arthritis-encephalitis virus (CAEV)[19]. Of these, only FIV shares the characteristics of HIV and SIV of a pronounced T cell tropism and the associated loss of $CD4^+$ cells, ultimately leading to a fatal immunodeficiency syndrome[20].

Genomic organization

The genomes of HIV and SIV consist of two positive single-stranded RNA molecules of about 9.2 kb[21]. The long terminal repeats, which flank the proviral DNA, contain signals for integration, transcription, and poly-adenylation. Three major open reading frames, *gag, pol* and *env*, code for the core proteins (p24, p17, p9 and p7), the viral enzymes (reverse transcriptase (RT), RNase H, protease and integrase) and the envelope glycoproteins (Env), respectively (Figure 2). The regulatory proteins Tat and Rev are encoded by two overlapping exons each. HIV and SIV differ in the composition of genes coding for the accessory proteins: HIV-1 encodes Vif, Vpr, Vpu and Nef, HIV-2 and SIV Vif, Vpx and/or Vpr and Nef, respectively.

Virion structure

Mature lentivirus particles are approximately 110 nm in diameter and consist of a lipid bilayer membrane or envelope that surrounds the cone-shaped nucleocapsid (Figure 3)[22]. The membrane of HIV and SIV particles contains approximately 72 spikes that show triangular symmetry[23]. Each spike probably comprises three heterodimers of the highly glycosylated viral envelope glycoprotein (Env). Each heterodimer is composed of a surface (SU) and a transmembrane (TM) subunit that are associated through non-covalent bonds[24]. In addition, membranes of HIV and SIV particles contain several cellular proteins acquired during the budding stage of virion assembly. These cellular proteins include several adhesion molecules, ß2-microglobulin and human histocompatibility leucocyte antigen (HLA) antigens[25,28]. A role in viral replication and/or pathogenesis for these cellular proteins incorporated into virions has been suggested[29], but has not been firmly established yet. Interestingly, immunization with human HLA-class I and II could protect monkeys against SIV which was grown on human cells[30,31].

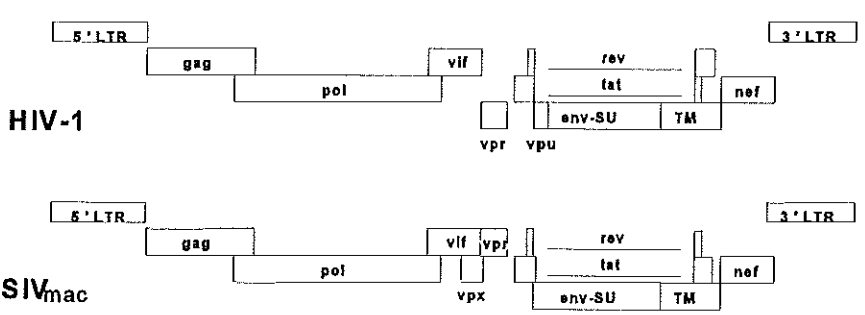

Figure 2. Genomic organization of HIV-1 and SIV_{mac}.

The nucleocapsid is composed of the major Gag (group specific antigen) protein p24 and contains the virus genome of two positive stranded RNA molecules. Closely associated with the genomic RNA are the RT and the Gag proteins p9 and p7. Other virus encoded proteins present in the mature virion are the protease, RNase H and integrase and the accessory proteins Vpr, Vpx, Vif and Nef[32,35]. The nucleocapsid is surrounded by an icosahedrycal layer composed of the p17 Gag matrix protein which is associated with the viral envelope[22].

Replication cycle

As the replication cycle of HIV-1 has been most extensively studied and that of SIV is not expected to contain major structural differences from that of HIV-1, the following section will discuss the major steps in the replication cycle of HIV-1.

VIRION ATTACHMENT AND ENTRY. The first step in infection of a target cell is the high affinity interaction of the virion with the primary cellular receptor, the CD4 molecule[36]. Subsequent conformational changes create a new recognition site on gp120 for the second receptor[37,38], which is one of the members of the C-X-C and C-C chemokine receptor family[39,40]. At some yet undefined point, the gp41 fusion domain is exposed, allowing penetration of the gp41 N-terminal stretch of hydrophobic amino acids into the opposing cell membrane followed by fusion of viral and host cell membranes. Initially, studies with laboratory adapted HIV-1 strains suggested that these conformational changes also induced shedding of gp120. However, this appears not or to a much lesser extent to be the case for primary virus isolates[41].

REVERSE TRANSCRIPTION AND INTEGRATION OF VIRAL DNA. After uncoating, the viral RNA is converted into linear double stranded DNA by the viral RT[42]. This process of reverse transcription is particularly error prone, since no proof reading is involved. In addition, template switching during reverse transcription resulting in retroviral recombination contributes to the high level of HIV-1 variability[43,44]. The newly formed linear double-stranded DNA is subsequently transported into the nucleus and integrated in the genomic DNA by means of the viral integrase. Both reverse transcription and integration can only be completed in dividing cells[45,46]. When quiescent, cells can still be infected, but new progeny can generally not be formed until the cells are stimulated. These latently infected cells may serve as a major reservoir for persistent chronic infection.

VIRAL GENE EXPRESSION. From the integrated proviral DNA, initially multiple spliced short viral mRNAs are formed coding for the regulatory proteins Tat, Rev and Nef. Tat and Rev subsequently regulate the production of full length and singly spliced mRNAs. The full length RNA transcripts serve either as genomic RNA to be encapsidated or as mRNA to translate the Gag and Pol precursor proteins. The single spliced mRNAs encode the Envelope and Vif, Vpu, Vpr, Vpx proteins. The latter proteins are generally not necessary for virus replication *in vitro*, but may modulate virus infectivity as well as viral and cellular gene expression *in vivo* [47,48]. Because of the early expression of the

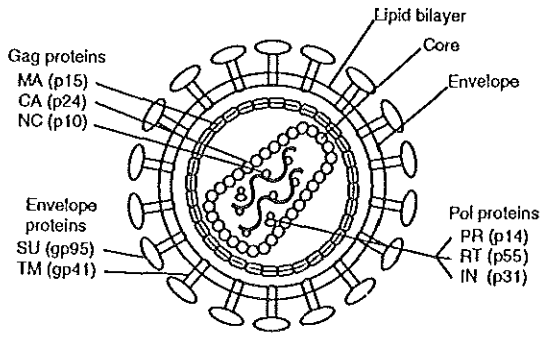

Figure 3. Schematic respresenation of the structure of HIV-/SIV_{mac} virus particles (reproduced with permission from K. Siebelink[147]).

Tat, Rev and Nef proteins, which is suggested to be at least 8 hours before Gag expression[49] and sometimes even longer since the replication cycle may be blocked at this point[50], it is speculated that CTL directed against these proteins may kill infected cells before the expression of structural proteins and before the release of infectious virus particles[51].

ASSEMBLY AND BUDDING. Incorporation of the viral genomic RNA is part of the capsid formation at the cell membrane. In the subsequent budding process, the viral capsid and the fully processed envelope protein associate, which results in a new-formed virus particle[52]. At the same time, envelope glycoproteins expressed at the cell surface of infected cells may interact with the CD4 receptor expressed at the surface membrane of adjacent cells. This may lead to membrane fusion similar to that in cell-free virus entry, resulting in the formation of multinucleated cells or syncytia[53]. Such interactions between neighbouring cells may also be at the basis of direct cell-to-cell spread of the infection[54].

SIV_{mac} and SHIV INFECTION OF MACAQUES AS MODELS FOR HIV-1 INFECTION

Several primate lentiviral models for HIV-1 infection in humans have been described (see Table 1). Of these, only infection of certain macaque species with certain strains of SIV cause an immunodeficiency syndrome similar to AIDS in humans. Most animal studies on pathogenesis and vaccine development have used infection of rhesus or cynomolgus macaques with SIV_{mac}. Recently, chimeras of SIV and HIV-1 called SHIV, which are infectious for macaques, have been generated by recombinant DNA techniques. In these chimeric viruses *tat, rev, env*, and in some cases other SIV

genes have been replaced by their HIV-1 counterparts[55,59]. By doing so, the testing of vaccines based on HIV-1 antigens has become possible in macaques.

Tropism

The cellular tropism of SIV_{mac}, SHIV and HIV-1 is mainly determined by the presence of their respective primary and secondary receptors. They infect $CD4^+$ cells, like helper T-lymhocytes, monocytes, macrophages, follicular dendritic cells and microglial cells[60]. Further differentiation between the respective viruses and their subtypes is based upon their second receptor usage. T-cell line tropic, syncytium inducing (SI) HIV-1 strains generally use the α-chemokine receptor CXCR4, while macrophage tropic, non-syncytium inducing (NSI) HIV-1 variants generally use the ß-chemokine receptor CCR5 as their second receptor[61]. Occasionally, virus variants have been shown to use additional receptors such as CCR2b[62] and CCR3[63] and possibly other new chemokine receptors[64]. All SIV strains tested until now use the CCR5 receptor for entry[65]. In addition, STRL33 has been shown to act as SIV co-receptor, and the use of other presently unknown coreceptors has been suggested[65,66].

Transmission and pathogenesis

Experimental SIV_{mac} infection of macaques has been carried out by the intravenous, intrarectal and intravaginal routes[6]. This is reminiscent of HIV being transmitted sexually, through intravenous drug abuse, by therapies utilizing blood and blood products, and vertically from mother to child[67,69]. The *in vitro* $TCID_{50}$ of SIV generally equals the *in vivo* MID_{50} when infection takes place via the intravenous route[70]. For intravaginal or intrarectal infection of monkeys, 10 to 1000 fold more virus is usually needed[71].

SIV_{mac} infection of macaques and HIV infection of humans are generally characterized by three phases: the acute, the asymptomatic, and the AIDS phase. The acute phase, which comprises the first 8-12 weeks post infection, is either accompanied by influenza like symptoms or runs subclinically[72]. Upon infection, high levels of cell-associated and plasma viraemia develop, which peak at about 2 weeks post infection. This rise in viraemia is accompanied by a transient drop in the number of $CD4^+$ cells[73]. Subsequently, the amount of circulating virus declines, reflecting either the onset of an effective immune response[74,75] or an exhaustion of permissive host cells[76]. In the mean time, infection has spread in the lymphatic tissues. Here, lots of virus particles are trapped in the follicular-dendritic-cell (FDC) network[77,78]. In the clinically asymptomatice phase plasma viral levels are low to undetectable, however, the virus is turning over very rapidly. As was recently revealed in HIV-1 infected persons, about 10^{10} virions are produced and cleared from the circulation each day[79,80]. More than 99 % of virus is thought to be produced by newly infected $CD4^+$ T cells, of which the half-life is about 1.6

Table 1. Primate models for HIV-1 infection in humans[1].

Virus	Host[2]	Pathogenicity/development of AIDS
HIV-1	chimpanzee	AIDS rare
	gibbon	none (?)
	pigtail macaque	transient infection
SIV_{cpz}	chimpanzee	none (?)
HIV-2	baboon	AIDS rare
	rhesus/cynomolgus macaque	occasionally AIDS after more than 4 years
SIV_{syk}	sykes monkey	none (?)
SIV_{mnd}	mandrill	none (?)
SIV_{tal}	talopoin	none (?)
SIV_{agm}	african green monkey	none (?)
	pigtail macaque	AIDS
SIV_{sm}	sooty mangabey	none (?)
	rhesus/cynomolgus/ pigtail macaque	AIDS in 1-4 year
SIV_{mac}[3]	rhesus/cynomolgus/ pigtail macaque	AIDS in 1-4 year
SIV_{mne}[3]	rhesus/cynomolgus/ pigtail macaque	generally low pathogenicity no AIDS within 4 year
SIV_{stm}[3]	stumptail macaque	AIDS
SHIV[4]	rhesus/cynomolgus/ pigtail macaque	depending on composition chimera and of *in vivo* passage history: induction of AIDS

[1] As described in[6,122,148].
[2] Latin names of the respective primate species: chimpanzee - *Pan troglodytes*;gibbon - *Hylabates lar*; baboon - *Papio hamadryas*; pigtail macaque - *Macaca nemestrina*; rhesus macaque - *Macaca mulatta*; cynomolgus macaque -*Macaca fascicularis*; stumptail macaque - *Macaca arctoides*; african green monkey -*Cercopithecus aethiops*; mandrill - *mandrillus spinx*; talopoin - *Cercopithecus talapoin.*
[3] These viruses probably result of a cross-species transmission of SIV_{sm} strains to macaques held in captivity and do not occur in nature.
[4] Chimera of SIV_{mac} and HIV-1

days[79,81]. This enormous viral turnover is thought to drive the eventual breakdown of the immune system and the gradual decline in $CD4^+$ cell numbers. In HIV-1 infected humans, the average time of the asymptomatic phase is 10-11 years. About 10% of HIV-1 infected people progress to disease within 3 years (rapid progressors; RP), while another estimated 5-15 % remains asymptomatic for more than 12 years (long-term non-progressors; LTNP)[82]. In rhesus and cynomolgus macaques, the average time from SIV_{mac} infection to disease is shortened to 1-4 years[83]. Both virus and host characteristics seem to play important roles in this respect. The SIV strain used for infection generally determines whether the infection will ultimately lead to AIDS or not[6,83]. However, individual variability in the rate of disease progression in identically SIV infected monkeys indicates an important role for the host immune response. In both SIV_{mac} and HIV-1 infection, the level at which the plasma viral load plateaus following seroconversion has been shown to be highly predictive for the length of the asymptomatic period[84].

Eventually, AIDS develops, which is generally accompanied by loss of body weight, persistent diarrhoea and opportunistic infections[82,83,85]. This coincides with loss of cellular immunity, a dramatic drop in the levels of antiviral antibodies and $CD4^+$ cells and a sharp increase in the levels of virus and infected cells in circulation[86]. The lymphnode architecture is disrupted by extensive fibrosis and fatty infiltration[82]. Infection of macaques with SHIV initially failed to induce disease[55,56,59]. However, pathogenic SHIVs have recently been obtained by serial *in vivo* passages in macaques and specific gene replacements [57,87,88].

An important role of the phenotype of the virus in the course of HIV-1 infection has been suggested. Macrophage tropic, NSI variants are generally found to establish the infection in humans, even if they constitute only a minority in the infecting inoculum[89,90]. These NSI variants can subsequently be isolated throughout the course of infection in virtually all individuals. With advancement to disease, in about 50 % of the cases, SI variants can be found[91]. It is still uncertain whether appearance of SI variants is the cause or the consequence of the clinical course of the infection. Similar observations on virus alterations have been reported in non-human primate models of HIV-1 infection, namely HIV-1 infection in chimpanzees and HIV-2 infection of rhesus macaques and baboons[92,93]. Interestingly, in theses species the appearance of cytopathic virus variants was not predictive for the development of AIDS[94].

IMMUNE CORRELATES OF PROTECTION

Immune response and correlates of long-term survival

HUMORAL IMMUNE RESPONSE. High antibody titers are usually found within a few weeks after infection. Neutralizing antibodies are, however, generally detected later and at a time that the primary viraemia has already been cleared[74,83]. Antibodies may, however, play a role in the containment of primary plasma viraemia by trapping of complement bound antibody-antigen complexes by follicular dendritic cells in the lymph nodes as well as by antibody dependent cytotoxicity (ADCC)[82,83].

In the asymptomatic phase antibodies against virtually all viral proteins are found in circulation. The VN antibody titre is high, and its specificity broadens over time[95,96]. Some investigators have reported that antibody titres, including the VN antibody titre, are higher in LTNP as compared to RP[97,98]. Evidence for a selective pressure of autologous VN antibodies on virus composition comes from the observation that viral isolates obtained early after seroconversion are neutralized by sera from the time point of isolation, while subsequent patient isolates are not neutralized[99,100]. In addition, administration of heat inactivated plasma from healthy infected individuals could slow the progression to AIDS in both humans and monkeys[101,103]. Doubts, however, about an *in vivo* protective role of HIV-1 antibodies have been raised by recent studies showing little or no virus neutralization of serum from infected individuals against primary HIV-1 isolates[104,105]. In addition, the presence of HIV and SIV infection enhancing antibodies has been reported, but their *in vivo* significance is still subject of debate[106,108].

CELLULAR IMMUNE RESPONSE. Studies in monkeys infected with SIV_{mac} showed the presence of $CD8^+$ CTL as soon as 4-7 days post infection[109,110]. Clearance of primary viraemia is associated with the outgrowth of $CD8^+$ CTL in circulation. Moreover, antiviral pressure by early HIV-1 specific CTL has been demonstrated by the rapid selection of CTL escape virus[75], indicating a role for CTL in the initial containment of virus replication. In addition, early SIV specific Th responses have been associated with long-term non-progression[111].

In most SIV infected monkeys, like in HIV-1 infected individuals, vigorous and broadly directed SIV specific $CD8^+$ CTL responses have been observed[112]. In one study, the presence of CTL against multiple SIV proteins was correlated with a better clinical outcome[112,113]. In HIV-1 infected individuals, the mere presence of CTL activity does not seem to distinguish LTNP from RP[114]. However, the presence of CTL precursors directed against the early proteins Rev and Tat was recently shown to correlate with the length of AIDS-free survival[115]. It has also been suggested that individuals with CTL activity against immunodominant HIV-1 epitopes have a more stable and effective immune response to HIV-1 than those whose CTL responses are against multiple, less dominant epitopes[116]. In addition, HIV-1 specific CTL from LTNP may differ from those of RP with regard to their epitope specificity[117,118] or T cell receptor usage [119]. A debate is still ongoing about the question whether higher levels of CTL activity indicate a stronger immune response controlling HIV infection, or whether increased CTL levels simply reflect the viral load. In addition, a role of CTL in immuno-pathogenesis has been

proposed[82].

Besides cytotoxic activity, $CD8^+$ lymphocytes may generate soluble factors capable of inhibiting lentivirus replication. This non-cytotoxic, non-MHC restricted suppression is mediated, at least in part, by as yet undefined soluble factor(s) ($CD8^+$ T cell antiviral factor or CAF)[120]. ß-chemokines[61] and Il-16[121], which can be produced by $CD4^+$ and $CD8^+$ cells, were demonstrated to have an inhibitory effect on HIV replication, although these factores could not fully account for the observed $CD8^+$-derived antiviral activity[122,123]. $CD8^+$ cell mediated HIV-1 suppression has been found not only in HIV-1/SIV infected individuals but also in naive humans and monkeys[124,125]. It is not yet clear how and to what extent these factors are induced by lentiviral infection or vaccination. Relatively high levels of non-cytotoxic HIV-1 suppressive factors in LTNP have been described[125,127]. However, more information is needed to establish their role in the control of HIV infection *in vivo*.

Resistance to infection

Several studies with monkeys exposed to SIV at mucosal sites, have shown evidence that transient, probably local, infection can occur in the absence of systemic virus dissemination[128]. Moreover, monkeys exposed to low-dose mucosal virus challenge who were not infected as determined on the basis of lack of seroconversion or detectable virus, were protected from subsequent high-dose mucosal SIV challenges[129,130]. These animals showed a greater *in vitro* proliferative response to SIV[129,130] and a higher degree of $CD8^+$ cell-mediated suppressor activity compared to non-infected control animals[129]. In addition, two out of four monkeys intravenously exposed to HIV-2 but not infected, were protected against rectal SIV_{sm} challenge, and protection correlated with the presence of CTL against RT and Nef[131]. These results are consistent with observations in exposed-uninfected humans: The presence of CTL in HIV-1 exposed-uninfected individuals, including children born from seropositive women, indicated that a transient infection can take place[132,134]. Again, it is not known whether the presence of these CTL responses simply reflects restricted viral infection at the site of exposure, or whether they were actually responsible for preventing viral dissemination. Since all HIV-1 exposed individuals who have remained uninfected, are HIV-1 antibody negative, it is assumed that it is not a B cell response that protects these individuals, although it can not be ruled out completely that a localized or low-level B cell response is involved[135]. A role for antibodies in resistance from infection is, however, suggested by the correlation found in some studies between autologous VN antibodies in the mother and protection from infection in the child[136]. Finally, also non-immune parameters, like CCR5 genotype, HLA-composition, virus load of the donor, integrity of the mucosal barrier and virus phenotype have been shown important factors in the efficiency of sexual and maternal-fetal HIV-1 transmission[136].

DEVELOPMENT OF A VACCINE AGAINST HIV INFECTION

The development of a safe and effective vaccine against HIV infections is complicated and flawed by several factors related to the nature of this virus including its ability to establish a persistent infection, to develop rapid and extensive variation in biological and antigenic properties, to infect cells of the immune system, and to be transmitted via multiple routes. The efficient induction of broadly neutralizing antibodies is generally considered an important prerequisite of a candidate HIV vaccine, since pre-existing antibodies may prevent transmission of cell-free virus or limit initial dissemination once the virus has been transmitted. The induction of a potent helper T cell response may be important for the generation of protective and long lasting antibody- and CTL responses. The latter may be of particular importance in clearance of virus infected cells, once the infection has been established.

To date, virtually all classical and experimental approaches for vaccine development have been or are being evaluated for their potential to induce protective immunity in macaque models of HIV-1 infection (for review see Chapter 5). Among these are the use of whole virus-, live attenuated virus-, live vector-, subunit- and nucleic acid based vaccine strategies. Although some studies indicate that the presence of either VN antibodies[47,96,137,139] or CTL[131,140,142] correlate with protection, it is clear that the reliable induction of protective immunity should probably not be dependent on a single protective mechanism. Furthermore, the observation that vaccine-induced antibody-mediated enhancement may occur in SIV, FIV, EIAV and CAEV infections, indicates that the induction of biologically active antibodies is not always beneficial[107,108,143,146]. So far little is known about the mechanisms underlying these enhancement phenomena or their *in vivo* relevance[106]. Clearly, the many failures that have been encountered on the way towards a safe and effective HIV vaccine indicate that its development will require more profound insights in the mechanisms of protective immunity against lentivirus infections.

OUTLINE OF THIS THESIS

The studies described in this thesis use SIV_{mac} or SHIV infection of cynomolgus macaques as a basis to evaluate different approaches of HIV-1 vaccine development, and more generally to study the pathogenesis of primate lentivirus infections. These viruses spread via cell-free and cell-associated routes. VN antibodies may prevent the transmission of cell-free virus, while cellular immune responses, in particular CTL, may eliminate virus infected cells. The envelope glycoprotein is a major target of both VN

antibodies and virus specific CTL. In Chapter 2, the antigenicity and immunogenicity of recombinant vaccinia virus produced envelope glycoproteins of SIV_{mac32H} with different *in vivo* passage histories is described with the intention to show their suitability as potential vaccine components (section 2.1). Since a vaccine based on recombinant proteins should be free of residual vector infectivity, the effect of paraformaldehyde and binary ethylenimine treatment used to inactivate recombinant vaccinia viruses, as well as their influence on the antigenicity and immunogenicity of the recombinant proteins they express, is documented in section 2.2. The potential of SIV-Env and HIV-1- Env proteins incorporated into iscoms to induce protection in cynomolgus macaques against SIV_{mac} or SHIV challenge, respectively, is described in Chapter 3. Furthermore, the additional protective effect of adding p27-Gag iscoms and Nef lipopeptides to SIV-Env iscoms is evaluated in cynomolgus monkeys. In Chapter 4, the role of CTL immunity in the control of SIV_{mac} infection in macaques is studied in SIV protein- or sham vaccinated macaques. For this purpose, different methods of CTL analysis were developed and compared for their sensitivity and reproducibility (section 4.1). Using these tools, the evolution of SIV protein- and epitope-specific CTL responses and their relationship with the kinetics of virus replication in SIV_{mac} infected cynomolgus monkeys is studied, as reported in sections 4.2 and 4.3.

In the summarizing discussion presented in Chapter 5, the results of the experiments described in this thesis are evaluated in relation to the overall progress made in the evaluation of current lentivirus vaccine designs in macaque models of HIV-1 infection.

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Chapter 2

CHARACTERIZATION OF CANDIDATE SIV VACCINES

ANTIGENICITY AND IMMUNOGENICITY OF RECOMBINANT ENVELOPE GLYCOPROTEINS OF SIVmac32H WITH DIFFERENT *IN VIVO* PASSAGE HISTORIES

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ABSTRACT

Shortly after infection of two rhesus monkeys (*Macaca mulatta*) either with a SIVmac32H challenge stock or with the same virus that had been passaged in another rhesus monkey for eleven months, SIV-envelope genes were cloned from their peripheral blood mononuclear cells and subsequently expressed by recombinant vaccinia viruses. The molecular weights and antigenicities of the thus produced envelope glycoproteins were largely identical to those of the native SIV. The envelope glycoprotein derived from the *in vivo* passaged virus proved to be poorly recognized by virus neutralizing monoclonal antibodies directed against one of the seven antigenic sites for which monoclonal antibodies were available. Immunization studies in rats showed that this protein was also less efficient in inducing antibodies against this antigenic site, and that it induced significantly lower levels of virus neutralizing antibodies than the other SIV-envelope glycoprotein. The immunogenicity of the SIV-envelope glycoprotein incorporated into immune stimulating complexes (iscoms) was compared to that of the same protein presented with Quil A or MDP-tsl.

INTRODUCTION

Simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV) are closely related lentiviruses which show similarities both in their genomic organization and in the disease they cause in their respective hosts[1]. Therefore SIV infection of macaques is considered to be a suitable model for HIV infection in humans to study pathogenesis and development of passive and active immunization strategies.

The envelope gene encoded surface (SU) and transmembrane (TM) glycoproteins of lentiviruses play a crucial role in virus attachment and fusion to host cells, and serve as targets for virus neutralizing (VN) antibodies and helper and cytotoxic T cells (for review see[2]). Therefore, the inclusion of these envelope glycoproteins in candidate vaccine preparations in a form that stimulates both cellular and humoral immunity is desirable.

In several studies SIV candidate vaccines have been tested for their ability to induce protective immunity against challenge with SIV. Although in many of these studies induction of protective immunity by candidate SIV vaccines was reported, it was shown that in most cases an anti-cell rather than an anti-virus response was at the basis of this protection[3]. We have shown that after vaccination with envelope glycoprotein enriched whole SIVmac, four of the eight vaccinated animals were protected against an intravenous challenge with SIV infected peripheral blood mononuclear cells (PBMC) [4,5]. So far, in SIV experiments there has been only one report describing protection induced with a recombinant vaccine preparation[6]. In this study priming with live recombinant vaccinia virus expressing the envelope protein followed by boosting with recombinant envelope protein expressed by baculovirus, resulted in protection of rhesus macaques against a homologous challenge with a molecular clone of SIVmne. In the SIVmac model system, immunization with recombinant SIV preparations led to a reduced virus load[7,8].

Successful vaccines against lentivirus infections should at least provide protective immunity against homologous viruses and related variants. This may be achieved by the induction of immune responses directed to conserved regions of the envelope protein involved in VN, or the simultaneous induction of immune responses to variable regions on the envelope protein of different variant viruses. In the present study, we describe the analysis of the antigenicity and immunogenicity of the envelope protein of SIVmac32H and its counterpart which had undergone an *in vivo* passage of eleven months. For this purpose, recombinant envelope proteins were obtained from PBMC of two monkeys shortly after infection with the respective viruses, to select for envelope sequences which are related to viruses which actually establish infection.

MATERIALS AND METHODS

Virus

SIV-*env* sequences were analyzed from two rhesus monkeys (*Macaca mulatta*), #8672 and #8789. These animals belonged to control groups of animals in a previous SIV vaccination study[5]. Macaque #8789 had been inoculated intravenously with 10 MID_{50} of the 32H isolate of SIVmac251 (11/88 pool)[9,10]. The monkey died from AIDS-like symptoms 26 weeks post infection. Macaque #8672 was inoculated intravenously with 10 MID_{50} of SIV infected PBMC derived from rhesus macaque #1XC. PBMC of #1XC were isolated eleven months post infection with an intravenous dose of 50 MID_{50} of the 32H isolated of SIVmac251 (pool 11/88)[11]. Macaque #8672 died from AIDS-like symptoms 39 weeks post infection. PBMC were isolated from macaque #8789 and #8672 two and four weeks post infection, respectively, and used for sequence analysis. At these time points no SIVmac32H specific VN serum antibodies had developed in the respective monkeys[5].

DNA template preparation

PBMC of rhesus macaques #8789 and #8672 were resuspended in a buffer containing 50 mM KCl, 10 mM Tris-Cl (pH 8.4), 1.5 mM $MgCl_2$, 0.5% Tween-20 and 100 µg/ml proteinase K at a concentration of $1x10^7$ cells/ml and incubated for 16 h at 54°C, followed by 10 min at 95°C. The resulting cell lysate was used as source of template DNA in subsequent PCR amplifications.

PCR amplification

As the number of infected cells in PBMC of SIV infected macaques early in infection was expected to be low, a nested primer PCR was developed to obtain sufficient quantities of DNA for cloning. Primers for PCR were chosen which recognized conserved sequences flanking the SIVmac envelope gene sequence (Los Alamos AIDS database). Outer set of primers: 5'-GGCCAACCTGGGGGAGGAAATCC-3' (6398); 5'-GCACTGTAATAATCCCTTCCAGTCCCCCC-3' (9481). Inner set of primers: 5'-*CGC*GTCGACGTAAGTATGGATGTCTTGGGAATCAGC-3' (6599); 5'-*GAC*CCCGGGCCCCTGATTGTATTTCTGTCCC-3' (9264). The positions of the 5'-nucleotide in the SIVmac32H-J5 molecular clone are indicated in parentheses. SmaI en SalI restriction sites (underlined above) were incorporated near the 5' ends of the inner primer set to facilitate subsequent cloning. These sites were chosen from a list of 'non-cutters' generated after analysis of the SIVmac251 sequence. Ten µl of the cell lysate was added to a PCR reaction mixture containing 10 mM Tris-Cl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µg/ml gelatin, 200 µM of each dATP, dCTP, dGTP and dTTP, 0.1 µM of each primer of one primer set and 2.5 units Taq polymerase (AmpliTaq, Cetus, Emeryville, CA). The total reaction volume of 100 µl was overlaid with mineral oil. DNA

was amplified by a 4 min 94°C denaturation step followed by 36 cycles comprising denaturation at 94°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 2 min. Reamplification of 10 µl of the first PCR was performed with the inner set of primers under the same reaction conditions for 40 cycles.

Molecular cloning and sequence analysis

PCR reaction mixtures were extracted once with phenol/chloroform and concentrated on an Amicon-30 filter (Amicon). DNA was subsequently analyzed on a 0.9% agarose gel, the presumed 2.6 kb envelope fragment was purified and digested with SalI and SmaI and cloned into SalI and SmaI digested cloning vector pBluescriptII SK(+) (Stratagene, La Jolla, CA). Sequencing was carried out using a multiwell microtitre plate DNA sequencing system (Amersham) based on the dideoxynucleotide chain termination sequence reaction. The nucleotide and protein alignments were determined with Lasergene software (DNAstar Inc., London, United Kingdom).

Nucleotide sequence accession numbers

Nucleotide sequences of clone 8672-20 and 8789-11 have been submitted to GenBank and are available under accession numbers L35596 and L35597, respectively.

***In vitro* mutagenesis**

In order to mutate the presumed primary and secondary cleavage site of the precursor envelope protein[12], amino acid substitutions were made by site-directed mutagenesis (primary cleavage site: RNKR to RNSA; secondary cleavage site: KR to KS) using three PCR reactions. The pBS+ constructs containing the respective envelope genes were used as template DNA. Primers 5'-mut (5'-GTGAAGAGcTACAC-TACTGGTGGCACCTCAAGAAATAgcgcAGGGGTCTTTGTGC (8135)) and 3'-NcoI (5'-GCATTTGGCCATGGTACAGTAGTGTGGC-3' (8478)) were used to obtain fragment 5'-mut/3'-NcoI. Using primer pair 3'-mut (5'-GCACAAAGACCCCTgcgcTATTTCTTGAGGT GCCACCAGTAGTGTAgCTCTTCAC-3', reverse complementary to 5'-mut) and 5'-ClaI (5'-GGCAGAACTATCGATTGGAATTGGG-3'(8059)) fragment 5'-ClaI/3'-mut was obtained. The positions of the 5'-nucleotide in the SIVmac32H (J5) sequence are indicated in parentheses. The lower case letters represent nucleotide changes that were introduced. Subsequently, the 5'-ClaI/3'-mut and 5'-mut/3'-NcoI fragments were used as template in a PCR using primers 5'-ClaI and 3'-NcoI. The PCR derived 5'-ClaI/3'-NcoI fragment including the nucleotide substitutions was purified by preparative gel electrophoresis, digested with ClaI and NcoI and cloned into ClaI and NcoI digested clones 8672-20 and 8789-11, respectively. The presence of the substitutions was confirmed by sequence analysis. The mutated envelope proteins were designated 8672-m and 8789-m, respectively.

Construction of recombinant vaccinia viruses

Both the wild type and the mutated 8672-20 and 8789-11 envelope genes were cloned under control of a synthetic early/late vaccinia promoter in the plasmid vector pSC65. Recombinant viruses were made by homologous recombination with vaccinia virus (WR strain) as previously described[13]. A control recombinant vaccinia virus, vSC65, was made by homologous recombination of the plasmid vector pSC65 with no insert[14]. Recombinant vaccinia viruses were plaque purified at least three times and virus stocks were grown on RK13 cells.

Production of recombinant *env* glycoproteins

Baby Hamster Kidney (BHK) cells were infected with recombinant vaccinia virus (rVV) at a multiplicity of infection of 4 and incubated for 20 h. Cells were collected and the vaccinia virus was inactivated in 1.5% paraformaldehyde for 30 min. Subsequently, cells were incubated in 0.2 M glycine for 1 h. Membrane proteins were solubilized in TN (10 mM Tris-HCl pH 7.4, 0.15 M NaCl) containing 4% RBT (n-octylpolyoxyethylene, Rosenbuch-Tenside; Bachem, Bubendorf, Switzerland) at a concentration of $2x10^7$cells/ml for 90 min at 4°C. The cell lysate was centrifugated for 15 min at 2500 rpm. Glycoproteins were allowed to adsorb to lentil lectin sepharose (Pharmacia LKB, Uppsala, Sweden) overnight. Subsequently, the lentil lectin sepharose was washed with buffer A (10 mM Tris-HCl pH 7.4/0.3 M NaCl/0.5% RBT) followed by washing with buffer B (buffer A supplemented with 10 µg/ml of the lipids cholesterol and phosphatidylcholine (Sigma, St. Louis, USA)). Bound glycoproteins were eluted with buffer C (buffer B containing 0.5 M α-methylmannopyranoside). Quantification of SIV-envelope glycoproteins was performed as described previously[5].

Preparation of antigen preparations and immunization schedule in rats

For the preparation of immune stimulating complexes (iscoms) lentil lectin enriched 8672-m or 8789-m proteins were mixed with the lipids cholesterol and phosphatidylcholine (Sigma; stock 10 mg/ml in 10% MEGA-10 (Boehringer Mannheim, Mannheim, Germany)) and Quil A (Spikoside, Iscotec, Luleå, Sweden) at a ratio of 1:1:5 (w/w/w). After ultrasonic treatment for 10 min the mixture was incubated for 1 h at room temperature (RT). Subsequently, the mixture was extensively dialysed against 10 mM Tris-HCl pH 7.4/0.3 M NaCl, layered over a linear (10-60%) sucrose gradient and centrifugated (18 h at 25,000 rpm: Beckmann SW28 rotor). The gradient was fractionated and fractions were analyzed for the presence of envelope protein by enzyme-linked immunosorbent assay (ELISA). Fractions containing peak levels of the envelope protein (coinciding with the presence of iscom-like structures, as judged by electron microscopy) were pooled.

For the Quil A and MDP-tsl antigen preparations, 3 µg of lentil lectin enriched SIV-*env* 8789-m was adjuvanted either with 10 µg Quil A (Spikoside, Iscotec) or 0.5 ml MDP-tsl (0.462 ml PBS containing 12.5 µg MDP (muramyl dipeptide) (Sigma), 1 µl Tween-80, 25 µl squalene and 12.5 µl of the plutonic block polymer L101 (Serva, Heidelberg, Germany))[15,16].

Female rats (RIV:tox) were divided into groups of four animals and were immunized intramuscularly (i.m.) with the different antigen preparations and boosted 4 weeks later with the same material. Each rat received 3 µg of rgp160 per immunization except for the rats immunized with both 8672-m and 8789-m incorporated into iscoms. These received 3 µg of each rgp160 construct per immunization. Serum samples were collected before and 2, 4, 5, 6, 8, and 14 weeks after the first immunization.

Western blotting

Procedures for western blotting were performed as described previously[5].

Binding of monoclonal antibodies (Moabs) to different SIV-envelope preparations

Multiwell plates (Costar, Cambridge, USA) were coated with 100 µl PBS containing 200 µg ConA and then incubated with the different SIV-envelope preparations at a concentration of 10 ng envelope protein in 100 µl PBS containing 1% RBT, for 16 h at RT. Wells were blocked with PBS containing 0.01% Tween-20 and 0.1% BSA (PTB) supplemented with 10% FCS, and subsequently incubated for 2 h at RT with 3-fold serial dilutions starting at 1:200 of one of the *env* specific Moabs (ascitic fluid, kindly provided by Dr. K. Kent). Plates were washed and incubated with biotin conjugated goat-anti-mouse IgG (Amersham) and horse radish peroxidase (HRPO) conjugated streptavidine (Amersham). The substrate reaction was carried out with 3,3',5,5'-tetra-methyl-benzidine[17]. The antibody titre was defined as the reciprocal of the dilution of the Moab giving 50% of the maximum OD_{450} obtained for that Moab and was determined from three independent experiments. Values two times that of similarly processed preparations of vSC65 infected BHK cells were considered positive.

Whole SIV ELISA to demonstrate envelope specific antibodies

ConA coated multiwell plates were incubated with 50 µl of SIV infected C8166 cell lysate[5] in PBS/1% triton containing 70 ng/ml SIV-*env*, for 16 h at RT. (Quantification of the SIV-envelope protein was performed as described previously[5]). Wells were blocked with PTB containing 10% FCS and subsequently incubated with 50 µl of 2-fold dilutions of rat serum in PTB containing 4% FCS. After 2 h incubation at RT, wells were washed and bound antibody was detected using rabbit-anti-rat Ig-HRPO antibody preparation (DAKO, Glostrup, Denmark). The substrate reaction was carried out as described above. Endpoint titres were calculated using a cut-off value twofold above the respective dilution of the pre-immune serum at OD_{450}. Antibodies with specificity for BHK cell components in the immunogen did not react with plate bound C8166 cell components. Comparison of titres between groups was conducted using a one way analysis of variance (Anova; Minitab Inc., State college, USA). Titres were considered significantly different if $p<0.05$.

SIV-envelope specific Moab inhibition ELISAs

Inhibition ELISAs were carried out as described previously[5] with minor

modifications. Briefly, ConA coated multiwell plates were incubated with 100 µl C8166-SIV cell lysate containing 70 ng/ml SIV-envelope proteins. Hundred µl of twofold dilutions of rat serum were incubated for 1 h at RT. Subsequently, 50 µl was taken out and wells were supplemented with 50 µl of Moab in PTB containing 4% FCS. Only Moabs with VN activity were used for these inhibition studies. It has been shown that Moabs KK5 and KK9 of competition group 1, which recognize a conformation dependent epitope, do not interfere with the binding of the SIV envelope protein to sCD4 as demonstrated in immunoprecipitation assays[18,31]. The Moabs were diluted to give an absorbance at OD_{450} of 50-70% of the maximum absorbance in an indirect SIV-ELISA. Plates were incubated with a biotin-conjugated goat-anti-mouse IgG antibody preparation (Amersham) in PTB containing 4% FCS and 5% Normal Rat Serum (NRS), which was pre-incubated in this buffer for 1 h at RT, and subsequently with HRPO conjugated streptavidine (Amersham). The anti-mouse biotin IgG antibody preparation did not cross-react with rat Ig in this assay. Inhibition titres were defined as the dilution of rat serum inhibiting 50% of the absorbance measured without competing antibody. The difference in titer between the different immunization groups was evaluated through a student's t-test. Differences of $p<0.05$ were considered significant.

SIV neutralization assay

The SIV neutralization assay using SIVmac32H was performed as described previously[5]. Briefly, 10 µl of different dilutions of heat inactivated sera were incubated (four replicates per dilution) with 10 µl of SIVmac32H diluted to give 10 infectious particles per 10 µl. After 30 min incubation at 37°C, 200 µl of media containing 2000 C8166 cells was added to each well of the microtiter plate. Plates were incubated for seven days at 37°C after which 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 using a SIV specific immunoperoxidase assay. Wells containing no infected cells were scored as negative and the number of negative wells was used to determine the neutralizing dose 50% end point (ND50) for that serum by the method of Spearmann-Karber. The difference in titer between the different immunization groups was evaluated through a student's t-test. Differences of $p<0.05$ were considered significant.

RESULTS

Selection of SIV-envelope glycoproteins and sequence comparison

PBMC were collected from two rhesus macaques, two weeks (monkey #8789) or four weeks (monkey #8672) after infection with SIVmac32H. The first animal had been

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          1                                                                                                  90

consensus MGCLGNQLLIAILLLSVYGI YCTQYVTVFYGVPAWRNATI PLFCATKNRDTWGTTQCLPD NGDYSELALNVTESFDAWEN TVTEQAIEDV
8789-11   -------------------- -------------------- -------------------- -------------------- ----------
8672-20   -------------------- -------------------- -------------------- ------------------n- ----------
          91                                  V1                                                180
consensus WQLFETSIKPCVKLSPLCIT MRCNKSETDKWGLTKSLTTT APT...APTAASKIDMVNET SSCITHDNCTGLEQEQMIGC KFNMTGLKRD
8789-11   -------------------- -------------------- ---...-------------- -------------------- ----------
8672-20   --------r----------- ------------------s--- -s-tttt--t---------- ------------------s- ----------
          181       V2                                                                      270
Consensus KTKEYNETWYSTDLVCEQGN STDNESRCYMNHCNTSVIQE SCDKHYWDTIRFRYCAPPGY ALLRCNDTNYSGFMPKCSKV VVSSCTRMME
8789-11   -------------------- -------------------- -------------------- -------------------- ----------
8672-20   -k------------------ -------------------- -------------------- -------------------- ----------
          271                                                 V3                                  360
Consensus TQTSTWFGFNGTRAENRTYI YWHGRDNRTIISLNKYYNLT MKCRRPGNKTVLPVTIMSGL VFHSQPINDRPKQAWCWFGG KWKDAIKEVK
8789-11   -------------------- -------------------- -------------------- -------------------- ----------
8672-20   -------------------- -------------s------ -------------------- -------------------- e---------
          361                                                 V4                                  450
Consensus QTIVKHPRYTGTNNTDKINL TAPGGGDPEVTFMWTNCRGE FLYCKMNWFLNWVEDRNTTN QKPKEQHKRNYVPCHIRQII NTWHKVGKNV
8789-11   -------------------- -------------------- -------------------- -------------------- ----------
8672-20   -------------------- -------------------- -------------------- ---n---------------- ----------
          451          V5                                                      SU|TM               540
Consensus YLPPREGDLTCNSTVTSLIA NIDWTDGNQTNITMSAEVAE LYRLELGDYKLVEITPIGLA PTDVKRYTTGGTSRNKRGVF VLGFLGFLAT
8789-11   -------------------- -------------------- -------------------- -------------------- ----------
8672-20   -------------------- -------------------- -------------------- --n----------------- ----------
          541                                                                                   630
Consensus AGSAMGAASLTLTAQSRTLL AGIVQQQQQLLDVVKRQQEL LRLTVWGTKNLQTRVTAIEK YLKDQAQLNAWGCAFRQVCH TTVPWPNASL
8789-11   -------------------- -------------------- -------------------- -------------------- ----------
8672-20   -------------------- -------------------- -------------------- -------------------- ----------
          631                                                                                   720
Consensus TPNWNNDTWQEWERKVDFLE ENITALLEEAQIQQEKNMYE LQKLNSWDVFGNWFDLASWI KYIQYGIYIVVGVILLRIV IYIVQMLAKLR
8789-11   -------------------- -----p-------------- -------------------- --------v---------- ----------
8672-20   --d----------------- -------------------- -------------------- --------m---------- ----------
          721                                                                                   810
consensus QGYRPVFSSPPSYFQQTHIQ QDPALPTREGKEGDGGRSGG NSSWPWQIEYIHFLIRQLIR LLTWLFSNCRTLLSRAYQIL QPILQRLSAA
8789-11   -------------------- ----------------g-- -------------------- -------------------- ----------
8672-20   -------------------- ----------------g-- -------------------- -------------------- ---f-----t
          811                                                       881
consensus LQRIREVLRTELTYLQYGWS YFQEAVQVGWRSATETLAGA WGDLWETLRRGGRWILAIPR RIRQGLELTLL
8789-11   -------------------- -------------------- -------------------- -----------
8672-20   -------------------- -------------------- -------------------- -----------
```

Figure 1. Predicted amino acid sequences of the envelope precursor protein of clones 8672-20 and 8789-11. They are aligned with the consensus seequence for the envelope glycoprotien present in the 32H-isolate of SIVmac251 (11/88 pool) as identified by Almond *et al.*[17]. A dash indicates that the amino acid is identical to the consensus sequence; variation in seqeuence is given in single letter code. Amino acid positions where no equivalent is present are indicated by a dot. The regions of the envelope proteins equivalent to the variable regions (V) of HIV-1 are indicated[34]. The predicted cleavage site between the SU and TM part of the envelope protein is indicated. Potential N-linked glycosylation sites (N-X-S or N-X-T) are indicated in bold type.

infected with 10 MID_{50} of the cell free virus (11/88 pool) and the second with 10 MID_{50} of the PBMC from a monkey 11 months after infection with the 11/88 pool[5]. Representative SIV envelope genes were derived from the respective monkeys. SIV envelope clone

8789-11 was selected with a sequence shared by five out of six clones in the variable regions 1 and 2 (V1 and V2)[19] and clone 8672-20 was selected from a panel of three clones which showed considerable similarities in this region with the exception of single amino acid differences. The predicted amino acid sequences of clones 8672-20 and 8789-11 are outlined in figure 1, where they are compared with that of the consensus sequence of the envelope glycoprotein present in the 11/88 stock of the 32H isolate of SIVmac251 as identified by Almond *et al.*[19]. As expected the 8789-11 sequence showed much more similarity to the consensus sequence of the envelope glycoprotein present in the 32H isolate than the 8672-20 sequence (3 and 19 amino acid differences, respectively). A 98% overall amino acid sequence identity was shown between clones 8672-20 and 8789-11. Most strikingly, in the V1 region of the 8672-20 envelope sequence an extended sequence was found that was rich in serine and threonine residues, leading to a sequence commonly found in heavily O-linked glycosylated proteins[20]. An extra potential N-linked glycosylation site was found in clone 8672-20 directly N-terminal of the start of the V1 region. In addition, two amino acid difference were found in the V2 region, one in the V4 region, four amino acid differences outside the variable regions of the SU and five in the TM part of the SIV-envelope precursor sequence (Figure 1).

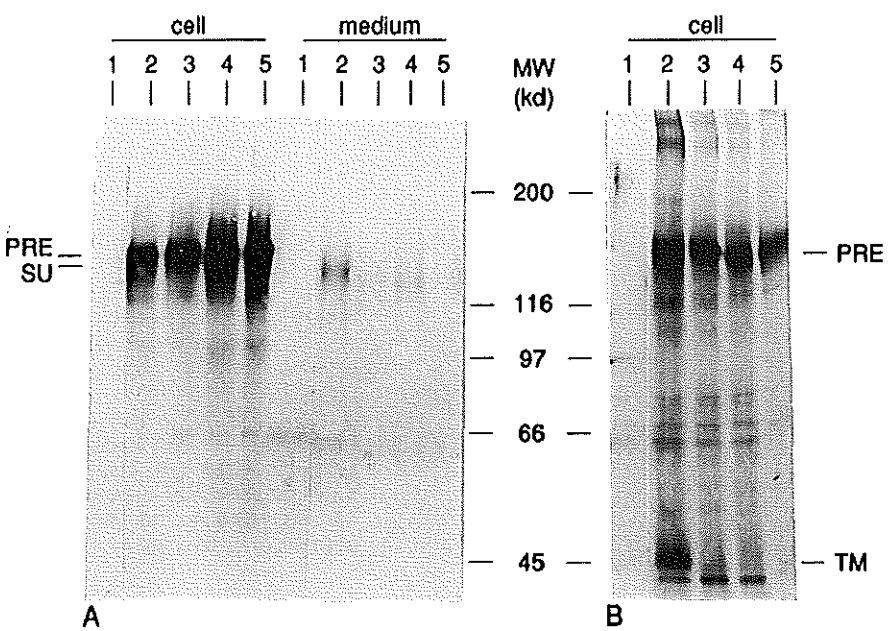

Figure 2. Western blot analysis of wild-type and mutant SIV envelope glycoproteins expressed by rVV in BHK cells using a gp120-specific monoclonal antiobdy (KK10) (A) or a gp41 specific monoclonal antibody (KK20) (B) with cell lysate (cell) and culture medium (medium). Lane 1, vSC65 (negative control); lane 2, v8672; lane 3, v8672-m; lane 4, v8789; lane 5, v8789-m. The locations of the envelope precursor proteins (pre), surface membrane protein (su) and transmembrane protein (tm), as well as the mobilities of molecular wight markers, are indicated.

Expression of SIV-envelope glycoproteins by rVVs

The RNKR sequence in clones 8672-20 and 8789-11, representing the cleavage site between the SU and TM part of the envelope genes, together with a second conserved cluster of basic amino acids which may serve as a secondary cleavage site[12], was modified by site directed mutagenesis. Subsequently, rVVs were generated from the parental 8672-20 and 8789-11 envelope genes (designated v8672 and v8789, respectively) and from the cleavage site mutated envelope genes (designated v8672-m and v8789-m, respectively).

Western blot analysis of BHK cells infected with v8672 using a TM specific Moab, revealed proteins with molecular sizes of 160 kD and 41 kD (Figure 2b, lane 2). The 160 kD protein was also recognized by the SU membrane specific Moab (Figure 2a, lane 2). In the culture medium a protein with a Mr of about 120 kD was recognized by the gp120 specific Moab (Figure 2a). These data indicate that, like in the native protein, a 160 kD SIV precursor protein is synthesized which is processed to a 41 kD TM protein and a 120 kD SU protein which is also released into the medium. In v8789 infected BHK cells the 160 kD envelope precursor protein was predominant. In the culture medium the SU envelope glycoprotein was detectable. In the culture medium of cells infected with v8672-m and v8789-m containing the mutated envelope genes, no indication for the presence of the SU envelope glycoprotein was found.

Incorporation studies with lentil lectin purified extracts from BHK cells infected with either of the rVVs, showed that iscoms prepared with the cleavage site mutated recombinant envelope proteins contained the gp160 precursor protein (Figure 3).

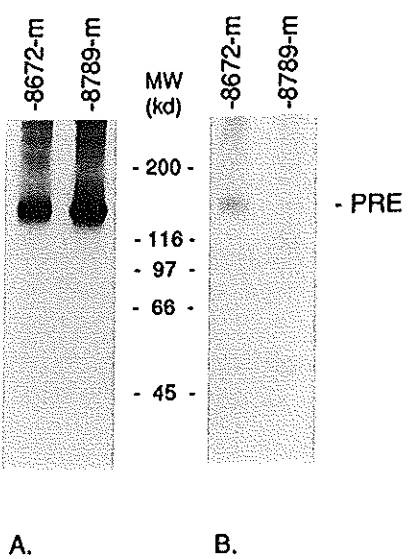

Figure 3. Western blot analysis of iscoms prepared with lentil-lectin purified proteins derived from v8672-m and v8789-m infected BHK cells using a gp120 specific monoclonal antibody (KK13) (A) or a gp41 specific monoclonal antibody (KK20) (B). The location of the envelope precursor protein (pre) as well as the mobilities of molecular weight markers are indicated.

Antigenicity of recombinant envelope glycoproteins 8672, 8672-m, 8789, 8789-m and envelope glycoprotein derived from SIVmac32H

Antigenicity of the envelope proteins expressed by v8672, v8672-m, v8789 and v8789-m incorporated into iscoms was assessed with a panel of 15 SIV-envelope specific Moabs divided over 7 competition groups[21,22] (see Figure 4). The antibody titre at which 50% of the maximum OD_{450} was obtained, was similar for all Moabs tested comparing the envelope glycoproteins derived from SIVmac32H infected C8166 cells and rVV 8789 expressed envelope glycoprotein . Envelope glycoproteins derived from v8672 were poorly recognized by Moabs of competition group 1 compared to envelope glycoproteins derived from v8789 or SIV itself. Interestingly, all Moabs of this competition group recognize a conformational epitope and have been shown to possess VN activity[21,22].

No differences were found in the binding of the Moabs to the 8672 and 8789 envelope proteins in their native or cleavage site mutated form, except for one of the Moabs of competition group 7, KK16, which hardly recognized the 8789-m envelope protein.

No changes in binding of the Moabs to the recombinant envelope glycoproteins were found as a consequence of either paraformaldehyde fixation, lentil-lectin purification, or incorporation into iscoms (data not shown).

Immunogenicity of recombinant envelope glycoproteins 8672-m and 8789-m

The immunogenicity of the 8672-m and 8789-m envelope glycoproteins incorporated into iscoms was analyzed by immunizing laboratory rats. The overall kinetics and levels of SIV specific antibody titres induced by immunization with two times 3 µg of one of the envelope glycoproteins proved to be similar when measured by a whole SIV ELISA. Rats immunized with both iscom preparations (3 µg envelope protein of each per immunization) showed a significantly higher antibody response (Figure 5a). Inhibition of the Moab KK56 could only be clearly demonstrated with sera from rats immunized with 8789-m envelope glycoproteins (Figure 5b). This inhibition pattern of KK56 was representative of all Moabs of competition group 1 (data not shown). Apparently there is a direct relationship between the recognition or presence of certain epitopes and their capacity to induce specific antibodies. VN serum antibody titres of 8789-m iscom immunized rats were also significantly higher than those of 8672-m iscom immunized animals (Figure 5c). Similar levels of VN serum antibodies and KK56 inhibition titre were found in rats immunized with the 8789-m envelope proteins alone or in combination with the 8672-m envelope protein (Figure 5b and 5c).

DISCUSSION

In the present study, we have shown that the antigenicity and immunogenicity of two

envelope glycoproteins derived from the same parent virus, SIVmac32H, with different in vivo passage histories differed considerably: The envelope protein derived from PBMC of a monkey infected with a virus which had undergone an extra *in vivo* passage for eleven months was not recognized by a group of VN antibodies able to bind to the envelope protein of the parent virus, SIVmac32H. Upon immunization, this envelope protein failed to induce antibodies which could compete with these monoclonal antibodies for binding to the envelope protein of SIVmac32H. Concurrrently, a lower total VN antibody titre against SIVmac32H was induced in comparison to that induced by the envelope protein derived directly from a SIVmac32H infected monkey.

There are several indications that conformational epitopes are involved in eliciting SIV neutralizing antibodies. SIV synthetic peptides proved to be poor inducers of VN antibodies, whereas immunization of macaques with recombinant envelope protein gp110 elicited strong VN anitbodies[23]. Furthermore, VN activity in sera from infected macaques could be absorbed out with native gp110, but not with reduced or denatured gp110[23]. Therefore, we chose the eukaryotic rVV expression system for the production of SIV-envelope glycoproteins to be incorporated in SIV candidate vaccines. Vaccinia virus infectivity was eliminated by paraformaldehyde treatment. Although paraformaldehyde is able to alter the antigenic structure of membrane proteins[24], no differences in antigenicity were found in the SIV-envelope protein before and after paraformaldehyde fixation (Hulskotte *et al.*, manuscript in preparation). The SIV 160 kD envelope precursor proteins, expressed by rVV v8672 and v8789, were similarly cleaved to a 120 kD SU and 41 kD TM protein as in SIV infected cells, although cleavage of especially 8789-*env* proved to be less efficient. No differences in antibody reactivities of a panel of 15 Moabs with envelope glycoproteins derived from SIVmac32H infected C8166 cells and v8789 expressed envelope glycoprotein were found indicating that the rVV expression system is able to produce SIV-envelope glycoproteins in their native conformation.

Comparison of the amino acid sequences of 8672-20 and 8789-11 envelope precursor proteins revealed a 98 % amino acid similarity. It is interesting to note that in the V1 region of the 8672-20 sequence - derived from the *in vivo* passaged SIV - a T-rich stretch was inserted compared to the 8789-11 sequence. In SIVmne such a T-rich stretch was observed to be more common late in infection[25,26]. Sequential sequence analysis of the envelope genes derived from these monkeys provided strong evidence that such T-rich stretches emerge later in infection by active selection, indicating expression *in vivo*[26]. The presence of this T-rich sequence in the envelope protein derived from PBMC of a monkey four weeks post infection with an *in vivo* passaged virus indicates that sequence variation has most likely occured during those 11 months of *in vivo* passage of SIVmac32H in monkey 1XC. In addition, at four weeks post infection a homogenous population of envelope sequences has been demonstrated in both HIV and SIV infection[27,28,29,30].

The 8672 envelope glycoprotein was poorly recognized by all VN Moabs directed to a conformation dependent epitope on the surface protein (KK5, KK9, KK56). A recent

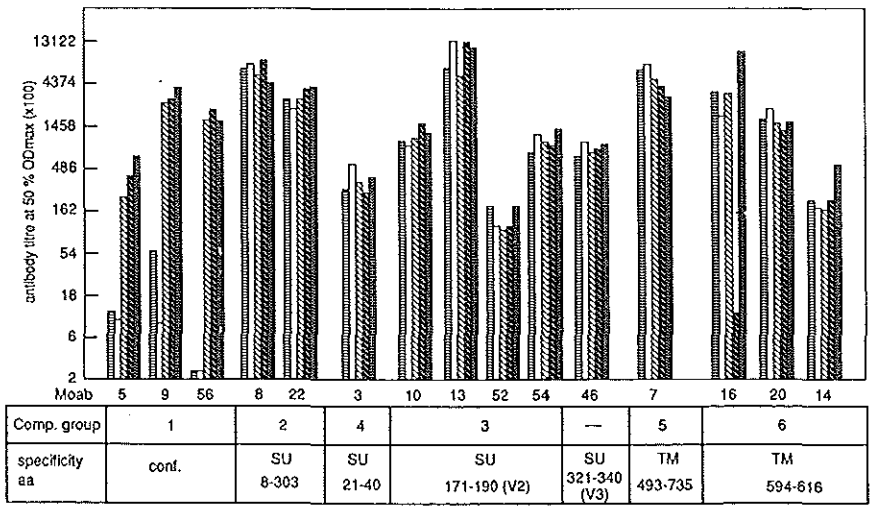

Figure 4. Binding of 15 SIV-envelope-specific mnoclonal antibodies to the 8672, 8672-m, 8789 and 8789-m envelope glycoprotiens incorporated into iscoms and SIVmac251(32H) produced envelope proteins on C8166 cells[5]. Competitions groups are defined in Kent et al. [19, 20]Aa; amino acid numbers BK28 SIV-envelope protein. Conf.; conformational epitope. Bars represent the dilution of the monoclonal antibody giving 50% of the maximum OD450 obtained using that monoclonal antiobdy.▯= 8672,▯= 8672-m,▯=8789,▯ =8789-m,▯= C8166-SIV

study suggests that the V4 region is involved in the recognition of the SIVmac239 envelope protein by Moabs KK5 and KK9, as demonstrated by an amino acid substitution at position 419 (equivalent position in SIVmac32H sequence, Figure 1), or deletions of four amino acids (position 421-424 and position 423-427) in this region[31]. We also found a difference in the amino acid sequence between the 8789 and 8672 envelope proteins in the same region in which the deletions were made[31] (K to N at position 423). However a possible role for the other amino acid differences found between clone 8672 and 8789 in the recognition by the respective Moabs can not be excluded and may be implicated in an escape mechanism from neutralization by antibodies which had developed in the macaque which donated the challenge material for monkey #8672.

No difference between the two envelope proteins was observed in binding of the other Moabs, including the VN antibodies KK10 and KK54[21,22]. However, in contrast to the VN antibodies of competition group 1, binding of the VN Moabs KK10 and KK54 could neither be inhibited by the respective rat sera, nor by sera from monkeys immunized with the envelope glycoproteins 8672-m and 8789-m incorporated into iscoms, nor by sera from SIVmac infected monkeys (data not shown). However, it has been shown by others that sera from some SIVmac infected macaques[32,33] and some macaques immunized with virus derived gp130 in incomplete Freund's adjuvant with or without prior priming with live rVV expressing the SIVmacBK28 envelope protein, react with the peptide epitopes of these Moabs[33].

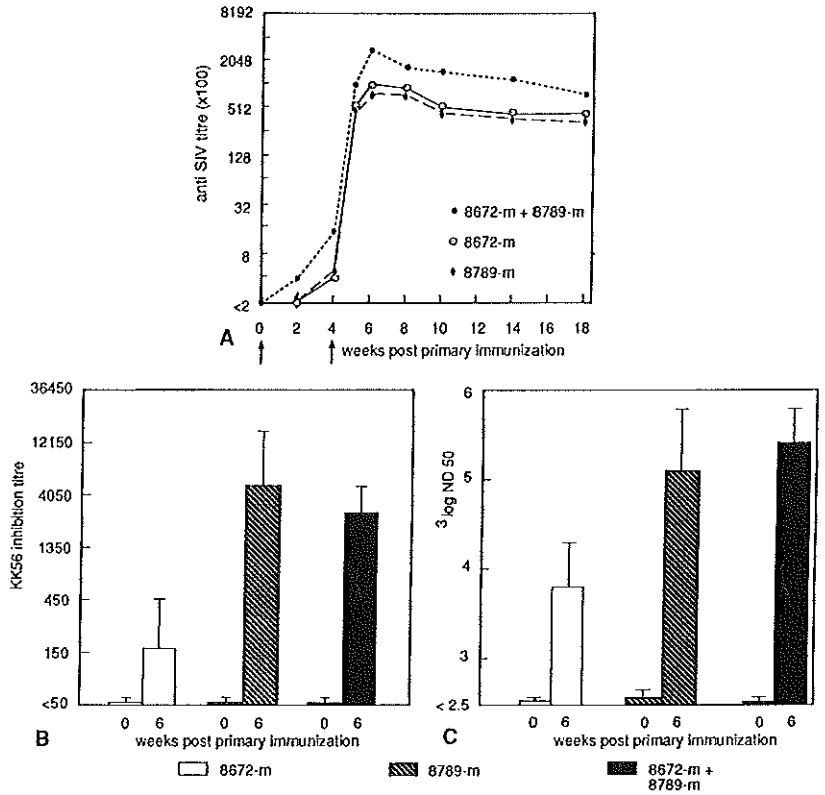

Figure 5. SIV envelope antibody development in rats immunized with 8672-m iscoms (3 µg per immunization), 8789-m iscoms (3 µg per immunization) or both (3 µg each per immunization). Mean titres were calculated from four rats immunized with the same envelope preparation. A. Anti-SIV antibody titre. Weeks of immunization are indicated by arrows. B. Response to a conformational epitope before immunization and at peak level of antibody response (week 6 post primary immunization) as measured by inhibition of binding of monoclonal antibody KK56. The standard error of the means are presented as a vertical line. C. SIV-VN antibody tires before immunization and at peak level of antibody response (week 6 post primary immunization). Serial dilutions of monkey plasma were incubated with SIVmac32H for 30 min at 37°C. Subsequently, C8166 cells were added. The number of negative wells was used to determine the neutralizing 50% endpoint (ND_{50}) for the relative serum. The standard error of the means are presented as a vertical line.

To facilitate incorporation of the hydrophillic SU protein into iscoms, cleavage site mutated envelope proteins were expressed by rVVs. As mentioned above, the 8789 envelope glycoprotein was inefficiently cleaved exhibiting most of the envelope glycoprotein as the uncleaved precursor. However, we decided to also mutate this construct to obtain a uniform and well defined product. Indeed, the cleavage site mutated envelope glycoproteins, 8789-m and 8672-m, were efficiently incorporated into iscoms, which has also been demonstrated for the cleavage site mutated precursor envelope glycoprotein of FIV[14].

No differences in antibody reactivity of a panel of 15 Moabs were found between the

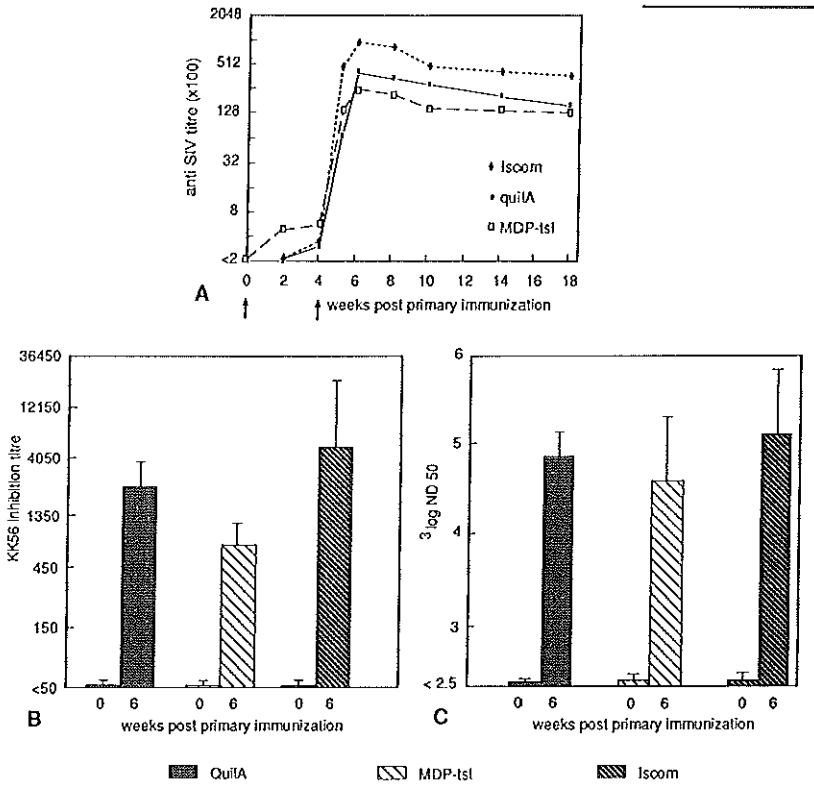

Figure 6. SIV-envelope antibody development in rats immunized with 8789-m envelope glycproteins using different adjuvant systems. Mean titres were caluclated from four rats immunized with the same adjuvant. Legends to Figure 6A, B and C are as for Figure 5A, B and C rescpecitively.

wild type and cleavage site mutated 8672 and 8789 envelope glycoproteins, except for the abrogated recognition of the 8789-m protein by Moab KK16. Sequence analysis showed that during PCR used for mutation of the cleavage site, a mutation had been introduced in the region where the reactivity of KK16 was mapped[21] (position 619, C to G mutation). This did not influence the recognition of all the other Moabs, including those that recognize a conformation dependent epitope, indicating that the overall conformational integrity of 8789-m had remained intact.

The capacity of different adjuvants to enhance the antibody response was compared using the 8789-m envelope glycoprotein. This protein was selected on basis of its reactivity with KK5, KK9 and KK56 and its ability to induce high VN titres (Figure 4 and 5). The highest titres of SIV specific antibodies were found in rats immunized with the 8789-m iscoms. This is in agreement with previous studies in which monkeys immunized with SIV proteins also showed a higher envelope antibody response with SIV-envelope proteins incorporated into iscoms than adjuvanted with MDP[5]. However, in rabbits the HIV-1 envelope protein adjuvanted with MDP-tsl was shown to induce a higher antibody response than HIV-1 envelope protein incorporated into the iscom matrix using a multiple immunization schedule[16]. This discrepancy may be due to the

quality of the respective preparations, the animal species or to differences in the immunization schedule used.

In conclusion, our results indicate that sequence changes occurring during *in vivo* passage, result in major changes both in antigenicity and immunogenicity of the envelope glycoproteins of SIV. The availability of envelope proteins with distinct variable regions may offer the possibility to study their protective value *in vitro* and *in vivo* against a wider range of virus variants, but may also offer the possibility of sequential immunization to prime and boost for an antibody response directed against conserved regions in the absence of a secondary immune response to variable regions.

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CHEMICAL INACTIVATION OF RECOMBINANT VACCINIA VIRUSES AND THE EFFECTS ON ANTIGENICITY AND IMMUNOGENICITY OF RECOMBINANT SIMIAN IMMUNODEFICIENCY VIRUS ENVELOPE GLYCOPROTEINS

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Vaccine, In press

ABSTRACT

The efficiency of paraformaldehyde (PFA) and binary ethylenimine (BEI) in inactivating recombinant vaccinia virus (rVV) present in baby hamster kidney cells expressing simian immunodeficiency virus envelope glycoproteins (SIV-Env), was measured in a series of inactivation studies. Both compounds were shown to be effective in reducing rVV titres. The use of standard 3-day titration assays proved to be inadequate to measure PFA inactivation, since upon prolonged incubation, residual rVV infectivity was detected in cultures negative at 3-days. Different procedures using PFA or BEI were selected to assess their influence on the antigenicity and immunogenicity of rVV expressed SIV-Env. Antigenicity, as defined by the ability to react with a panel of monoclonal antibodies recognizing major antigenic sites, and immunogenicity, as defined by the ability to induce SIV envelope specific and virus neutralizing serum antibodies in rats, proved to be preserved after either inactivation procedure. These data show that both protocols using PFA or BEI can be used successfully as part of the procedures to remove residual rVV infectivity.

INTRODUCTION

For the development of vaccines against human and animal lentiviruses, the use of viral subunits produced by recombinant DNA techniques is currently being evaluated. For this purpose expression of viral proteins by recombinant vaccinia viruses (rVVs) is attractive for several reasons (for review see[1]). One of the conditions for the use of antigens produced in this way, is the adequate removal of rVV infectivity from the final vaccine formulation. To this end several chemical and physical procedures are available which should, however, not affect the antigenicity and immunogenicity of the proteins expressed.

Inactivation by formaldehyde is one of the procedures most frequently used to inactivate viruses in viral vaccines, although infections resulting from residual virus infectivity in formaldehyde inactivated vaccine formulation have been reported[2]. Formaldehyde reacts with amino, imino, amido, sulphydryl and hydroxy groups and with peptide linkages within proteins. One of its principal advantages is that, as a result of cross-linking, the gross three-dimensional architecture of proteins is conserved. In fact, the oligomeric form of the envelope glycoprotein of human immunodeficiency virus (HIV) has been shown to be stabilized by formaldehyde treatment[3]. However, one drawback in the use of formaldehyde is the possible alteration or destruction of epitopes, which may result in an immunogen eliciting ineffective or even deleterious immune responses[4,5]. The antigenic structure of several membrane proteins has been shown to be best preserved by paraformaldehyde (PFA), the polymeric form of formaldehyde[6]. In addition, antigen presenting cell function has been shown to be maintained after PFA fixation[7-9].

Alternatively, one can hypothesize that compounds which inactivate viral infectivity without interaction with proteins would better preserve the antigenic structure of a protein. One of the most widely used compounds in this respect is binary ethylenimine (BEI), an aziridine compound reacting with nucleic acids, which has been shown to efficiently inactivate several RNA and DNA viruses (for review see[10]).

In the present paper we evaluate the effects of both PFA and BEI treatment on the infectivity of rVVs in relation to their influence on the antigenicity and immunogenicity of the recombinant SIV-Env which they express.

MATERIALS AND METHODS

Recombinant vaccinia viruses

The rVVs used in this study were: vSC65, a control rVV made by homologous

recombination of the pSC65 vector with vaccinia virus (WR strain)[11]; v8672-m and v8789-m, rVVs containing the cleavage site mutated 8672-20 and 8789-11 SIV envelope gene, respectively[12]. These SIV envelope genes were derived from SIVmac32H after different *in vivo* passages[12].

Inactivation experiments

VACCINIA VIRUS INFECTION. RVV was incubated in 0.25 mg/ml trypsin for 20 min at 37°C and vortexed regularly. Baby hamster kidney (BHK) cells were infected with the vaccinia virus preparation at a multiplicity of infection of 1. After 24 h of incubation in complete medium (CM: Dulbecco's Modified Eagles Medium, penicillin (100 IU/ml), streptomycin (100 G/ml), L-glutamine (2 mM), ß-mercaptoethanol (2 x 10^{-5} M)) supplemented with 5% FCS, cells were collected by centrifugation.

PFA INACTIVATION PROCEDURE. A PFA (Merck, Darmstadt, Germany) stock in PBS (pH 7.3) was freshly prepared before each experiment. To solubilize the PFA, 0.1% 4 N NaOH was added, followed by neutralization with an equal molar amount of 4 M HCl.

Twenty four h after vaccinia virus infection, BHK cells were harvested by centrifugation and resuspended in PBS at a concentration of 10^7/ml. A one ml sample was taken to quantify vaccinia virus in the starting material. The remaining cell preparation was mixed with an equal volume of an appropriate PFA solution giving a final concentration of either 0.3% or 1.5% or 7.5% (w/v) PFA. Samples were incubated at room temperature (RT) for various lengths of time as indicated. Thereafter, 10 (w/v)% 1 M glycine (Merck) in PBS was added to stop the inactivation. Cells were centrifuged and resuspended in 0.2 M glycine in PBS. After an 1 h incubation at RT, cells were collected by centrifugation, resuspended in CM at a concentration of 2 x 10^7 cells/ml, and stored at -70°C until used for titration.

BEI INACTIVATION PROCEDURE. BEI was prepared freshly before each experiment according to a procedure described elsewhere[10]. Briefly, 2-Bromo-ethylamine (BEA, Sigma, St. Louis, Mo. USA) was dissolved in 0.175 N NaOH to a concentration of 0.1 M and incubated at 37°C for 30-60 min to allow cyclization. The formation of BEI was verified by measuring the pH which dropped from 12.5 to 8.5.

As initial studies performed with 1.5 mM BEI in DMEM containing 1 mM ethylene-diamine-tetra-acetic acid (EDTA, Merck), 2 mM phenyl-methyl-sulfonyl-fluoride (PMSF, Sigma), 2 µg/ml leupeptin (Sigma) and 2% FCS did not allow consistent vaccinia virus inactivation, further experiments were conducted in PBS (pH 7.6) containing 1 mM EDTA, 2 mM PMSF and 2 µg/ml leupeptin at varying BEI concentrations and incubation periods. Twenty four h after vaccinia virus infection, BHK cells were harvested by centrifugation and resuspended at 10^7/ml in PBS (pH 7.6). A one ml sample was taken to quantify vaccinia virus in the starting material. The remaining cells were mixed with 0.1 M BEI at a final concentration of either 1.5 mM or 7.5 mM. To control for decline in vaccinia virus infectivity in time, a control sample without BEI was run in parallel. After

incubation at 37°C for various lengths of time as indicated, virus inactivation was stopped by the addition of 1 M sodium-thiosulphate at 10% of the volume of the BEI solution used. Samples were stored at -70°C until used for titration.

INFECTIVITY ASSESSMENT BY 3-DAY PLAQUE TITRATION. Trypsin (Gibco BRL, Life Technologies, Paisley, Scotland) was added to 100 µl of each sample at 0.25 mg/ml and incubated for 20 min at 37°C. Ten-fold serial dilutions in CM were made and 100 µl aliquots were added in quadruplicate to rabbit kidney cells (RK13 cells) seeded in 24-well plates (Costar, Cambridge, USA). In addition, 100 µl aliquots of each undiluted sample were added in quadruplicate to RK13 cells. Samples were incubated on a rocker for 90 min at RT, diluted with one ml of CM containing 2.5% FCS and further incubated at 37°C/5% CO_2 for 3 days. To count plaques, cells were stained with 0.8% crystal violet in 50% ethanol/5% formalin. Virus titre was defined as the number of plaque forming units per ml (pfu ml^{-1}) containing 10^7 vaccinia virus infected BHK cells. The detection threshold was 2.5 pfu ml^{-1}.

LONG TERM (21 DAYS) INFECTIVITY ASSESSMENT. Samples were prepared as described above in 'Infectivity assessment by plaque titration'. Two hundred µl of undiluted or 500 µl of ten-fold serial diluted, trypsinized sample was added to RK13 cells seeded in 25 cm^2 flasks and incubated on a rocker for 90 min at RT. Subsequently, 4 ml of CM containing 2.5% FCS was added and the flasks were further incubated at 37°C/5% CO_2 for 3 weeks. Cultures were split 1 in 5 twice a week.

Production of recombinant SIV-Env and preparation of iscoms

The production of SIV-Env and their incorporation into iscoms was performed as described previously[12]. Briefly, BHK cells were infected with either rVV v8672-m or v8789-m[12]. Subsequently, cells were collected and treated with either 1.5% PFA in PBS for 30 min at RT or with 1.5 mM BEI in DMEM containing 1 mM EDTA, 2 mM PMSF and 2 µg/ml leupeptin and 2% FCS for 24 h at 37°C. The envelope proteins were solubilized from the membrane by incubation in 4% Rosenbuch-Tenside (RBT, n-octyl-polyoxy-ethylene; Bachem, Bubendorf, Switzerland), enriched by lentil-lectin chromatography and incorporated into iscoms by the dialysis method.

Immunization of rats

Two groups of four female rats (RIV:tox) were immunized intramuscularly at weeks 0 and 4 with 3 µg of 8789-m SIV-Env incorporated into iscoms. The Env was solubilized from rVV infected BHK cells treated with either 1.5% PFA for 30 min at RT or 1.5 mM BEI for 24 h at 37°C. Serum samples were collected 0, 2, 4, 5, 6, 8, and 14 weeks after the first immunization.

Binding of monoclonal antibodies (Moabs) to different SIV-Env preparations

The reactivity of SIV-Env 8672-m and 8789-m, derived from either non-inactivated

or PFA or BEI inactivated rVV infected BHK cells, with a panel of Moabs (kindly provided by Dr. K. Kent, in part through MRC) was tested by ELISA as described[12]. The panel consisted of 14 murine Moabs which could be divided in 6 competition groups[13,14] located at the surface and transmembrane part of the SIV-Env (see also *Figure 3*). The Moabs of competition group 1 recognize conformation dependent epitopes. They all cross-compete but differ in their ability to react with different strains of SIV, to compete with Moabs recognizing the V3 region of SIV, and to inhibit CD4-gp120 binding[15-17]. Further mapping has shown that the conformation dependent epitope recognized by KK9 involves the V3 and the V4 domain, that the epitope of KK5 involves V4 but not V3 and that KK56 recognizes a conformation dependent epitope involving the CD4 binding site[15].

Detection of SIV-Env specific antibodies

Envelope specific antibodies were detected by a whole SIV ELISA as described[12]. Briefly, concanavalin-A coated 96-well plates were incubated with a SIV-infected C8166 cell lysate. After overnight incubation wells were blocked and incubated with two-fold dilutions of rat serum. Bound antibody was detected by rabbit-anti rat Ig-horse radish peroxidase (HRPO) antibodies (DAKO, Gostrup, Denmark). The substrate reaction was carried out with 3,3',5,5'-tetramethyl-benzidine. Endpoint titres were calculated using as cut-off value twice the OD_{450} given by parallel dilutions of the pre-immune serum. Comparison of titres between the immunization groups was conducted using a one way analysis of variance (Anova; Minitab Inc., State College, USA). Titres were considered significantly different if $p<0.05$.

Inhibition of a virus neutralizing antibody (KK56)

To demonstrate the presence of antibodies capable of inhibiting the virus neutralizing (VN) antibody KK56[16], an inhibition ELISA was used as described[18]. Briefly, concanavalin-A coated 96-well plates were incubated with 100 µl of SIV-infected C8166 cell lysate. Two-fold dilutions of rat serum in 100 µl were added for 1 h at RT. Subsequently, 50 µl was replaced with 50 µl of KK56 diluted to give an absorbance at OD_{450} of 70% of the maximum absorbance in an indirect SIV-ELISA. Plates were incubated with a biotin-conjugated goat-anti-mouse IgG antibody preparation (Amersham), and subsequently with HRPO conjugated streptavidine (Amersham). Inhibition titres were defined as the dilution of rat serum inhibiting 50% of the absorbance measured without competing antibody. The difference in titre between the two immunization groups was evaluated through a student's *t*-test. Differences of $p<0.05$ were considered significant.

SIV neutralization assay

The SIV neutralization assay was performed with SIVmac32H as described[18]. Briefly, 10 µl of serial dilutions of heat-inactivated sera were incubated in four replicate wells with 10 µl of SIVmac32H at 10 infectious particles per 10 µl. After a 30 min

incubation at 37°C, 2 x 10^3 C8166 cells in 200 µl were added to each well. Cells were incubated for seven days at 37°C, transferred to poly-L-lysine flat-bottom microtiter plates, fixed in methanol, and examined for SIV infection by an SIV specific immunoperoxidase assay. The number of negative wells was used to determine the neutralizing dose 50% end point (ND50) for that serum by the method of Spearmann-Karber. The difference in titre between the two immunization groups was evaluated through a student's *t*-test. Differences of $p<0.05$ were considered significant.

RESULTS

Inactivation of vaccinia viruses in BHK cells

(I) PFA INACTIVATION. Three concentrations of PFA, 0.3%, 1.5% and 7.5%, were tested for their ability to inactivate rVVs present in BHK cells. The kinetics of inactivation were determined by assessing residual infectivity in a 3-day plaque titration assay (*Figure 1A*). Complete inactivation of rVVs appeared to be achieved with all three concentrations of PFA, within about 0.5, 8 and 50 min, respectively. The rate of inactivation was found to increase with the concentration of PFA (*Figure 1B*). However, when cultures were maintained for prolonged periods, residual infectivity could be detected in all PFA treated samples within three weeks (see *Table 1*). In none of the concentrations tested the effective inactivation with PFA therefore exceeded 10^6 pfu ml^{-1} rVV infected BHK cells.

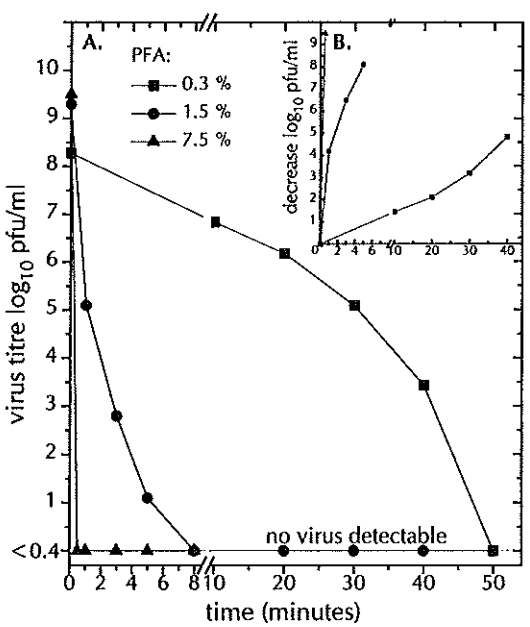


Figure 1. Inactivation of rVVs present in BHK cells with 0.3%, 1.5% and 7.5% PFA at RT as measured by assessing residual infectivity in a 3-day plaque titration assay. (A) Kinetics of inactivation of vaccinia virus. (B). Extrapolation of the decrease of vaccinia virus infectivity in time. The data shown are representative for at least two independent experiments. Inactivation curves were generated using control rVV vSC65, expressing no recombinant protein, or rVVs v8672-m or v8789-m expressing SIV-Env.

As PFA interacts with proteins, we subsequently tested the effects of different concentrations and incubation times of PFA inactivation on the yields of SIV-Env obtained from rVV infected cell preparations by detergent solubilization. As shown in *Table 1*, the recovery of SIV-Env proved to be inversely related to PFA concentration and duration of treatment.

(II) BEI INACTIVATION. In pilot experiments, inactivation of vaccinia virus with BEI was shown to be more efficient at 37°C compared to 20°C (data not shown). Therefore, two concentrations of BEI, 1.5 mM and 7.5 mM, were tested for their ability to inactivate vaccinia virus at 37°C using different incubation periods (*Figure 2A*). Complete inactivation of rVVs was found with both concentrations of BEI, within 8 and 24 h, respectively. The rate of inactivation was found to increase with the concentration of BEI (*Figure 2B*). In contrast to inactivation with PFA, inactivation with BEI did not result in recovery of residual infectivity upon prolonged incubation (*Table 1*).

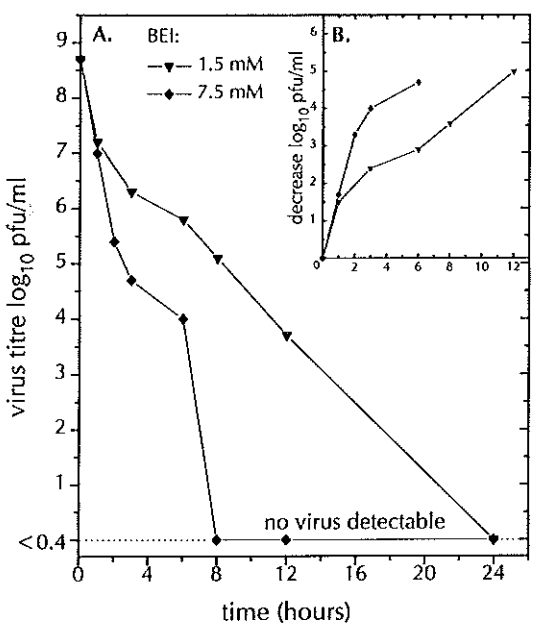


Figure 2 Inactivation of rVVs present in BHK cells with 1.5 mM and 7.5 mM BEI at 37°C as measured by assessing residual infectivity in a 3-day plaque titration assay. (A) Kinetics of inactivation of vaccinia virus. (B) Extrapolation of the decrease of vaccinia virus infectivity in time. The data shown are representative for at least two independent experiments. Inactivation curves were generated using control rVV vSC65, expressing no recombinant protein, or rVVs v8672-m or v8789-m expressing SIV-Env.

Antigenicity of rVV expressed SIV-Env after PFA and BEI treatment

On basis of their inactivation kinetics and for practical reasons, the following procedures using PFA and BEI were selected to evaluate their effects on antigenicity and immunogenicity respectively: 1.5% PFA for 30 min at RT; 1.5 mM BEI for 24 h at 37°C. The effect of PFA and BEI inactivation on the antigenicity of rVV v8672-m and rVV v8789-m expressed SIV-Env were assessed by evaluating their reactivity with a panel of 14 SIV-envelope specific Moabs recognizing major antigenic sites[13,14,17] (*Figure 3*). These Moabs included the virus neutralizing Moabs KK10 and KK54 recognizing

linear epitopes and KK5, KK9, and KK56 recognizing different conformation dependent epitopes[13-17]. With all the Moabs tested, the reactivity of PFA or BEI treated SIV-Env was essentially the same as that of SIV-Env recovered without prior use of an inactivation method.

Immunogenicity of rVV expressed SIV-Env after PFA and BEI treatment

To investigate the immunogenicity of SIV-Env, rats were immunized with rVV v8789-m expressed SIV-Env incorporated into iscoms after inactivation either with PFA or BEI. The overall levels and kinetics of SIV specific antibody titres were virtually identical in rats immunized with either PFA or BEI treated SIV-Env (*Figure 4A*). In addition, no significant difference was found in titres of serum antibodies capable of inhibiting the VN antibody KK56, as well as total VN serum antibodies (*Figure 4B and C*). Similar results were obtained using rVV v8672-m expressed SIV-Env incorporated into iscoms for immunization (data not shown).

Table 1. Measurement of residual vaccinia virus infectivity using a 3- or 21-days titration assay and recovery of SIV-Env from differently inactivated rVV infected BHK cells.

compound	incubation period	decrease in infectivity (log pfu ml^{-1})		yield[c]
	(min)	3-days[a]	21-days[b]	(µg SIV-Env ml^{-1})
7.5% PFA	0.5	>8	6	nd
	1.5	>8	nd	0.7
	3	>8	6	0.4
1.5% PFA	15	>8	5	3.1
	30	>8	6	2.8
0.3% PFA	60	>8	3	nd
	180	>8	nd	4.2
	360	>8	6	2.4
1.5 mM BEI	24 h	>8	>8	nd

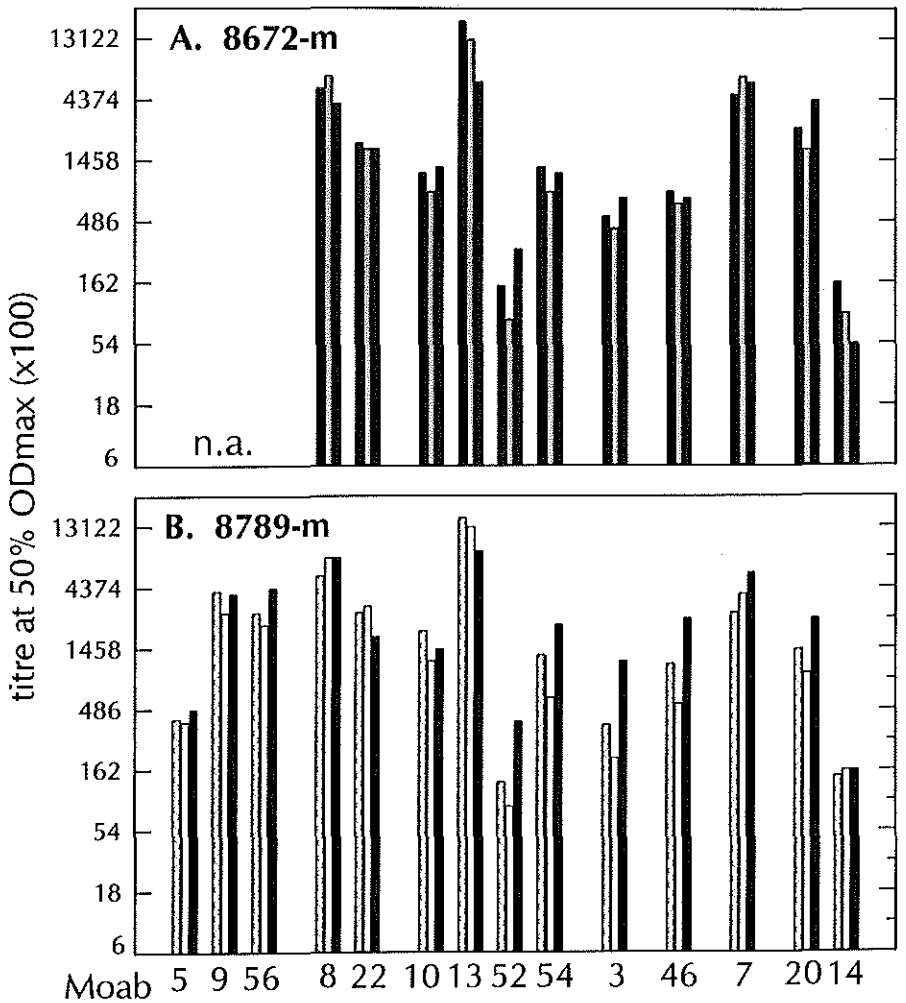
[a] Decrease in vaccinia virus infectivity as determined in a standard 3-day plaque titration assay.
[b] Decrease in vaccinia virus infectivity as determined by maintenance of cultures for 21 days.
[c] Yield of SIV-Env obtained from rVV v8789-m infected BHK cell preparations by solubilization with 4% RBT.

DISCUSSION

In the present study, we have shown that both PFA and BEI inactivate vaccinia virus in a dose- and time-dependent fashion. However, in samples which scored virus negative in a standard 3-day plaque titration assay, residual infectivity could be detected in PFA but not BEI treated samples when cultures were maintained for up to 3 weeks. Treatment of rVV infected BHK cells with 1.5% PFA for 30 min at RT or 1.5 mM BEI for 24 h at 37°C preserved the reactivity of rVV expressed SIV-Env with a panel of 14 Moabs recognizing the major antigenic sites on the SIV envelope glycoprotein. The immunogenicity of SIV-Env, as defined by SIV specific serum antibody titres, inhibition to a VN monoclonal antibody and total VN antibody titre against SIVmac32H, proved to be comparable using either inactivation method.

Formaldehyde is widely used to inactivate viral vaccines[19]. However, accidents due to residual virus infectivity in formaldehyde inactivated vaccine preparations have been reported[2]. Residual infectivity has been attributed to non-linearity of the inactivation curves, reversion of the process of formaldehyde mediated inactivation, slow adsorbtion of formaldehyde treated virus to cells, and retarded penetration of the virus into the cell [2,20-24]. In the present study, using PFA for inactivation, residual vaccinia virus infectivity was found in cultures that had scored virus negative in a standard 3-day plaque titration assay, after additional culturing for up to 3 weeks. Apparently, the conditions used did not result in an inactivation of more than 6 logs. In contrast, the calculated inactivation which would result from a hypothetical inactivation curve based on extrapolation of the data obtained in the 3-day plaque titration assay would have been more than 10^{30} pfu ml^{-1} (1.5% PFA for 30 min at RT). It therefore seems most likely that the late recovery of infectivity is due to a delay in regaining virus infectivity rather than to an asymptotic nature of the inactivation curve. However, more detailed experiments are needed to elucidate the exact underlying mechanism, and whether for instance the relapse in infectivity could be overcome by changing the conditions used, such as even longer incubation periods, higher concentrations of PFA, or different incubation temperatures. Inactivation of vaccinia virus in this study may also have been complicated by the fact that inactivation is carried out with intact vaccinia virus infected cells and not with cell free virus.

From these data it has become clear that inactivation based solely on PFA treatment may not be sufficient. In the procedure to make the experimental SIV-Env vaccine preparation used for immunization, additional steps to isolate and purify the protein may have contributed to inactivating or removing vaccinia virus. These steps include detergent extraction, which reduces infectivity with at least 6 logs (data not shown), lentil-lectin affinity chromatography and iscom preparation including dialysis and sucrose gradient centrifugation which in general give additional reductions in virus titre of about 3-4 logs each[25]. We could never demonstrate residual infectivity in iscom



comp. group	1	2	3	4	-	5	6
specificity	conf.	SU	SU (V2)	SU	SU(V3)	TM	TM
aa		8-30	171-190	21-40	321-340	493-735	594-616

Figure 3 Binding of fourteen Moabs to rVV v8672-m (A) and rVV v8789-m (B) expressed SIV envelope glycoproteins recovered either without prior use of an inactivation procedure for vaccinia virus or after inactivation with 1.5% PFA for 30 min at RT or with 1.5 mM BEI for 24 h at 37°C. Competition groups are defined by Kent *et al.* [13,14,16]. The Moabs of competition group 1 recognize conformation dependent epitopes. They all cross-compete but differ in their ability to react with different strains of SIV, to compete with Moabs recognizing the V3 region of SIV and to inhibit CD4-gp120 binding [15-17]. SU; surface glycoprotein. TM; transmembrane glycoprotein. Aa; amino acid numbers BK28 SIV envelope protein. conf.; conformation dependent epitope. na; not appliciable, 8672-m envelope glycoprotein is hardly recognized by Moabs of competition group 1[12]. Bars represent the dilution of monoclonal antibody giving 50% of the maximum OD450 using that monoclonal antibody. ■ =8672-m, ▣ = 8672-m PFA, ▣ = 8672-m BEI ☑ =8789-m, □ = 8789-m PFA, ▣ = 8789-m BEI. Binding of Moabs to untreated 8672-m and 8789-m envelope glycoproteins has been published before in reference 12.

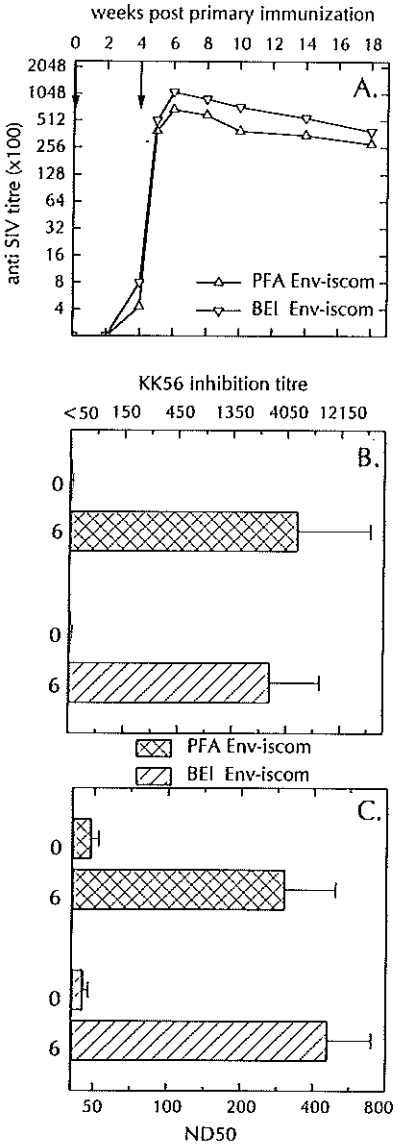


Figure 4 Induction of SIV-Env specific antibodies in rats immunized with rVV v8789-m expressed SIV-Env incorporated into iscoms after inactivation either with PFA or BEI. Data on SIV-Env specific antibody induction after PFA treatment have been published before in reference 12. (A) Mean SIV antibody titre from four rats immunized with the same SIV-Env preparation. Weeks of immunization are indicated by arrows. (B) Inhibition of binding of the virus neutralizing monoclonal antibody KK56 by monkey sera and (C) SIV virus neutralizing antibody response before immunization (week 0) and at peak level of antibody response (week 6 post primary immunization). Mean titres from four rats are shown with the standard error of the means presented as a horizontal line.

preparations produced in this way: neither *in vitro* (data not shown) nor *in vivo* upon immunization of rats (present study and[12]) or monkeys[26] with recombinant SIV-Env derived from rVV infected BHK cells originally containing about 0.3 to 1 x 10^9 pfu per immunization dose, respectively.

In several studies BEI has been shown to efficiently inactivate RNA and DNA viruses[10]. Inactivation was found to be linear in time with inactivation rates ranging from 0.5 to 4.1 log h^{-1} using 1 mM BEI at 37°C[10]. In our study, the calculated inactivation rate of vaccinia virus with 1.5 mM BEI at 37°C was on average about 0.4 log h^{-1}. A more detailed analysis of the inactivation curves will be needed to precisely determine the inactivation rate at all stages.

There are several indications that the SIV envelope protein did not dramatically

change its conformation by either the PFA or the BEI treatment. First, the recognition of the envelope protein by a group of VN antibodies specific for conformational epitopes was preserved. Second, VN antibodies were induced by immunization with PFA or BEI treated SIV-Env. As large parts of VN antibodies present in sera from SIV infected monkeys[27] and HIV infected humans[28,29] are conformation dependent, this finding may indicate a rather native conformation of the SIV Env studied. Our results with PFA treated SIV-Env are also in agreement with the findings of Sattentau *et al.*[30] showing the conservation of four neutralizing epitopes on HIV-1 gp120 after formaldehyde treatment.

Overall, the results generated in the present studies are encouraging for the proposed inclusion of recombinant proteins expressed by rVVs in experimental viral vaccines, since they show that antigenicity and immunogenicity of the highly glycosylated SIV-Env glycoprotein are preserved. Treatment of rVV infected cells with 1.5 mM BEI at 37°C resulted in complete inactivation. PFA treatment was shown to give a delayed appearance of residual vaccinia virus infectivity after prolonged culture, and therefore additional procedures are needed if this compound is to be used in the preparation of vaccines for human use.

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Chapter 3

TESTING OF CANDIDATE VACCINES IN CYNOMOLGUS MACAQUES

VACCINE-INDUCED VIRUS-NEUTRALIZING ANTIBODIES AND CYTOTOXIC T CELLS DO NOT PROTECT MACAQUES FROM EXPERIMENTAL INFECTION WITH SIMIAN IMMUNODEFICIENCY VIRUS SIVmac32H (J5)

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ABSTRACT

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 CTLp 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.

INTRODUCTION

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 of 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 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, 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 after infection 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 hrs 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 *EcoR*1 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 [WAGLWNWFSITNWLWYIKIFIMIVGGLVG]) was amplified by PCR and cloned C-terminal of the Gag-p27 gene. The resulting plasmid, pMALc-Gagp27, expresses in *Escheria 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 MgCl2) containing 0.25% MEGA-10, 10 µg of cholesterol per ml and 10 µg of phosphatidylethanolamine per ml and 2 µg 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 108-123), LRTMSYKLAVDMSHFI; 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 (MID50) 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.

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 lipopeptides	1
		108-123 (LRTMSYKLAVDMSH)	1
		155-169 (DWQDYTSGPGIRYPK)	1
		164-178 (GIRYPKTGWLWKLV)	
C	K66, K71, K73, K79	FIV Env iscoms	10

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

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 contol. After 16 h of incubation at room temperature (RT), wells were blocked with PBS containing 0.1% Tween-20 and 1% bovine serum albumine (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 OD450. One-way analysis of variance (Minitab, Inc., State College, Po.) 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 a 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 than 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 titre 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 horsradish 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 x 103 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 difference in titre among the different groups at each time point were evaluated by Student's *t* test. Treshold of significance was $P < 0.05$.

Determination of frequencies for CTL precursors (CTLp) frequencies

(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 x 10^4 to 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 x 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 perwell. 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 isothiocyanaat-conjugated anti-CD4 (Okt4, Ortho Diagnostic System, 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 Cr^{51}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 and spontaneous release were set up in 18 replicate wells. Spontaneous was <25% of maximum ^{51}Cr release in all assays.

(IV) CALCULATION OF CTLP FREQUENCIES. Individual wells were considered positive when 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 x 10^6 to 1.6 x 10^3 were seeded in duplicate wells in the presence of 3 µg 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 106 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

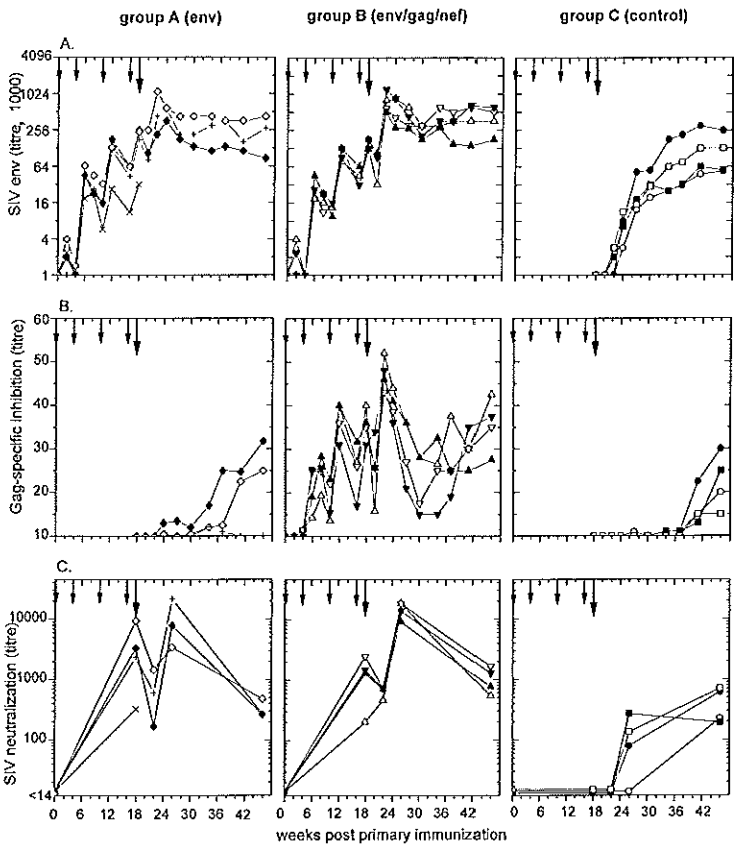

Figure 1. Pre- and postchallenge SIV specific antibody responses in monkeys of groups A, B and C. A. SIV Env specific serum antibodies, as determined by indirect ELISA using SIVmac32H-derived Env as immobilized antigen; B. SIV Gag specific antibodies, as determined by inhibition ELISA. Bacterial-produced $p27^{gag}$ was used as immobilized antigen. A biotinylated polyclonal serum of a SIVmac infected monkey was used for inhibition. C. SIVmac32H neutralizing 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 neutralizing dose 50% end point titre for the relative plasma. Weeks of immunizations are indicated by small arrows. Day of challenge is indicated by a large arrow. Group A monkeys (Env-immunized): ♦ 769; ◇ K70; + K81; x K2. Group B monkeys (Env-Gag-Nef-immunized): △ K77; ▽ K80; ▼ K83; ▲ K88. Group C monkeys (control): ○ K66; ■ K71; ● K73; □ K79. Monkey K2 (group A) died during recovery from anaesthesia on the day of challenge.

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.

(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 of group B after the second immunization (Fig. 1B). Titers increased after the second and third immunization, 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 of groups A and C.

(III) SIV NEUTRALIZING 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 of the 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 at 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; K88: Env- and Gag-specific CTLp), albeit at lower levels (2 to $15/10^6$ PBMC). In the monkeys from group A, only macaque 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 target cells and MHC class I-mismatched rVV Env infected target cells. Lysis of the autologous 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 $p27^{gag}$ specific plasma antibody titers declined 2 weeks after challenge but showed an anamnestic response peaking 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 could be demonstrated in any monkey from group A.

Cell-associated virus loads

After challenge with 50 MID50 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 respective groups. The mean value of virus load for the monkeys from the 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.

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

Group	Macaque	Frequency (CTLp/10^6 PBMC)					
		Day of challenge			Wk 9 or 12 postchallenge		
		Env	Gag	Nef	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 rIL-2. Split-well ^{51}Cr release assays were performed on day 14 of culture: specific cytotoxicty was assayed with autologous B-LCL infected with either rVV expressing the SIV protein under investigation or wild-type control vaccinia virus. Data from LDA 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

DISCUSSION

In this paper, we have shown that SIV subunit vaccines consisting of Env glycoproteins incorporated into iscoms either 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 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 using 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 analyses 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-resticted 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 of 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 (4 of 7 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). A 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-positive 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-specific

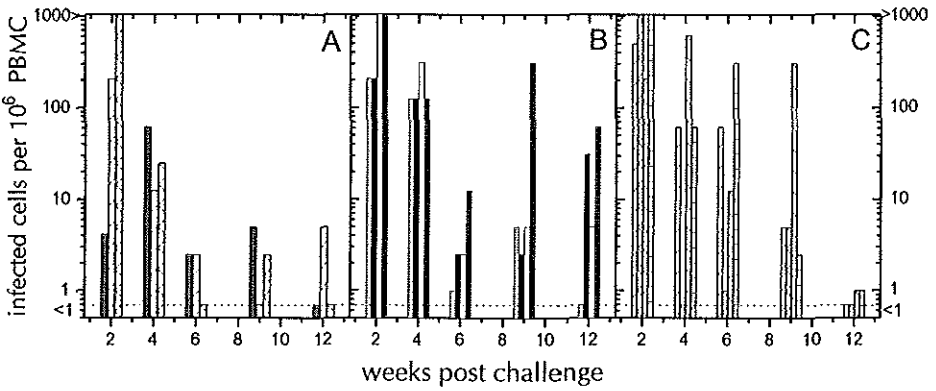

Figure 2. Cell-associated virus load in PBMC of SIV vaccinated (groups A and B) and control monkeys (group C) during a 12 week follow-up period. Serial 5 fold dilutions of PBMC ranging from 106 to 960 for the 2 week postchallenge and from 10^5 to 1600 for the other time points were incubated with C8166 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. A. Group A (Env) of monkeys: ■ 769; ▨ K70; ▨ K81. B. Group B (Env/Gag/Nef) of monkeys: ▨ K77; ▨ K80; ▨ K83; ▨ K88. C. Group C (control) of monkeys: ▨ K66; ▨ K71; ▨ K73; ▨ K79.

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 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 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 OF CYNOMOLGUS MACAQUES WITH ENVELOPE GLYCOPROTEINS FROM PRIMARY HIV-1 ISOLATES INDUCES LIMITED PROTECTION AGAINST INTRAVENOUS SHIV CHALLENGE

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ABSTRACT

Chimeric simian-human immunodeficiency viruses (SHIVs), used as challenge viruses in macaques, provide a new tool for testing the protective potential of candidate human immunodeficiency virus (HIV-1) vaccines. Here, four cynomolgus macaques were immunized with iscoms containing recombinant envelope glycoproteins 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 a HIV-1 envelope glycoprotein 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 envelope 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 animals replication of the challenge virus had not been prevented completely. These data show that although vaccination with HIV-1 envelope glycoprotein-based vaccines may be effective in limiting virus replication, it fails 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 different 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 a series of major drawbacks including limited availability, ethical concerns and limited HIV-1 pathogenicity (for review see Heeney, 1996). The construction of chimeric simian-human immunodeficiency viruses (SHIVs), which carry the envelope glycoproteins (Envs) of HIV-1 and are able to replicate in macaques, now 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 have been shown 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 which predominate in the asymptomatic stages of the infection, and syncytium inducing (SI) - usually T cell-line tropic - variants, which are predominantly found in later stages of infection (Zhu *et al.*, 1993; Zhang *et al.*, 1993). 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, the predominance of NSI strains in the asymptomatic phase is followed by a predominance of SI strains in about 50% of the HIV-1 infected individuals progressing to AIDS (Koot *et al.*, 1992). These differences 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 vaccinated macaques to protect against SIV_{mac} challenge (Hulskotte *et al.*, 1995a). Although no protection was observed, the role of the cytotoxic T cell response in the containment of SIV_{mac} infection was clearly illustrated in this study (Hulskotte *et al.*, 1995a; Geretti *et al.*, 1997; A.M. Geretti 1997, unpublished). SIV-Env differs in several structural, biological, and immunologic

properties from HIV-1 Env (Burns & Desrosiers, 1994). Here, we present a study in which cynomolgus macaques are immunized with different HIV-1 Envs before challenge with SHIV. To investigate potential differences in the immunogenicity of SI and NSI derived envelope proteins, two groups of monkeys were immunized with Envs derived from either a NSI or a SI HIV-1 biological clone, originating from PBMC of the same seropositive individual. The antigens were presented in iscoms to allow efficient induction of both VN antibody and cytotoxic T lymphocyte (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.

METHODS

Generation and characterization of HIV-1 Env iscoms.

(I) CONSTRUCTION OF RECOMBINANT VACCINIA VIRUSES (RVV). Envelope 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 envelope proteins 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), on which basis 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 envelope precursor protein was verified by western blot analysis.

(II) PREPARATION OF HIV-1 ENV ISCOMS. HIV-1 envelope proteins 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). Envelope 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 $HIV\text{-}1_{IIIB}$ (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} (kindly provided through the 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).

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 thymocytes 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 human 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), following the instructions of the manufacturer.

RESULTS

Antigenicity of recombinant HIV-1 Env preparations

The antigenicity of the 320.NSI and SI recombinant HIV-1 envelope preparations was studied by determining the relative affinities of a panel of HuMabs that had been selected for their ability to neutralize primary HIV-1 isolates (Muster *et al.*, 1993; Burton *et al.*, 1994; Schutten *et al.*, 1995; Trkola *et al.*, 1995; Schutten *et al.*, 1997) (Table 2). Both envelope preparations showed reactivity 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 the 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 monoclonal antibodies for these glycoproteins.

Vaccine-induced HIV-1 Env specific serum antibody responses.

(i) KINETICS OF HIV-ENV SPECIFIC SERUM ANTIBODY RESPONSE. After the second immunization, all the four HIV-1 Env immunized monkeys developed serum antibodies that recognized the envelope glycoprotein of the $SHIV_{320.3.1}$ (Fig. 1). Antibody titers increased after the third immunization, decreased subsequently, but increased again after the fourth immunization. The monkeys vaccinated with 320.SI-Env developed

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

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

* Concentrations of monoclonal antibody (ng/ml) giving 50% of maximal binding.
† cm, after cleavage site mutation ‡ PFA, after PFA fixation
§ Hardly any binding: OD_{450} values remained just above background levels at the concentrations tested.

about ten times 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).

(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 different 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. In contrast to sera from the 320.NSI-Env immunized monkeys, those from 320.SI-Env immunized monkeys barely recognized the T cell line adapted $HIV\text{-}1_{IIIB}$-Env. Sera from both groups of monkeys showed reactivity with 168.NSI and 168.SI envelope proteins, which are derived from a NSI and SI inducing primary HIV-1 isolate respectively. The overall binding of the sera was lower than that to the respective #320 envelope proteins used for immunization. The sera from 320.NSI-Env immunized monkeys generally bound better to the NSI Envs 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

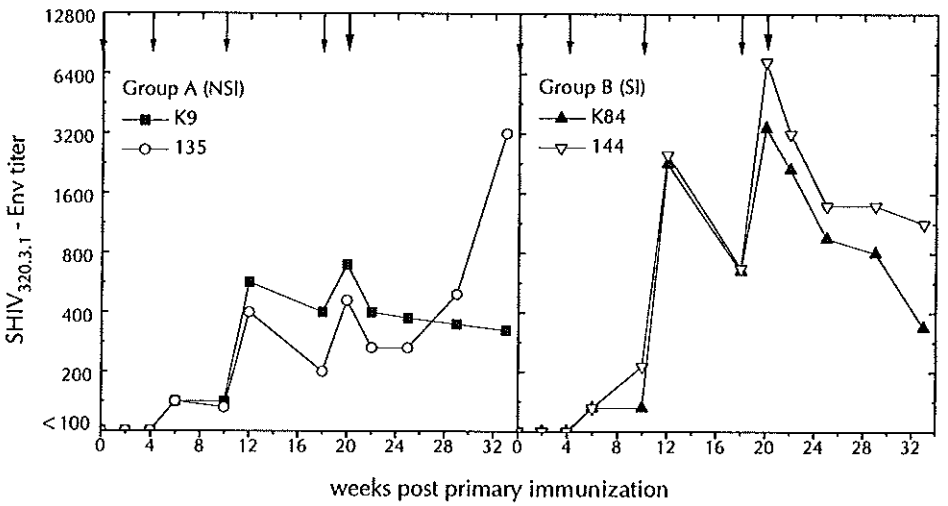

Figure 1. Pre-and post-challenge $SHIV_{320.3.1}$-Env specific serum antibodies, determined by indirect ELISA. Immunizations are indicated by small arrows. The day of challenge is indicated by a large arrow.

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.

(iv) Inhibition of HIV-1 CD4bd specific HuMab binding. On the day of challenge, serum reactivity with the CD4bd of HIV-1 was studied by testing their ability to inhibit the 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 and 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 abolishment of the CD4bd antigenicity of 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 $HIV\text{-}1_{IIIB}$ 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 $HIV\text{-}1_{IIIB}$ 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 $HIV\text{-}1_{IIIB}$.

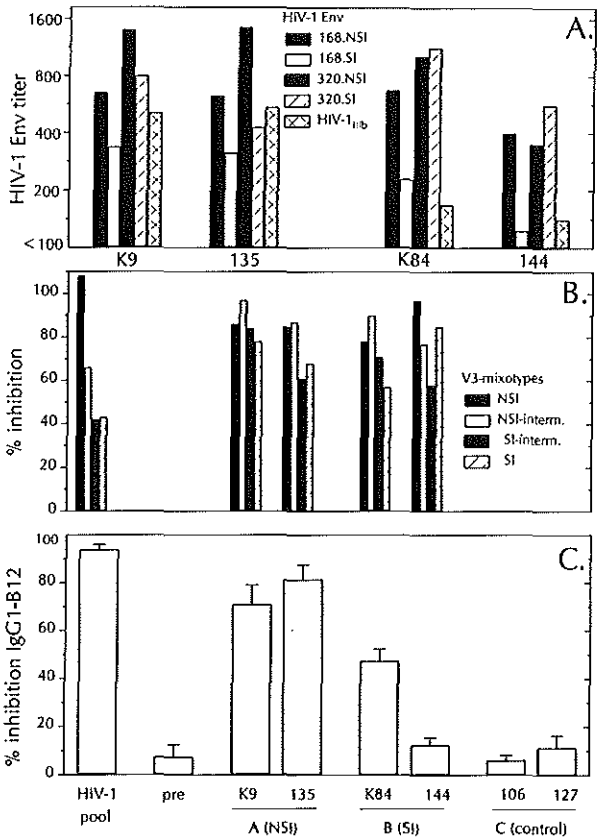

Figure 2. Envelope specific antibody responses of monkey sera collected on the day of challenge or prior to vaccination (pre), and of pooled sera of HIV-1 seropositive individuals (HIV-1 pool). (*a*) Envelope specific serum antibody responses against different HIV-1 envelope glycoproteins. HIV-1 envelope glycoproteins were produced by recombinant vaccinia viruses expressing the envelope glycoproteins of primary isolates (320.NSI- and 320.SI-Env, derived of seropositive individual #320 and also used for immunization (Andeweg *et al.*, 1992; Groenink *et al.*, 1991), and 168.NSI and 168.SI, derived of seropositive individual #168 (de Jong *et al.*, 1992b; Tersmette *et al.*, 1989)), and of the T-cell line adapted HIV-1_{IIIB}. (*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.

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).

HIV-1 Env and SIV Gag specific serum antibodies after challenge

After intravenous challenge with 1500 $TCID_{50}$ of $SHIV_{320.3.1}$, infection of one of the 320.NSI-Env vaccinated monkeys (i.e. #135), and both control monkeys (i.e. #106 and #127) was indicated by the development of SIV Gag specific serum antibodies (Fig. 4). In addition, the envelope specific antibody titer of monkey #135, but not those of the other HIV-1 Env vaccinated monkeys showed an anamnestic response after 4 weeks post challenge (Fig. 1). During the 13 weeks of follow up no detectable $SHIV_{320.3.1}$-Env specific antibodies developed in the control monkeys of group C (not shown).

$SHIV_{320.3.1}$ specific CTLp frequencies after challenge

As an indirect measure of virus replication, we studied the emergence of virus specific CTL upon $SHIV_{320.3.1}$ challenge. To this end, the frequencies of CTLp specific for HIV-1 Env, Tat, Rev, and 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). As was already expected on basis of 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 other three immunized monkeys also developed CTLp of multiple SHIV specificities, indicating low-level virus replication. More specifically, at 13 weeks 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 responses against HIV-1 Tat and Rev were detected in five out of six and two out of six animals, CTL responses 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 measured was 32/10 . 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 $SHIV_{320.3.1}$ 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.

Virus detection after challenge

After challenge, $SHIV_{320.3.1}$ was recovered from PBMC (Table 3) and lymphoid tissues (Table 4) of the 320.NSI-Env vaccinated monkey #135 and control monkey #127

Table 2. SHIV specific CTLp frequencies (CTLp/10^6cells) on day of challenge (doc) and week 13 postchallenge (pc)*.

Group		Sample	doc	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 control	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

*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^2goodness-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 method.

nd, not done

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.

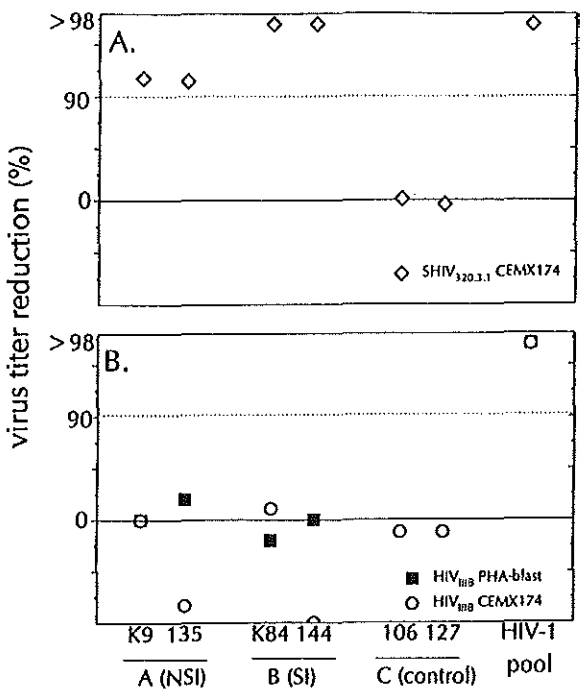

Figure 3. VN-antibody responses against $SHIV_{320.3.1}$ (A) and $HIV\text{-}1_{IIIB}$ (B) measured by a virus neutralization reduction assay. For $HIV\text{-}1_{IIIB}$ reduction in virus titer on CEMX174 cells and PHA-blasts was measured. As a consequence of low-level replication in human PHA-blasts,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

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

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 (indicated first) and virus isolation (indicated second). The presence of $SHIV_{320.3.1}$ DNA in PBMC was assayed by a nested PCR amplifying a 500 bp fragment of SIV Gag-p27. $0.5x10^5$, 106 or $2x10^6$ PBMC were present in the starting material of the samples taken until week 7, at week 9 or at week 13 pc, respectively. Virus isolation was done with a viral load assay at day 3 to week 5. For this purpose a total of $2x10^6$ freshly isolated PBMC were cocultivated with human PHA blasts at 10^5 and $3.3x10^4$ cells in fifteen replicate wells. From week 7 onwards, virus isolation was performed by cocultivation of freshly isolated PBMC (week 7; 10^6 PBMC) or their CD8 depleted fractions (weeks 9 and 13; CD8 depleted fraction of 6 and $10x10^6$ PBMC, respectively) with CEMX174 cells for 6 weeks.
+, virus detected; -, no virus detected; nd, not done.

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 $HIV\text{-}1_{IIIB}$ (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 $HIV\text{-}1_{IIIB}$ was observed in the sera of the vaccinated monkeys (Fig. 3). This may be due to the high amino acid sequence difference between the $HIV\text{-}1_{IIIB}$-Env and the immunizing Envs (Myers *et al.*, 1993). However, $HIV\text{-}1_{IIIB}$ was effectively neutralized by pooled sera from seropositive individuals infected with viruses probably as distant from $HIV\text{-}1_{IIIB}$ 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.

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 (pc).

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 (indicated first) and virus isolation techniques (indicated second). The presence of proviral $SHIV_{320.3.1}$ DNA by PCR was studied as described in Table 3. The starting material contained $2x10^6$ cells. Virus isolation of freshly isolated thymocytes, lymph node (LN, axillary (ax), pooled axillary and inguinal (in) or mesenteral (mes)), splenic or bone-marrow (BM) mononuclear cells (week 7; $3x10^6$ cells, week 13; $6x10^6$ cells) was done by cocultivation with CEMX174 cells for 6 weeks.

+, virus detected, - no virus detected, nd, not done.

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

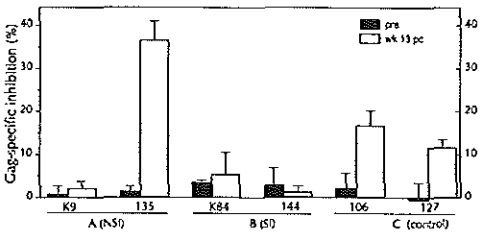

Figure 4. Gag specific antibodies, determined by inhibition ELISA. Bacterial p27-gag was used as immobilized antigen. The percentage inhibition of a biotinylated polyclonal serum of a SIVmac-infected monkey by a tenfold dilution of monkey sera collected at 13 weeks post challenge (wk 13 pc) or prior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

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 4

CTL AND THE CONTAINMENT OF SIV INFECTION

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

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ABSTRACT

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 paraformaldehyde-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.

INTRODUCTION

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 SIVmac 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 more than three 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 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-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 paraformaldehyde (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 penicillin, 100 µg/ml 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% 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' 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 non shown).

Immunofluorescence analysis

Cell samples in PBS with 0.1% BSA were incubated for 30' at room temperature with anti-CD2 (Leu-5b-FITC, Becton Dickinson, Montan View, CA) and anti-CD8 (Leu-2a-PE, 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 analyzed with a

FACScan (Becton Dickinson).

Preparation of antigen presenting cells (APC)

Autologous B-LCL were infected overnight with rVV (10 MOI) expressing either the Gag p56 gene of SIVmac32H (kindly provided by Prof. 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 ($2.5 \cdot 10^4$/well) in R-10 were cultured for 14 days in 96 well round-bottomed plates with SIV Gag or Tat APC (10^4/well) and autologous irradiated (2500 rad) feeder PBMC (10^4/well). Cultures were supplemented with 10 U/ml recombinant IL-2 (rIL-2) from day 3, and restimulated with 10^4/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) were cultured for 10 to 14 days with autologous irradiated PHA-blasts infected with SIVmac32H(J5) (10^5/ml), as described by Gotch *et al.*[17]. For mitogenic stimulation, PBMC (10^6/ml) were cultured for three days with ConA (5 µg/ml), washed, and expanded with rIL-2 (20 U/ml) for 4 to 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 pre-determined 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 MOI of SIV Gag or Tat rVV. Autologous B-LCL either infected with 186-poly rVV (containing a polycloning

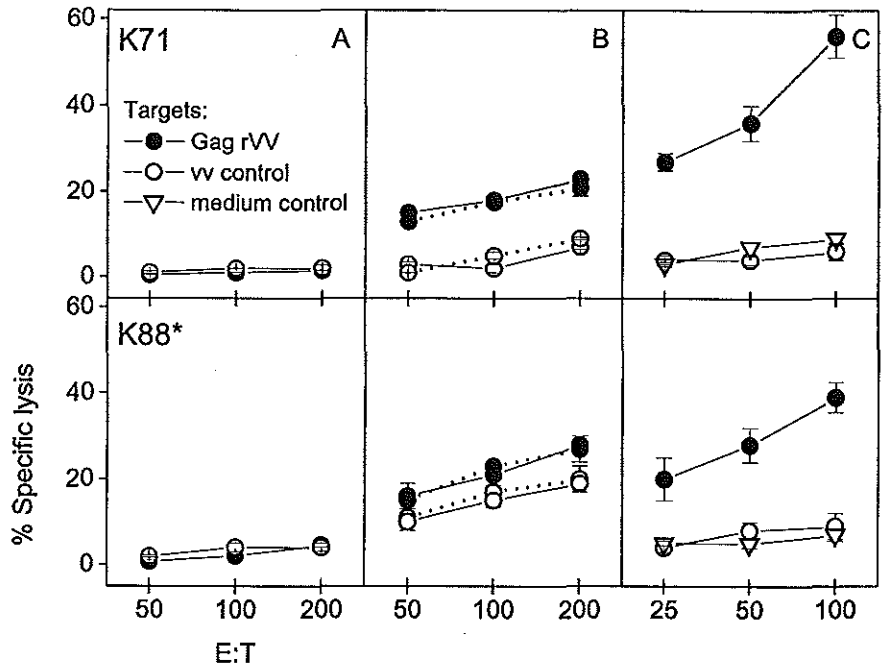

Figure 1. *In vitro* restimulation is required to detect SIV Gag-specific CTL in PBMC of immunized and non immunized cynomolgus macaques infected with SIVmac32H(J5). Data are shown for two monkeys. Monkey K88* had been immunized with an SIV Env-Iscoms, Gag-Iscoms, and Nef-lipopeptides subunit vaccine 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 (dotted line) and 14 (solid line) of culture (monkey K71), or on days 7 (dotted line) and 10 (solid line) 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.

site without insert and kindly provided by Dr M. P. Kieny, Transgene) or incubated with medium alone served as control. Targets labelled for one hour with 100 μCi of ^{51}Cr, washed three times, resuspended in R-10, and added to effector cells at $5 \cdot 10^3$ cells/well in 96 well round-bottomed plates (Costar, Cambridge, UK). After a 5 hour 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. SR was <30% of MR.

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 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 (SD). 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/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.

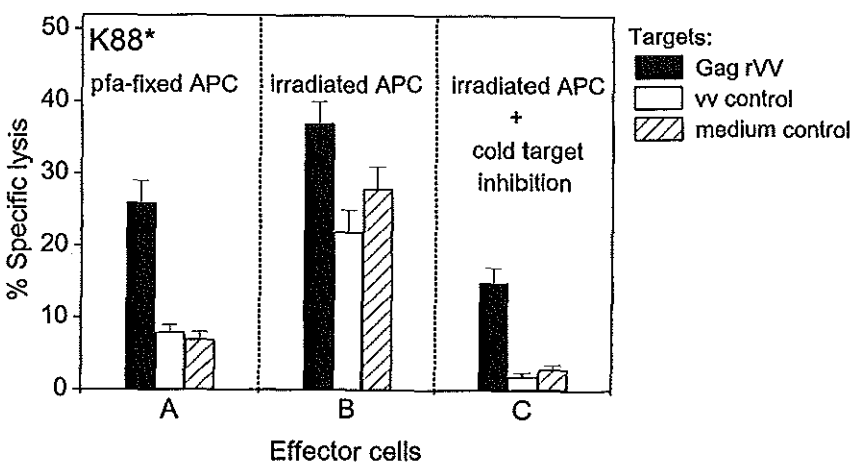

Figure 2 Stimulation efficiency of SIV Gag APC. Effector cells were generated by stimulation of PBMC from monkey K88* with either pfa-fixed (A) or irradiated (B and C) SIV Gag APC. Targets were autologous B-LCL either infected with SIV Gag rVV or vaccinia virus (vv) control, or uninfected (medium control). 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 ± SD from duplicate well estimations at an E:T ratio of 40:1.

RESULTS

Measurement of SIV Gag-specific CTL in bulk assays

SIV Gag-specific CTL responses were studied in eight cynomolgus macaques during the first four 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 1a*, 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 1b)*, 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 1c)*.

Previously we[13] and others[20] have shown that after pfa-fixation, EBV-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[13,20]. 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 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 3a)* or Tat *(Figure 3b)* APC mediated significant lysis of targets infected with the inducing rVV, but not of targets infected with either the discordant rVV or 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.

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 pre-determined 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*

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 wk (peak)	4 months	1-4 wk[d]	6-12wk[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	± 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	ND	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-lipopetides 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/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/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 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.

1c), after two 1-week cycles of SIV Gag-specific stimulation, effector cells in most wells mediated killing of SIV Gag rVV-infected targets *(Figure 5a)*, 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)/10^6 PBMC *(Figure 5c, circles)*. In contrast, in the absence of PBMC restimulation, SIV Gag-specific CTL were detected at a frequency of only 2 (0.3-3; χ^2: 1)/10^6 PBMC *(Figure 5c, triangles)*. Similarly, four 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)/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 (<10/10^6 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 stimulation, 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 4 (2-6; χ^2: 1)/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 X the standard deviation 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 <1/10^6 PBMC, data not shown), indicating that only primed CTLp were responsive to SIV Gag-specific stimulation.

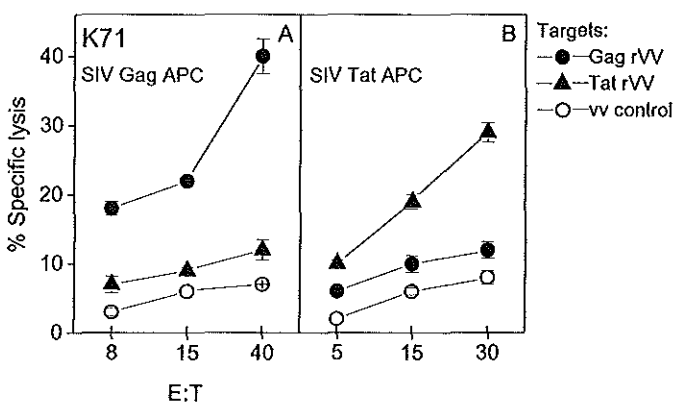

Figure 3. Selective expansion of antigen-specific CTL. Effector cells were generated by stimulation of PBMC from monkey K71 with either SIV Gag (A) or Tat (B) APC. Targets were autologous B-LCL infected with either SIV Gag or Tat rVV or with vaccinia virus (vv) control. Results are expressed as mean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

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 13/10^6 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, four 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)*.

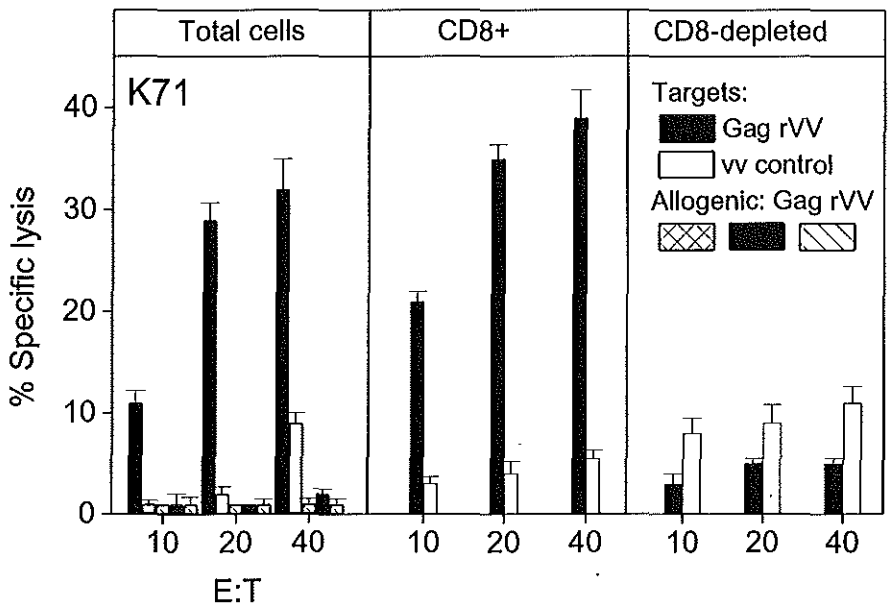

Figure 4. $CD8^+$ cell-mediated and MHC-restricted cytotoxic responses against SIV Gag. Effector cells generated by stimulation of PBMC from monkey K71 with SIV Gag APC were assayed as total cell population, positively isolated $CD8^+$ cells (>99% $CD8^+$), and CD8-depleted cell population (<2% $CD8^+$).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

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 dilution 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)*.

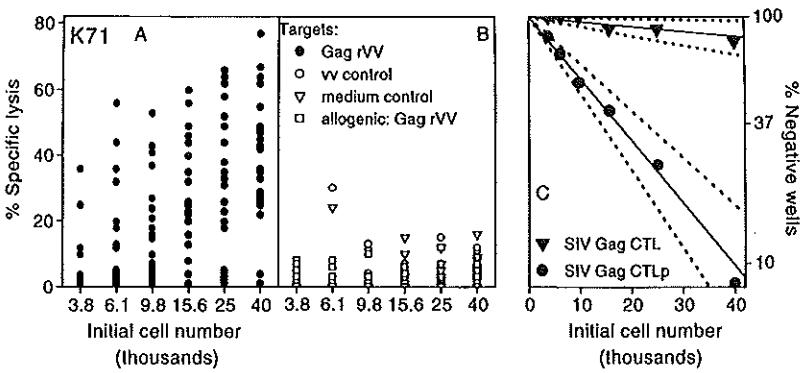

Figure 5 Limiting dilution analysis of cytotoxic responses against SIV Gag. PBMC isolated from monkey K71 at week 12 after infection were cultured in six dilutions (range: 40000 to 3815 cells/well), each including 24 replicate wells. After two 1-week cycles of SIV Gag-specific stimulation, individual 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-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/10^6 PBMC. Dotted lines indicate 95% confidence intervals. The frequency of SIV Gag-specific CTL was 2 (0.3-3; χ^2: 1)/10^6 PBMC. The frequency of SIV Gag-specific CTLp was 58 (44-72; χ^2: 1)/10^6 PBMC

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[4]. 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 *(Figures 3 to 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[3,9]. 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], theuse 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 six 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 vaccination strategies for the prevention and control of lentiviral infections.

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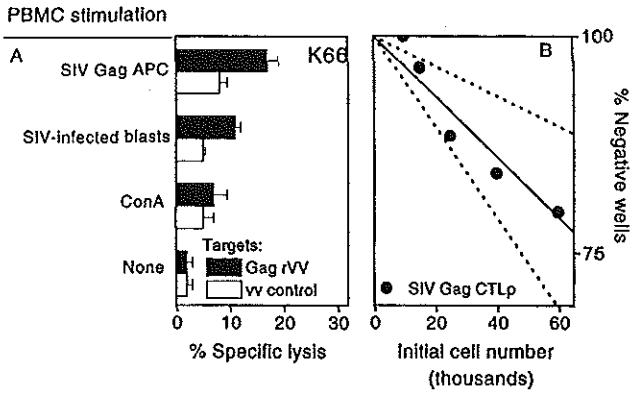

Figure 6 Bulk and limiting dilution assays of PBMC isolated from monkey K66 at week 12 after SIV infection. In bulk assays (A), PBMC that were unstimulated or restimulated with either ConA, or SIV-infected blasts, or SIV Gag APC, were assayed in duplicate at an E:T ratio of 80:1. In limiting dilution 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)/10^6 PBMC.

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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

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SUMMARY

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 defense 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 four 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).

Effector cells were generated from PBMC under limiting dilution conditions. Cells in R-10 medium (RPMI 1640 containing 10% FCS) were cultured with 10^4/well autologous paraformaldehyde-fixed B-lymphoblastoid cell lines (B-LCL) infected with a recombinant vaccinia virus (rVV) expressing Gag p55 of SIVmac32H, and with 10^4/well autologous irradiated (2500 rad) feeder PBMC, 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 polycloning site without insert, Transgene, Strasbourg, France), 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 (1µg/ml), irradiated (2500 rad) allogenic PBMC (10^5/well), irradiated (3500 rad) allogenic human B-LCL (10^4/well) (Van de Griend, 1994) 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 three months by alternate (every 14 to 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 SIVmac251. As shown in Fig. 1 *a* for 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

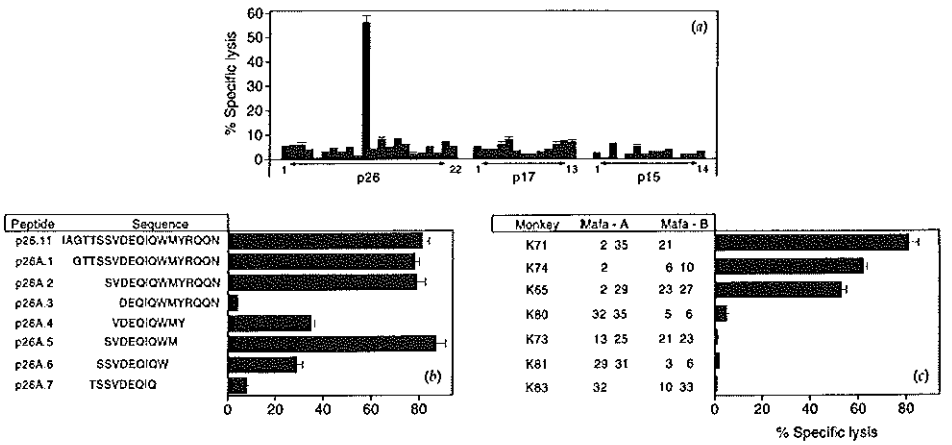

Fig. 1. Fine mapping of an MHC class-I restricted epitope in SIV Gag p26. CTL line K71/E26 was tested against: *(a)* autologous target B-LCL either pulsed with 30 µl of synthetic peptides (20 residues long, with a 10 amino acid overlap) covering the p26 (peptides 1-22), p17 (peptides 1-13), and p15 (peptides 1-14) sequences of SIVmac251, or infected with SIV Gag rVV or vaccinia virus (vv) control, or incubated with medium alone; *(b)* autologous target B-LCL sensitized with 30 µM of synthetic peptides (9 to 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 of peptide p26A.5. Results are expressed as mean specific lysis with standard error from duplicate well estimations at an E:T ratio of either 5:1 *(a)*, or 10:1 *(b and c)*.

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

In reciprocal experiments, PBMC ($2X10^4$/well) were cultured for 10 days with autologous irradiated (5000 rad) B-LCL (10^4/well) sensitized with 30 µM of peptide p26A.5, autologous irradiated feeder PBMC (10^4/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,

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

Effector cells*	E:T	% Specific lysis			
		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

* 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 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 percentages of specific lysis from duplicate well estimations with standard error <10%.

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, Oslo, Norway), 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 and 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 to H). However, comparison of the SIVmac251 and $HIV\text{-}1_{SF2}$ 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 $HIV\text{-}1_{SF2}$ p24 (GSDIAGTTSTLQEQIGWMTN), or infected with a rVV expressing $HIV\text{-}1_{LAI}$ Gag (TG1144, Transgene), were not recognized by CTL line K71/E26. These effector cells

also failed to recognize targets infected with a 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.

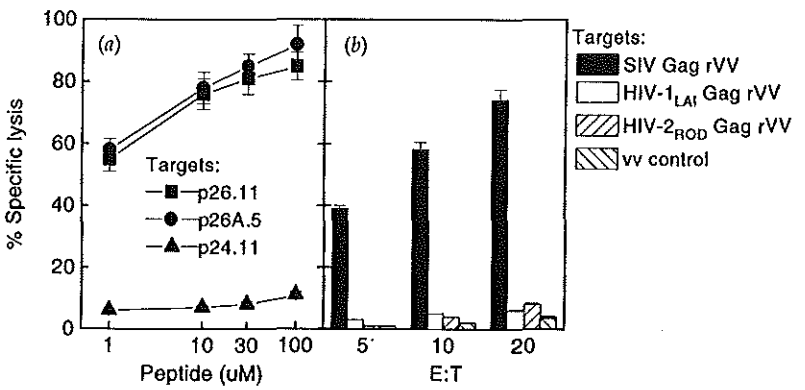

Fig. 2. Effects of sequence variation on CTL recognition. CTL line K71/E26 was tested against: *(a)* autologous target B-LCL pulsed with either peptide p26.11, or peptide p26A.5 or peptide p24.11 (GSDIAGTTSTLQEQIGWMTN, amino acids 235-254 of HIV-1_{SF2} p24), at an E:T ratio of 10:1; *(b)* autologous 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 specific lysis with standard error from duplicate well estimations.

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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

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ABSTRACT

The evolution of the 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. At a later stage, a dissociation emerged between PBMC and secondary lymphoid organs: virus loads 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

poorly recognized 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 cytotoxic T lymphocytes (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 the 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 *(Macaca fascicularis)* 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 from a minimum of 13 months (monkeys K73 and K79) up to 22 months (monkeys 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, Mountain 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% paraformaldehyde (pfa), and analyzed 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 from a 100 μl solution were resuspended in a final volume of 100 μl containing 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100), $MgCl_2$ (1.5 mM), dNTP (0.2 mM), 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 analyzed on a 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 analyzed 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 a 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 ($TCID_{50}$/ml) in the absence of serum to the titre in the presence of the sample serum.

Recombinant vaccinia viruses

Recombinant vaccinia 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 Mimms, 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 the p26 (ADP714/1-22), p17 (ADP775/1-13), and p15 (ADP776/1-14) sequences 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 pfa-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 cell dilutions, and 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 (10^6/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.

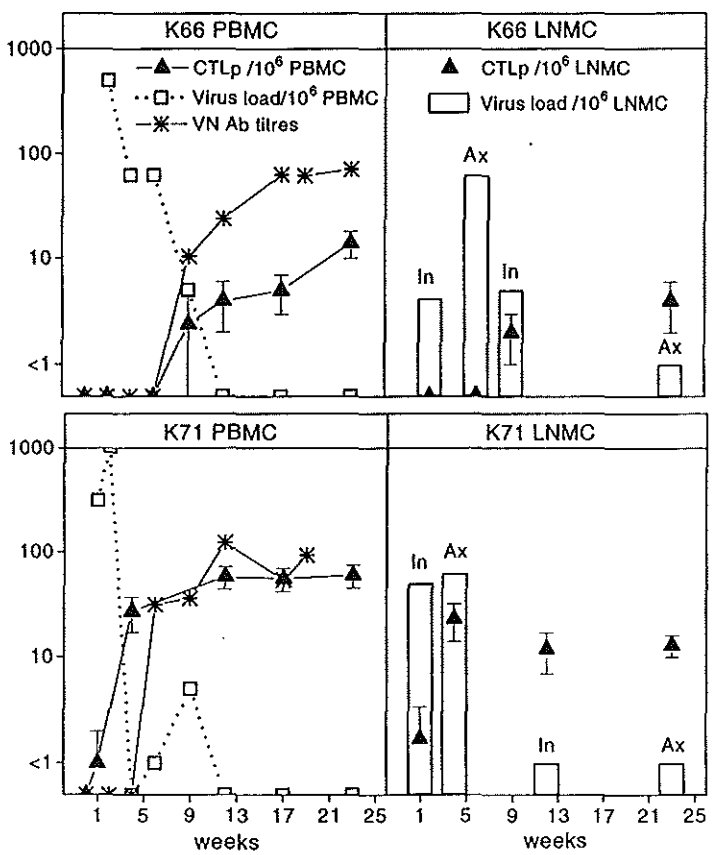

FIG. 1. Kinetics of cell-associated virus load (number of infected cells/10^6 mononuclear cells), virus neutralizing antibody (VN Ab) titres (yield reduction values) and Gag-specific CTLp frequencies (number of CTLp/10^6 mononuclear cells) during the first 23 weeks of infection with SIVmac32H-J5. Data from PBMC were compared to those from axillary (Ax) and inguinal (In) lymph node mononuclear cells (LNMC). Error bars indicate 95% confidence intervals.

RESULTS

Virological and clinical status of SIV infected macaques

As reported 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 could be 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 the other five infected monkeys (K66, K73, K79, K83* and K88*) for the entire observation period (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 of 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 ≤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 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.

Table 1. Virological status of $CD4^+$ 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 fraction. 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

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 both 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 seen in 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 the $CD4^+$:$CD8^+$ 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 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. 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

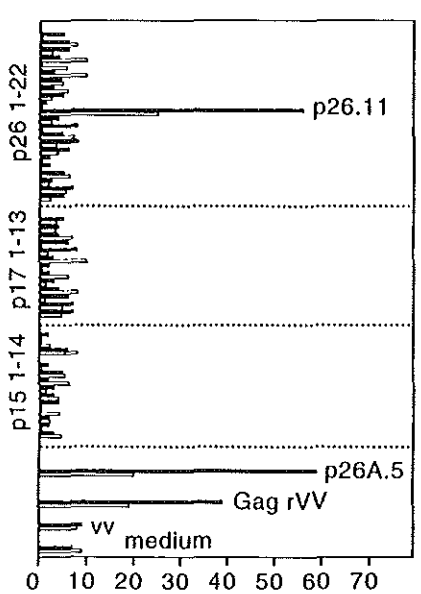

FIG. 2. Gag-specific CTL of monkey K71 recognize a nine-mer peptide in SIV p26. Effector cells derived from PBMC by stimulation with either SIV Gag rVV (solid bars) or with ConA (open bars) recognized targets either pulsed with 30 µM of peptide p26A.5 (SVDEQIQWM) or with peptide p26.11 (IAGTTSSVDEQIQWMYR QQN), or expressing endoge-ously 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 ether 5:1 (solid bars) or 20:1 (open bars).

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)

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

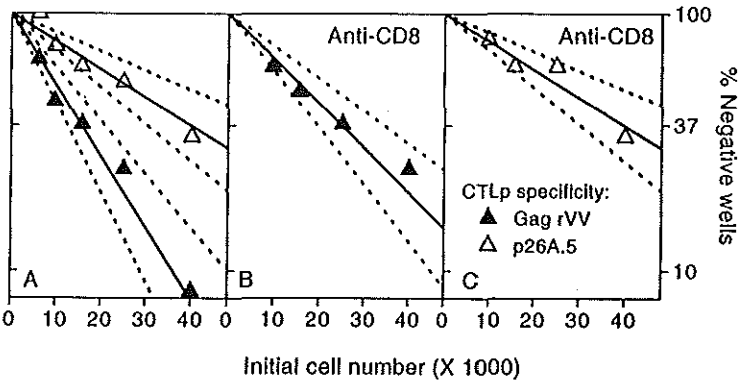

FIG. 3. P26A.5-specific CTL responses of monkey K71 are not inhibited by anti-CD8 mAb. Effector cells were generated from PBMC by Gag-specific stimulation. Autologous target B-LCL either infected with SIV Gag rVV or with vaccinia virus control, sensitized with 30 μM of peptide p26A.5, or incubated with 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.

percentages of circulating $CD8^+$ cells. 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; $\chi^2 = 4$). In the same LDA, p26A.5-specific CTLp showed also a rapid but less pronounced increase and 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 also 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 frequences in PBMC also showed a moderate increase (Table 4).

Comparison of peripheral blood and lympoid 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 burdens were 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 both 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 were below or barely above detection levels. CTLp

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

Month	Gag-specific CTLp		p26A.5-specifc CTLp	
	Gag-stimulation		Gag-stimulation	p26A.5-stimulation
	Exp I	Exp 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.
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 p26A.5 epitope may generate CTL escape mutants.

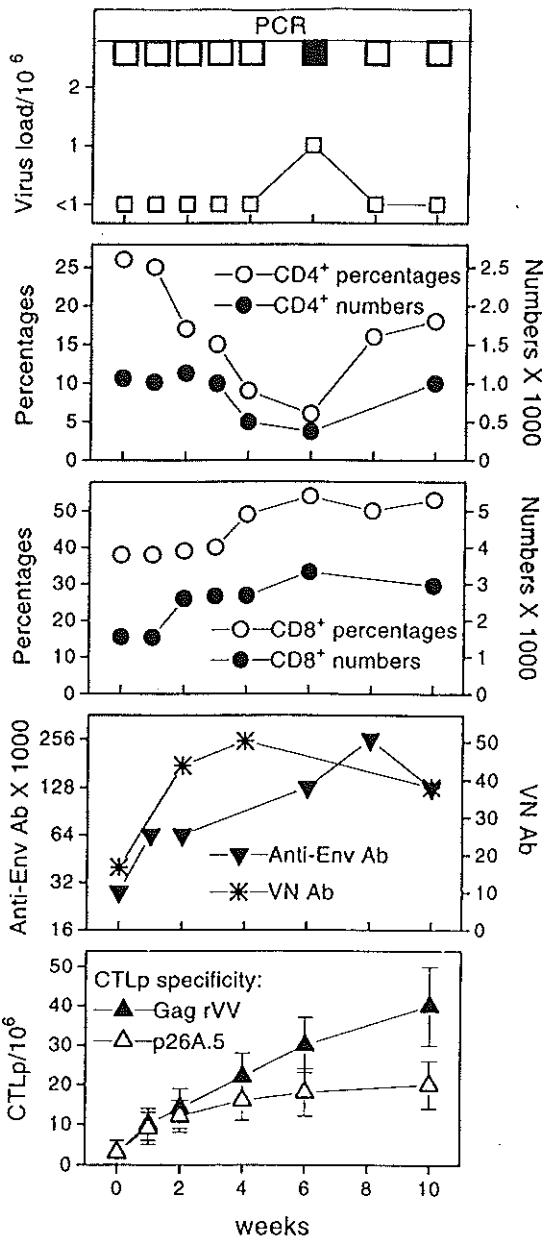

FIG. 4. Re-challenge of monkey K71. Cell-associated virus load (number of infected cells/10^6 PBMC) was measured in whole PBMC and their CD8-depleted fractions. Nested-Gag PCR was used to amplify provirus DNA from PBMC, their CD4-enriched fractions, and the same cells after co-culture with C8166 cells. 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.

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).

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-	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-	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.

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 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

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	---------	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. 6. Genetic variation within the p26A.5 epitope. DNA samples were analyzed 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.

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 both before and after re-challenge, and in SPMC isolated at the end of observation. In bulk CTL assays, the variant peptide was effective in inducing CTL expansion from SPMC, but was poorly immunogenic for PBMC and LNMC (Fig. 7a). Consistent with these findings, 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 one 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.

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

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)

[a] 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 p26A.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.

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 $CD8^+$ and 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 generally 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 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 loads 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 previously with both HIV-1-infected humans (4, 24) and

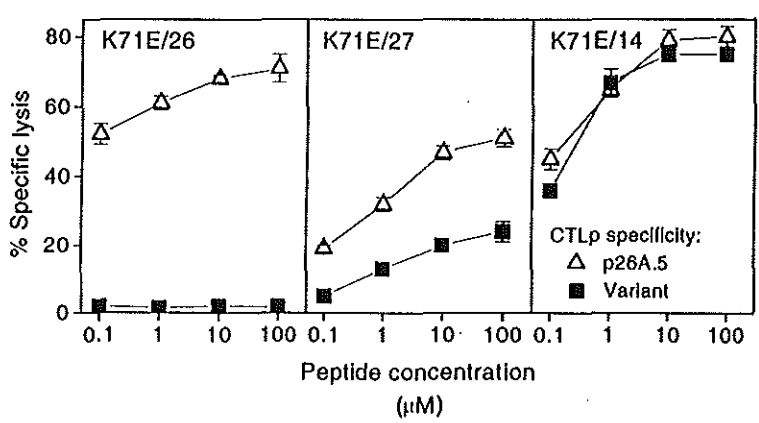

FIG. 6. Recognition of the prototype (SVDEQIQWM) and variant (SVEEQIQWM) p26A.5 epitope by CTL clones generated from PBMC of monkey K71 at three and four months after primary infection. Results are expressed as mean specific lysis from duplicate well estimations at an E:T ratio of 5:1.

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 (i.e., 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 compromised CTL function. Transient cell-associated viraemia, rising SIV-specific antibodies 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 super-infection 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 higher 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).

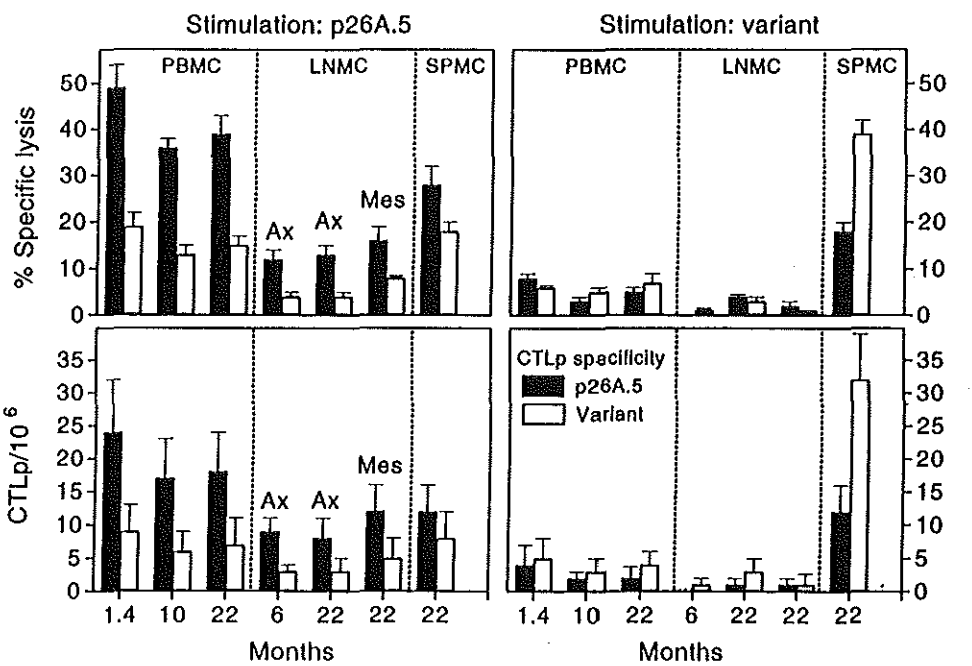

FIG. 7. Bulk (top) and limiting dilution (bottom) assays of CTL responses against prototype (SVDEQIQWM) and variant (SVEEQIQWM) p26A.5 epitope. PBMC, axillary (Ax) and mesenteric (Mes) LNMC, and SPMC were isolated from monkey K71 both before and after re-challenge. Effector cells were generated by stimulation 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.

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 micro environment 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 we 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 newly induced 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 also 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

DISCUSSION AND SUMMARY

TOWARDS AN HIV-1 VACCINE: LESSONS FROM STUDIES IN MACAQUE MODELS

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Vaccine, in press

ABSTRACT

The development of a safe and effective vaccine for the prevention of AIDS has thus far proven to be extremely difficult, at least in part due to complexities associated with HIV-1 and its pathogenesis. The recent description of individuals transiently infected with HIV-1, as well as persons who survived HIV-1 infection for more than 15 years, indicates the ability of the immune response of certain individuals to control HIV-1 infection. Moreover, vaccination-challenge experiments in macaques infected with simian immunodeficiency virus have shown that protection against infection or development of disease may be achieved in the absence of sterilizing immunity, suggesting that the goals for AIDS vaccine development may have to be redefined. In addition, evaluation of new lentivirus vaccine strategies may largely benefit from the use of the newly developed chimeric simian-human immunodefiency viruses, allowing the tesing of HIV-1 antigen based vaccines in macaques.

INTRODUCTION

Since its discovery in 1983, human immunodeficiency virus type 1 (HIV-1) continues to spread at an alarming rate. It is estimated that by the turn of the century, more than 30 million individuals will have been infected with this virus worldwide[1]. Recent successes with antiviral treatment have raised optimism about a possible cure of HIV-1 infection[2]. However, the actual use of such treatments in developing countries, in which more than 90 % of HIV-1 infected persons live, is highly unlikely due to high costs, intricate dosing regimens, and the severe adverse effects involved. It is therefore generally believed that the development of an effective vaccine is required to eventually control the HIV-1 pandemic.

From the beginning, it became clear that development of an HIV-1 vaccine would be a major challenge. Progress in HIV-1 vaccine development has been slowed

considerably by several problems specifically related to properties of HIV-1 and the infection it causes. Amongst these are its high variability, its ability to infect cells of the immune system via both cell-free and cell-associated routes, incorporation of viral DNA into the host genome and the establishment of viral latency. The apparently unavoidable progression of the vast majority of HIV-1 infected individuals to immunodeficiency, despite the often vigorous virus specific humoral and cellular immune responses, further complicates HIV-1 vaccine design.

Simian immunodeficiency virus (SIV) infection of macaques has proven to be of great value in testing the value of novel lentivirus vaccine strategies. Recently, new models using chimeras of SIV and HIV-1 called SHIVs, have been shown to allow the testing of vaccines based on HIV-1 antigens in macaques. Here we present an overview of the progress made in the evaluation of current vaccine designs in macaque models of HIV-1 infection. This not only illustrates past and ongoing efforts, but also highlights critical issues for future developments in this field.

MACAQUE MODELS OF HIV-1 INFECTION.

Although HIV-1, which is responsible for the majority of AIDS cases worldwide, can infect apes like chimpanzees (*Pan troglodytes*) and gibbons (*Hylobates lar*), infection of these species generally does not result in AIDS[3,4]. Furthermore, high cost, restricted availability and ethical objections limit the use of these models for HIV-1 vaccine development. HIV-1 infection of pig-tailed macaques (*Macaca nemestrina*) has also been described, however, virus repliation is highly restricted in this species[5,6].

Infection of rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) monkeys with several strains of SIV resembles the course of HIV-1 infection in humans more closely (see Table 1). SIV has a genomic organization and biology similar to HIV-1 (reviewed in[7]). Three major open reading frames, *gag*, *pol* and *env*, code for the core, the viral enzymes and the envelope glycoproteins, respectively. Furthermore, the HIV-1 genome encodes the regulatory proteins Vif, Vpr, Vpu and Nef, while SIV contains reading frames for Vif, Vpx and/or Vpr and Nef. Like for HIV-1, the primary receptor for SIV is the CD4 molecule[8]. Recently, it has been shown that genetically divergent strains of SIV use CCR5 as coreceptor[9]. CCR5 is a receptor for the ß-chemokines RANTES, MIP-1α, and MIP-1ß, and is also used by macrophage-tropic HIV-1 strains[10]. Clinically, SIV infection may cause an immunodeficiency syndrome that shares many features with AIDS in humans[11]. However, the course of the infection is usually more rapid in macaques, and most animals develop a fatal AIDS-like syndrome within 1-4 years. The second human immunodeficiency virus, HIV-2, which is less prevalent and possibly also less pathogenic in humans[12], infects rhesus and cynomolgus monkeys but generally does not cause disease in these animals[13].

One of the notable shortcomings of SIV/HIV-2 macaque models has been the inability to test the protective potential of candidate vaccines based on HIV-1 immunogens. The use of the recently developed SHIVs as challenge viruses, may solve this problem. In these chimeric viruses *tat, rev, env*, and in some cases other SIV genes

have been replaced by their HIV-1 counterparts[14,18]. Infection of macaques with SHIVs initially failed to induce disease[14,15,18]. However, more pathogenic SHIVs have recently been generated by serial *in vivo* passages in macaques[16,19,20]. However, this has also resulted in amino acid changes in the HIV-1 derived envelope glycoprotein[21].

MINIMAL REQUIREMENTS FOR AN EFFECTIVE HIV-1 VACCINE.

Initially, it was widely believed that a successful HIV-1 vaccine should provide complete protection from infection ("sterilizing immunity"). Otherwise, a permanent threat of virus escape from integrated proviral DNA would remain, even in the presence of a vigourous anti-viral immune response. This concept of sterilizing immunity, by definition, implicated neutralizing antibodies as key mediators of protection. As a consequence, the envelope glycoproteins gp120 and gp41, which harbour the neutralizing epitopes, were considered the key components of a safe and efficacious HIV-1 vaccine.

Several lines of evidence now suggest that a successful HIV-1 vaccine may not necessarily completely prevent infection. (1) Clearance of HIV-1 infection in newborns has been documented[22]. Moreover, a small number of adults who have been repeatedly exposed to HIV-1 and have apparently remained uninfected, carry HIV-1-specific cytotoxic T lymphocytes (CTL)[23], suggesting that transient infections may occur. (2) The description of long-term non-progressors, who remain healthy for more than 12 years and have little or no detectable virus in circulation, indicate that effective immune containment of HIV-1 may be possible[24,25]. In addition, it has been shown that HIV-1 levels in plasma reflect a steady-state balance between the daily production and clearance of large amounts of virus[26,27]. In the light of these findings, it is gradually appreciated how close the human immune system may come to success in its fight against HIV-1 infection. (3) The plateau in plasma viral load seen after seroconversion is prognostic for the rapidity of progression towards AIDS[28]. This indicates that early control of HIV-1 infection may at least extend the asymptomatic period, or even prevent disease development completely. As a consequence, a vaccine that does not completely prevent infection but clears the virus or keeps the viral load low, may be a valid option. In this light, the additional induction of cellular immune responses, in particular $CD8^+$ CTL, which are able to eliminate infected cells[29], is considered of major importance. Accordingly, viral proteins other than Env may be considered to be important targets of a candidate HIV-1 vaccine.

The final goal of the vaccination strategy needs to be defined both at the level of the individual and of the population at large. If sterilizing immunity is not feasible and prevention of disease development in the infected individual proves the second best option, transmission of HIV-1 from vaccinees to naive individuals needs to be prevented. This may perhaps not be a major problem, since both disease progression and transmission appeared to be inversely related to virus load[22].

Besides being efficacious, an HIV-1 vaccine should be safe, inexpensive and easy to administer, and provide long-term protection against all subtypes of HIV-1, regardless

of the route of exposure (rectal, vaginal, oral or intravenous). Moreover, the vaccine used should preferably allow discrimination between vaccinees and infected persons.

PASSIVE TRANSFER STUDIES

Protection from lentiviral infections is likely to involve multiple arms of the host immune response. The relative contributions of the different arms can be studied independently, by either adoptive transfer experiments or by selective deletion of an effector arm. With respect to the cellular arm of the immune response, adoptive transfer of primed T cells is not currently feasible in outbred macaques. Attempts to achieve sustained *in vivo* depletion of specific cell populations using monoclonal antibodies have generally been unsuccessful[30]. Instead, transfer of well defined antibody pools is feasible and this approach has been explored by several investigators. The outcome of such passive transfer experiments has been highly dependent on the nature of the challenge virus and the primate species used. In chimpanzees, it was shown that transfer of polyclonal or monoclonal antibodies specific for HIV-1 Env may confer protection against T-cell line adapted HIV-1[31,32]. In macaques, transfer of serum from a monkey immunized with an inactivated whole HIV-2 vaccine and resistant to HIV-2 challenge, conferred protection in naive monkeys against infection with HIV-2 grown in monkeys cells[33]. Similarly, nine out of eleven rhesus macaques receiving plasma from a healthy SIV_{mne}-infected monkey were protected from intravenous SIV_{mne} infection[34]. Protection from infection with the more pathogenic SIV_{sm} was achieved by the transfer of serum from a SIV_{sm} infected macaque[33]. In a recent study, macaques were injected at day 1 and at day 14 after intravenous SIV_{sm} infection with serum from a healthy SIV_{sm} infected monkey. Four out of six animals showed a marked decrease in viral load and remained asymptomatic for at least 15 months. In contrast, nine out of ten control monkeys, which had received no treatment or non-immune serum, did progress to AIDS[35]. So far, no protection has been achieved against SIV_{mac} by passive transfer of either immune serum from asymptomatic SIV_{mac}-infected macaques, or monoclonal antibodies neutralizing SIV_{mac}[36,37]. In fact, evidence of enhanced infection has been reported[38], which correlated with antibody levels to a putative enhancing region in gp41[39,40]. It is not clear yet whether the origin of the Env (HIV-1 or 2, SIV_{mne}, SIV_{sm} or SIV_{mac}), the animal species used, or both are the reason for the conflicting results obtained in these passive transfer experiments. Also the presence of soluble factors in the serum used for passive transfer, such as the CD8-suppressive factor[41], chemokines[42] and cytokines[43,44], may have played a role in the achievement of protection.

WHOLE VIRUS VACCINE STRATEGIES

Whole inactivated virus vaccines

The use of whole inactivated virus vaccines was amongst the first strategies tested. This approach has been shown to be quite successful in conferring protection against

several other viral infections (for review see[45]). In addition, a vaccine containing all structural virus proteins could be considered to have a fair chance of inducing adequate protection. The protective component could then be determined by 'stripping' of the virus preparation.

Early experiments in macaques indicated that this concept was valid. Using whole-inactivated vaccines, hundreds of macaques proved to be protected from pathogenic SIV infection by the intravenous[46], intrarectal[47], or intravaginal[48] route. Absence of both detectable virus and anamnestic antibody responses indeed indicated the induction of sterilizing immunity. However, initial excitement turned to disappointment when the observed protection proved, at least in part, mediated by responses against human cellular antigens. The latter were shared by vaccine- and challenge viruses, since both had been propagated in the same human cells. The fact that protection was indeed due to such xeno-antigens became obvious when animals resistant to SIV grown in human cells, appeared to be fully susceptible to the same virus grown in macaque peripheral blood mononuclear cells (PBMC)[46,49,50]. Moreover, immunization with human cells ,[51] purified HLA-DR[52], or HLA class I [53]antigens, also induced protection against human- but not against monkey-cell grown SIV.

Other particle based designs.

Pseudovirions have been made from cells expressing HIV-1 Env and Gag, but lacking the packaging signals for the inclusion of HIV-1 RNA and the regulatory genes critical to HIV-1 replication[54,55]. Pseudovirions are considered a non-infectious and therefore safe variant of whole-inactivated viruses. Immunization of HIV-1 like particles in macaques has been shown to induce virus neutralizing (VN) antibodies and CTL[56]. In the SIV/macaque model, however, only one out of twelve monkeys immunized with a trivalent vaccinia virus expressing Env-Gag-Pol and boosted with SIV pseudovirion particles was protected against SIV_{mac} infection[57].

SUBUNIT VACCINE STRATEGIES

Despite the protective antigen being of human cell- rather than of viral origin, the successes obtained with whole virus vaccines stimulated the development of subunit vaccine approaches based on Env. Provided that they would induce qualitatively correct immune responses at sufficiently high levels, Env-based vaccines were believed to be able to equal the protection induced by cellular antigens. As subunit vaccines generally induce only antibody and $CD4^+$ cell-mediated responses, and are not able to access the major histocompatibility (MHC)-class I processing pathway leading to the induction of $CD8^+$ CTL, their use primarily aims at inducing antibody mediated sterilizing immunity. In contrast to whole inactivated virus vaccines, subunit vaccines do not suffer from the danger that incompletely inactivated virus or potentially hazardous nucleic acid are present.

In the SIV/macaque model, one of three monkeys immunized with virion derived

Table 1: Macaque models for HIV-1 infection[1].

Virus	Productive replication	Pathogenicity
HIV-1[2]	-	none
HIV-2	+	low
SIV		
SIV_{sm}	+	high
SIV_{mac}	+	high
SIV_{mne}	+	low
SHIV	+/-[3]	high/low[3]

[1] As described in[7,155,156].
[2] Infection of HIV-1 has only been observed in pig-tailed macaques.
[3] The replicative potential and pathogenicity of SHIV depend on the genetic background and *in vivo* macaque passages.

gp130 oligomers, and protected against SIV_{mac} grown on human T-cell lines, also resisted a second challenge with monkey cell grown virus[58]. In addition, different recombinant Env preparations suppressed SIV replication upon challenge and prolonged survival time of thus infected macaques[59,61]. Although VN antibodies can be expected to mediate protection afforded by Env subunit vaccines, no such correlation was found[59,61]. This does not necessarily exclude a role for VN antibodies in protection, as in most cases neutralization of the challenge virus was not tested. Furthermore, for neutralization assays often T-cell lines were used as target cells and for propagation of the virus. Results obtained with such assays have now been shown to differ from those obtained with assays using primary rather than laboratory adapted virus strains and PBMC as target cells[62,63]. The latter approach can be expected to reflect more closely the *in vivo* situation.

During the last years, adjuvant systems have been developed for subunit vaccines that not only increase the magnitude but also modify the nature of the induced immune response. Among these, immune stimulating complexes (iscoms) have been shown to access the MHC-class I restricted antigen-processing pathway and thus to induce specific $CD8^+$ CTL[64,65]. Disrupted whole virus- or native HIV-2 Env-iscoms have been shown to induce long-lasting protective immunity against challenge with cell-free HIV-2 in macaques[66,67]. We showed that, gp120-enriched SIV preparations adjuvated by MDP or iscoms, induced protection against cell-associated SIV in four out of eight macaques[68,69]. All protected monkeys shared the MHC class-I allele Mamu-A26 with the SIV infected monkey cells used for challenge, suggesting MHC class I-associated protection[70]. In subsequent studies, immunization with recombinant SIV Env-iscoms alone, or in combination with p27-Gag iscoms and Nef lipopeptides, did not protect monkeys from intravenous cell-free SIV_{mac} challenge, despite the induction of VN antibodies and CTL[71]. Furthermore, in our hands HIV-1 Env iscoms induced protection

against SHIV challenge in three out of four vaccinated monkeys, as indicated by the absence of both detectable virus and anamnestic antibody responses[72]. The presence of SHIV-specific CTLp, however, indicated that virus replication was probably limited but not prevented. Similarly, no SHIV was detected after immunization of macaques with an iscom based vaccine containing gp120, p24 and V2,V3-peptides[73], or after immunization with homologous recombinant gp120[19]. In none of these experiments, a clear correlation was found between protection and humoral or cellular parameters of anti-viral immunity. Further studies are needed to elucidate whether the induced immunity can confer protection against heterologous and more pathogenic SHIV challenge.

Lehner *et al.*[74] developed an approach to target immunity to sites where it might be needed most[74]. They immunized monkeys in the vicinity of the iliac lymph node to prevent rectal SIV transmission. Delivery of alum adjuvanted SIV gp120 and p27-Gag by targeted lymph node immunization, protected four out of seven macaques from rectal challenge with SIV_{mac}. The remaining three macaques showed either a transient viraemia or a decrease in viral load. Protection was associated with the presence of anti p27-IgA antibody-secreting cells, CD8-suppressor factor and the chemokines RANTES and MIP-1ß in the iliac lymph node. Strikingly, all macaques immunized by either the intradermal route through scarification of the inguinal skin, or by the nasal route followed by the rectal and intramuscular gluteal route, became infected. These results indicate that the control of sexual transmission of HIV-1 could best be initiated locally, at the mucosa and draining lymph nodes. Systemic immune responses may come too late, when the virus has already replicated to uncontrollable levels.

LIVE-ATTENUATED VIRUS VACCINE STRATEGIES

Live-attenuated virus vaccines have been the most successful vaccines of those in use today. Due to their capacity to replicate, a full range of immune responses is induced, including a MHC-class I restricted cellular response. Although safety issues may eventually limit the use of an attenuated vaccine against lentivirus infections, a considerable amount of information can be gained in animal models about the limits and correlates of immune protection thus induced.

Live-attenuated SIV vaccines have shown the most long lasting and impressive protection against SIV until now. Protection has been achieved against high doses of highly pathogenic strains of SIV[63,75,76], heterologous SIV strains[63,77], cell-associated virus[76], and against challenge via the intravenous, mucosal and oral route[78,79]. The initial report that Nef-deleted live-attenuated SIV can induce protective immunity in macaques has now been confirmed by several groups[63,76,80]. Similarly, macaques infected with attenuated viruses deleted in the Nef, Vpr and upstream sequences of the LTR, have been protected against wild type SIV_{mac} infection[63]. Interestingly, protection induced by both Nef- and Nef-Vpr-^U3 - deleted viruses appeared time dependent: either full, partial or no protection has been achieved in monkeys challenged at 79, 20 and 8 weeks after primary SIV_{mac} infection, respectively[63]. In another study, full protection against an uncloned primary SIV_{mac} isolate was observed at 42 weeks after primary infection, but

only partial protection was observed at 22 weeks[81,82]. Similarly, an attenuated macrophage tropic strain of SIV provided protection against SIV infection at about 35 weeks but not at 25 or 27 weeks after administration[77].

In some studies, attenuated viruses that replicated less vigorously *in vivo* were also less effective in inducing protection against SIV_{mac} challenge[63,83,85]. Similarly, an attenuated SIV_{mac} that was unable to establish permanent infection did not protect against infection with wild type virus[86]. Along the same line, macaques previously infected with sub-infectious doses of SIV resisted SIV challenge via the rectal[87,88], but not via the intravenous route[89]. In these animals CD8-mediated suppression [88]and T helper cell responses[87,89], but no antibodies could be detected. At the same time, previous infection with pathogenic SIV_{mac} did not always prevent virus replication after homologous rechallenge[90]. The identification of individuals dually infected with different clades of HIV-1 also suggests a very narrow window of protective immunity induced by live viruses[91]. Concerns about the safety of attenuated HIV-1 vaccines increased by the observation that reactivation of the vaccine strain is possible after challenge with the pathogenic virus[92,93]. Attenuated viruses carrying small Nef deletions have been shown to revert to wild-type virus, and the immune response induced against the Nef-deleted virus appeared not sufficient to prevent the onset of immunodeficiency[94]. However, in this case, the time of reversion, taking place from 17-45 weeks post administration, may have been too soon to develop full protective immunity. In new-born macaques, infection with live-attenuated virus has been shown to lead to fatal disease[95].

Until now, no clear immunologic correlate of protection induced by live-attenuated SIVs has been identified. The long time required for full protection to develop is difficult to correlate with either antibody or CTL development, as both are usually detected within 1-6 weeks post infection[77,83,90,96]. However, delayed quantitative or qualitative changes in these responses may correlate with the onset of protection. In fact, a recent study by Cole *et al.*[97] showed that the evolution of protective immunity in monkeys inoculated with attenuated SIV strains was associated with a complex and lengthy maturation of antibody responses over the first six to eight months postinoculation, as reflected in progressive changes in the dependence of antibodies on antigen conformation as well as avidity properties. A protective role for virus specific antibodies was also suggested by Clements *et al.*[77]. Here, passive transfer of serum from animals infected with attenuated SIV and resistant against pathogenic SIV challenge, protected two out of four naive animals. Unfortunately, the difference in protective capacity of sera collected at week 25 or 27, when the vaccinated monkeys were still unprotected from challenge, and week 35, when monkeys were protected, was not evaluated in this study. Further support for a protective role of neutralizing antibodies comes from the study by Wyand *et al.*[63], in which the titer of VN antibodies only correlated with protection when measured against a primary stock of SIV_{mac}251 and not when measured against a laboratory-passaged one. The fact that many vaccine studies have used laboratory

adapted strains to test for VN antibodies, may explain, at least in part, the failure to observe a correlation between neutralizing antibodies and protection.

Although these studies collectively provide strong indications that neutralizing antibodies play a role in the protective immunity induced by live-attenuated viruses, other studies suggest that they are at least not the only determinant. In particular, the protection observed against challenge viruses that contain unrelated envelope glycoproteins can hardly be explained by VN antibodies. This is the case, for example, with SIV infected monkeys that were fully protected against a SHIV challenge containing antigenically unrelated HIV-1 Env[76,78,93]. In reciprocal experiments one out of four SHIV infected macaques resisted intravenous SIV_{mac} challenge[98], while three out of five and two out of four animals were protected against intravaginal SIV_{mac}[99] and intrarectal SIV_{sm}[100] challenge, respectively. Similar experiments showed that pre-infection with HIV-2 prevented or delayed the development of SIV-induced immunodeficiency[92,101]. Moreover, two out of four macaques which had remained seronegative after intravenous exposure to HIV-2, were protected against SIV_{sm} challenge[102]. In humans, cross-protection against HIV-1 infection in a group of high risk HIV-2 infected women has been suggested on the basis of epidemiological data[103].

In some studies[78,99,102] but not in others[83,100], protection was associated with the detection of CTL. However, as not all studies included CTL analyses, and a lot of different detection methods with variable sensitivities are used[104], the role of CTL in the protection induced by live- attenuated viruses remains unclear. A recent study by Zou *et al.*[105] showed differences in cytokine- and chemokine gene expression in animals infected with different attenuated SIV strains, which may influence the vaccine efficacy measured in vaccination-challenge experiments. Alternatively, protection may be related to mechanisms of viral interference, similar to those described for murine retroviruses[106]. Here, infection of only a fraction of the susceptible host cell population could confer retroviral interference in vivo, leading to the inability of the second virus to infect the animal. Again, the mechanism of interference is difficult to relate to the apparent time-dependent protection induced by live-attenuated virus vaccines observed in the SIV/macaque model.

LIVE VECTOR VACCINE STRATEGIES

The use of a replicating vector carrying the genes coding for the virus antigens under investigation, has the advantage of inducing both antibody and cytotoxic responses, and allows presentation of proteins to the immune system in a more natural way. In addition, epitopes inducing irrelevant or unwanted immune responses can be left out. Furthermore, many of the safety concerns related to the use of whole virus vaccines can be avoided. While candidate live-attenuated lentivirus vaccines establish systemic and permanent infection, live viral vector vaccines generally replicate locally and for a short period of time, yielding a high antigen load per cell at the site of replication.

Poxviruses are amongst the most extensively evaluated live vectors in lentivirus

vaccine research. Although useful in animal models, the use of conventional vaccinia viruses in immunosuppressed individuals is considered unsafe due to their inherent pathogenicity in the immunocompromised. Attenuated vaccinia viruses, like "NYVAC" and modified vaccinia virus Ankara (MVA), or avian poxviruses, like "ALVAC", which do not or very restrictedly replicate in mammalian cells, may provide good immunity with improved safety when used as poxvirus vectors for lentivirus antigens[107,109]. As recombinant poxvirus vectors by themselves are poor inducers of neutralizing antibodies, a prime/boost combination is often used, in which immunization with recombinant live poxvirus is followed by a subunit protein boost[107,109].

In the macaque model, protection induced by such prime/boost protocols has been limited, and only against viruses of low pathogenicity. Protection from infection against homologous HIV-2 was shown in animals immunized multiple times with ALVAC and NYVAC-based vaccines expressing HIV-2 Env-Gag-Pol, and boosted with HIV-2 Env or V3 peptides[110,112]. Protection was long-lasting as five out of seven protected monkeys resisted homologous rechallenge six months later without the need for additional boosts[110]. No correlation was observed between protection and VN antibody titers or lymphoproliferative and CTL responses. Monkeys immunized with ALVAC expressing HIV-2 Env-Gag-Pol alone were not protected against infection[111]. A simplified, three-immunizations regimen administered over a shortened period of five rather than eighteen months, delayed but did not prevent infection[112]. These findings indicate that a longer immunization regimen, use of larger priming doses, additional subunit boosts, or a boost with native rather than recombinant HIV-2 Env[113], may all be advantageous for the induction of protective immunity. In addition, cross-protection against HIV-2 challenge was shown in three out of eight rhesus macaques immunized with a prime/boost protocol using HIV-1 Env or Env-Gag-Pol[114].

A prime/boost regimen with SIV_{mne} Env protected macaques against challenge with homologous SIV_{mne}[115]. However, protection was only partial, as the detection of a CTL response to SIV proteins not included in the vaccine, indicated that infection was not prevented[116]. The same immunization protocol also failed to protect animals against uncloned SIV_{mne}[117]. When similar vaccine strategies were tested against SIV_{mac} or SIV_{sm}, which are more pathogenic than SIV_{mne}, at best abortive infection or a reduction in viral load were observed[58,60,118,122]. No beneficial effect of vaccinia virus Env priming[58,59,122] or concurrent immunization with SIV-Gag[59] was observed, when compared to vaccination with recombinant Env alone, suggesting a significant role for VN antibodies in protective immunity. Monkeys immunized with non-replicative, attenuated MVA expressing Env-Gag-Pol, showed significantly lower levels of plasma viraemia than those immunized with a replication competent conventional vaccinia virus expressing the same SIV genes[118]. This counterintuitive result may be explained by the absence in MVA of immune-suppressive genes present in conventional vaccinia virus[109].

The most compelling evidence so far that SIV-specific CTL responses suppress virus replication comes also from a study that used recombinant vaccinia virus for immunization. Partly on the basis of the observation that Nef-specific CTL had been

detected in animals vaccinated with attenuated Nef-deleted SIV[123], macaques were immunized three times at five weeks intervals with a Nef encoding recombinant vaccinia virus[124]. Although six out of seven vaccinated animals became infected after homologous SIV_{mac} challenge, there was a significant inverse correlation between the frequency of Nef-specific CTL precursors before challenge and cell-associated viral load after challenge. The animal with the highest Nef-specific CTL precursor frequency was protected from challenge. Another study, in which Env was also excluded from the vaccine preparation and consequently the induction of VN antibodies was prevented, used *Mycobacterium bovis* BCG-SIV Gag for priming and a Gag-CTL epitope in liposomes for boosting[125]. In this case no protection was observed from SIV_{mne} infection. Early expression of Nef in the viral replication cycle, higher CTL precursor frequencies and the induction of multiple rather than single epitope specific CTL may all explain the difference in protection. Nevertheless, there is evidence to suggest that CTL responses with limited specificities may influence the course of HIV-1 or SIV infection[90,126,128].

Only few other live vector systems have been tested in SIV vaccine studies. Immunization with recombinant Semliki Forest Virus (SFV) expressing SIV-Env protected all four pig-tailed macaques from acute lethal $SIV_{smmPBj14}$ disease. There was no apparent correlation between survival and levels of cell-associated virus- or provirus load, or antiviral immunological parameters. Finally, immunization with *Salmonella typhimurium* expressing HIV-2 Gag and Env, either alone or in combination with recombinant gp160, did not protect against intravenous HIV-2 challenge[110].

NUCLEIC ACID BASED VACCINE STRATEGIES

Perhaps the most intriguing amongst the new candidate vaccines are those based on nucleic acid, or naked DNA alone (reviewed in[129]). Direct intramuscular or intradermal injections of DNA have been shown to allow the production of lentiviral antigens which apparently enter directly into the MHC class-I restricted antigen-processing pathway, resulting in the induction of specific $CD8^+$ CTL[130]. The induction of humoral immune responses has been observed to be much more inconsistent. In addition, the ability of nucleic acid vaccines to induce mucosal immune responses has been demonstrated[131]. Although not yet proven, integration of the retroviral DNA in the host genome as well as *in vivo* recombination, may be a serious drawback of the use of nucleic acid based lentivirus vaccines in humans[129].

Lu *et al.*[132] immunized rhesus macaques with a cocktail of five DNA plasmids encoding defective SIV_{mac} particles and several variants of SIV_{mac} Env. Following immunization by combinations of different routes, immunized animals generated $CD8^+$ MHC class I restricted CTL responses directed against Gag and Env, as well as antibodies neutralizing laboratory-adapted SIV strains. Although CTL activity could be detected up to eight weeks after the last immunization, neutralizing antibodies declined rapidly over this period. The animals were not protected against challenge with pathogenic SIV_{mac}. Recently, however, a study by Letvin *et al.*[133] demonstrated that priming with $HIV\text{-}1_{HXBc2}$ Env DNA, followed by a boost with the same Env DNA plus

recombinant HIV-1_{IIIb} Env, protected monkeys from SHIV containing homologous HIV-1 Env. Clearly, nucleic acid immunization should not only be considered promising as a new vaccination strategy, but also as an instrument to elucidate immune mechanims leading to protective immunity.

COMMON CORRELATES OF PROTECTION AND FUTURE PROSPECTS

The present belief that the host immune response may be able to control HIV-1 replication even if it fails to prevent infection, has shed new light on previous vaccine studies and stimulated the development of new vaccine designs. Immunization with live-attenuated viruses has resulted in total protection, transient infection, decreased viral load, protection from disease or no protection at all. Variables which appeared to influence the challenge outcome included time of challenge relative to time of vaccination, as well as the degree and nature of the attenuation. Different levels of protection have also been induced by either prime/boost protocols based upon recombinant vaccinia virus priming followed by a subunit protein boost, or by subunit immunizations alone. In these experiments, the presence of Env was generally required and multiple immunizations over a prolonged period of time were associated with higher levels of protection. The protection observed in these systems is probably the result of a fine balance between the induced immune responses and characteristics intrinsic to the challenge virus. This is particularly evident with protocols yielding total protection against low-dose, low-pathogenic viruses, which gave at best only reduction in virus load using high-doses, or pathogenic viruses. A complicating factor in drawing conclusions from all these macaque experiments, is the inevitable low number of monkeys with diverse genetic backgrounds per experiment.

An association between high VN antibody titers and protection has been observed in several studies[58,63,77,134]. Overall data, however, indicate a lack of correlation between neutralizing antibodies and protection from infection with either HIV-2 or SIV. This also holds true for studies which used vaccines based on Env alone[21,59,61,110,111,114] or in which the presence of Env was a prerequisite for protection[117]. Whether there is a true lack of correlation between protection and induction of VN antibodies or that technical limitations such as test insensitivity, use of laboratory adapted virus strains rather than primary virus isolates, and of T-cell lines rather than PBMC as target cells, have obscured the possible correlation remains to be determined.

Another question that remains unanswered is why, if at al Env is essential for the induction of protection, recombinant vaccines often do not protect against SIV challenge while live-attenuated vaccines, which induce about 10 fold lower Env specific antibody titers do. One explanation may be that native Env induces a better quality of antibody responses with respect to affinity and epitope specificity than recombinant produced Env. Several approaches may improve the immunogenicity of recombinant Env:

* Immunization with oligomeric Env. HIV-1/SIV Env is present on both viral and cellular membranes as an oligomeric structure[135,136], while, in contrast, monomeric recombinant Env is often used for immunization. Differences in conformation and epitope exposure

between monomeric and oligomeric Env may lead to induction of a different spectrum of antibody responses upon immunization[137,139]. For HIV-1, the relative affinity of antibodies with oligomeric but not monomeric Env has been shown to correlate with their ability to neutralize primary isolates[140]. Immune responses against regions showing similarities in structure rather than sequence may possibly also play a role in the heterologous protection observed in some vaccine experiments[63,76,78,93,101].

* Deletion of epitopes that lead to enhanced infection[39,40,141,142] or mask important neutralizing epitopes[143].

* Immunization with the epitopes recognized by HIV-1 monoclonal antibodies that neutralize primary isolates[144,145]. Such a strategy may direct the immune response to *in vivo* relevant antigenic sites.

* The use of multiple booster immunizations or highly effective adjuvants. Such an approach is supported by the observation that the magnitude and breadth of neutralizing antibody responses seem to increase over time in HIV-1 and SIV infection[77,97]. Furthermore, the success of immunization regimens tends to increase with the use of longer immunization schedules[63,77,112,118].

In light of these considerations, it would be advantageous to use HIV-1 Env rather than SIV Env for the evaluation of the protective capactity of candidate vaccines in macaques. Firstly, the structure of HIV-1 Env is better defined than that of SIV Env. In addition, more tools are available to characterize the humoral HIV-1 Env specific immune response. Finally, important differences in antigenic make-up have been observed between HIV-1 and SIV Env[147]. With the advent of the newly developed SHIV/macaque models, HIV-1 Env may indeed be used as an antigen in challenge experiments in macaques. This model may also addres the finding that VN antibody responses generally correlate with protection in the HIV-1/chimpanzee model[148], but not in the SIV - HIV-2/macaque model.

Although increasing evidence indicates a role for CTL in controlling HIV-1 infection[23,149,150], their role in vaccine-induced protection remains unclear. As both a decrease in initial viral load and prevention of disease development have become newly defined, additional goals for lentiviral vaccine development, induction of CTL which may eliminate virus infected cells, does attract increasing attention. The most compelling evidence for a beneficial role of CTL in SIV/macaque models comes from the work of Gallimore *et al.*[124]. They showed that the SIV Nef-specific CTL precursor frequency induced by vaccinia virus immunization, correlated with the virus load after challenge. This is, however, in contrast with data we have generated, showing that induction of SIV Nef, as well as Env and Gag specific CTL precursors, did not improve the outcome of a similar challenge. Although the two studies detected similar Nef specific CTL precursor frequencies on the day of challenge, frequencies generated in different laboratories using different assay systems can not directly be compared. Other explanations for the observed difference in protection may be found in the genetic background of the monkeys, immunization with vaccinia viruses expressing the entire Nef protein versus immunization with Nef lipopeptides, or the induction of additional CD8-associated virus

suppressive factors. In general, proteins expressed early in the viral replication cycle, may be particularly good targets for protective CTLs, because they may be expected to kill infected cells before newly generated virus is released. Interestingly, we have recently found evidence for an association between Rev and Tat specific CTL precursor frequency and long-term survival in both HIV-1 infected individuals[150] and SIV_{mac} infected macaques[151]. Clearly, more detailed analyses of CTL responses with respect to epitope specificity and precursor frequencies, as well as inclusion of the early expressed proteins in CTL analyses, may be relevant to unravel potential correlates of protection.

Recently, the contribution of soluble factors such as CD8-associated suppressive factors[41], chemokines[42] and different cytokines[43,44,152] to the control of virus replication has become the subject of intense investigation. In one study, protection against mucosal SIV_{mac} challenge was shown to correlate with the presence of CD8-derived suppressive factors and ß-chemokines[74]. However, it is not clear to what extent these factors can be induced by immunization, and whether their presence may be sufficiently long-lasting. $CD8^+$ cell mediated suppressor activity has also been detected in naive monkeys and seronegative humans[153]. This may even raise the possibility that stimulating non-HIV-specific immune responses may induce protective immunity against HIV-1 infection. Efforts should be made to examine the role of these soluble factors *in vivo* and possibilities to induce them by vaccination. In addition, co-administration of cytokines and the use of novel adjuvant formulations may largely improve our ability to activate the respective specific arms of the immune response[152,154].

CONCLUSION

During the past years, macaque models have provided important information about requirements of protective immunity against immunodeficiency viruses. Vaccine strategies including immunity against multiple antigens and targeting multiple arms of the immune system, should be considered the most promising. As most HIV-1 infections are sexually transmitted, the development of protective mucosal vaccine strategies seems to be urgently needed. In this respect, the induction of potent local cell mediated immunity seems crucial. In addition, new insights in HIV-1 second receptor usage, Env structure and mechanisms of virus entry may lead to improved designs of future vaccine strategies. The newly developed chimeric SHIV macaque models may help to test HIV-1 antigen based vaccines, before their application in clinical phase 1 and 2 human trials. Finally, the use of plasma viral load as a surrogate marker for disease progression may speed up the evaluation of new vaccine strategies, which do not necesarily focus on the induction of "sterilizing immunity".

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SUMMARIZING REMARKS

The studies described in this thesis have used SIV_{mac} and SHIV infection of cynomolgus monkeys as models to study HIV-1 vaccine development and pathogenesis. They have taken advantage of the similarities between the genomic organization and biology of SIV and HIV-1, and of the possibilities to define certain parameters of SIV infection in macaque experiments, such as virus strain, dose, route of inoculation. In addition, organ materials and blood samples can be taken at regular intervals.

In the design of an HIV-1 vaccine, several problems directly related to the biology of lentivirus infections should be taken into account (see Chapter 1). The virus is predominantly transmitted by sexual intercourse, blood-blood contact, and vertically from mother-to-child. Vaccination should ideally prevent all these different modes of transmission. At the cellular level, virus infection is initiated by the interaction between the viral envelope glycoproteins with their primary and secondary receptors. After entry, the replication cycle can be arrested at several stages (i.e. prior to or directly after integration into the host genome, or after expression of the early proteins Rev, Tat, Nef but prior to the expression of structural proteins). By "hiding" itself in the cell, the virus interferes with the complex process of recognition and elimination by the host immune system. In the infected individual, cells of the immune system are infected and the virus spreads via cell-associated and via cell-free routes. The result is a systemic infection, which can even reach immune-privileged sites like the brain.

A vaccine development approach that targets both the cellular and the humoral arms of the immune system seems the most promising: VN antibodies may prevent the transmission of cell-free virus, whereas cellular and in particular CTL responses, may eliminate cells that have already become infected. The envelope proteins are the only viral proteins which are the targets of both arms of the immune system. Therefore, we first evaluated the protective effect of an SIV Env-based subunit vaccine. *In vivo*, a large variation is found in the Env amino acid sequence, even within a patient[157]. Furthermore, *in vitro* culturing has shown to cause changes in both the sequence and the biological characteristics of Env[62]. In addition, Env is highly glycosylated, has a complex secondary and tertiary structure, and its conformation changes during the successive stages of virus entry[158,159]. Moreover, most of the VN antibodies detected in sera from infected humans and monkeys recognize conformational epitopes on Env[160].

In an attempt to address these issues in the design of an Env-based vaccine, the following strategy was adopted:

* Envelope sequences were obtained from PBMC of macaques shortly after infection with SIV_{mac32H}, in order to select for envelope sequences that are related to viruses actually establishing the infection.
* Envelope sequences were obtained by direct PCR on SIV infected PBMC, without an

in vitro culture step.

* The antigenicity and immunogenicity of two related envelope proteins of SIV_{mac32H} with different *in vivo* passage histories was analyzed.

* In an attempt to express Env in a native form, an eucaryotic expression system, rVV, was chosen.

Using this strategy, two SIV envelope sequences were selected and expressed by rVV. These sequences were representative for the envelope sequences present shortly after infection in either an SIV_{mac32H} infected monkey or a monkey infected with the same virus that had been passaged in another monkey for 11 months. (Chapter 2, section 2.1). Using a panel of 15 monoclonal antibodies recognizing both conformational and linear epitopes, the antigenicity of the first Env was shown to be similar to that of virus-derived Env. The Env derived from the *in vivo* passaged virus proved to be poorly recognized by a group of VN monoclonal antibodies directed to a conformation dependent epitope on the surface protein. It may be speculated that this envelope sequence was involved in escape from virus neutralization by antibodies, developed during the 11 months of *in vivo* infection. Immunization with recombinant SIV-Env presented in iscoms induced slightly higher antibody titers in rats than Quil A or MDP-tsl adjuvanted SIV-Env. The use of iscoms was considered to have the additional advantage of inducing $CD8^+$ CTL. Therefore, this preparation was selected for further vaccination studies.

To obtain a vaccine preparation free of residual vaccinia virus infectivity, paraformaldehyde (PFA) and binary ethylenimine (BEI) treatment was applied. Both treatments had no detectable effect on the antigenicity of the expressed SIV Env (section 2.2). In addition, it was shown that the anti-viral antibody titers in rats, including the VN antibodytiter, induced by either PFA or BEI treated SIV-Env incorporated into iscoms, were of the same order of magnitude.

In the subsequent vaccination-challenge study (Chapter 3, section 3.1), cynomolgus monkeys were immunized with the SIV-Env iscom preparations. Vaccine induced total- and VN antibody titers, were comparable to those found in SIV_{mac} infected macaques. However, upon intravenous challenge with the J5 molecular clone of SIV_{mac32H}, none of the monkeys were protected against infection, or exhibited a significant reduction in cell-associated virus load immediately after infection, as compared to sham vaccinated monkeys. To broaden the cellular immune response, monkeys were co-immunized with p27-Gag iscoms and Nef-lipopeptides in an additional vaccination group. Although relatively high Env-, Gag- and Nef-specific CTLp frequencies were detected at the day of challenge, also in this group of monkeys protection from challenge could not be demonstrated, illustrating the difficulty in conferring protection against lentivirus infection.

The SIV-Env differs in certain important structural and biological properties from the HIV-1 Env, and differences exist in the immune response to the respective Envs[147]. Therefore, we performed an additional vaccination-challenge experiment in which cynomolgus monkeys were immunized with different HIV-1 Envs and challenged with a

SIV - HIV-1 chimeric virus (Chapter 3, section 3.2). In this experiment, HIV-1 Envs of an SI and an NSI virus recovered from the blood of an HIV-1 seropositive individual were used for immunization. Upon challenge with a SHIV carrying the Env of an HIV-1 molecular clone derived from the same seropositive individual, one NSI-Env immunized monkey 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-specific antibody responses and the inability to detect SHIV by virus isolation and PCR. However, the pattern of SHIV-specific CTLp in the apparently protected animals after challenge, indicated that also in these animals virus replication had not been prevented completely. In both control monkeys, SHIV infection induced specific antibodies and CTLp. However, levels of virus replication were low, as during the 13 weeks of follow-up, SHIV could only be detected in one of the two control monkeys. These data show that although vaccination with HIV-1 Env-based vaccines did not prevent infection, it may have been effective in limiting virus replication. As recent data have shown that the viral load early after seroconversion is directly related to disease development[28], the degree of protection observed in these expemiments may be relevant for future HIV-1 vaccine development studies. In addition, most HIV-1 transmissions occur via the mucosal route with a relatively low efficacy of transmission. This also indicates that even the limited degree of protection found in these experiments, may be important to control virus replication and thus spread of HIV-1 between individuals.

The concept of a vaccine that, once infection has been established clears the virus or keeps the viral load low rather than completely preventing infection, receives growing support. Such concept implies that the induction of cellular immune responses and in particular CTL responses should be a major target in vaccine development. In order to develop a better understanding of the role of CTL in controlling lentivirus infections, we have investigated the relationship between SIV replication and CTL responses (Chapter 4). In general, the CTLp frequencies found proved to be a reflection of the observed virus loads. In line with this observation, sequestration of the virus within lymphoid organs was mirrored by compartmentalization of antiviral CTL to the sites of infection. Effective virus containment, as indicated by the absence of detectable virus in PBMC, was associated with declining CTLp frequencies. However, it remains to be determined whether CTL activity is responsible for the observed virus containment, or conversely, whether the kinetics of CTL only reflect *in vivo* antigenic stimulation. In any case, the observed association between viral load on the one hand and CTLp on the other hand, suggests that SIV specific memory CTL decline in the periphery under conditions of limited antigenic stimulation. This finding may have important implications for the design of a vaccine aiming at the induction of long-lasting protective CTL responses.

In the course of these studies, a monkey showing effective virus containment after primary infection was re-challenged with the same virus 20 months later. Upon rechallenge, limited virus replication was shown. Either reactivation of sequestered virus, as is also seen in studies using live attenuated virus vaccines[92,93], or replication of

the virus used for re-challenge may be at the basis of this observation. In both cases, however, it remains remarkable that an animal infected for 20 months and disposing a whole series of both humoral and cellular SIV specific immune responses, is at least partially susceptible to reinfection. Again this is a clear indication of the difficulties encountered on the way to induce protection against lentivirus infections. In the same animal, a variant virus carrying a mutation in the sequence of a functionally immunodominant Gag-specific CTL emerged. This variant virus was only poorly recognized by the prototype epitope specific CTL, indicating *in vivo* CTL pressure on virus replication.

Collectively, the results we and others have generated in vaccination-challenge experiments in macaque models, support the idea that the successfull induction of protective immunity, should probably take advantage of several the humoral and cellular immune responses that may contribute to this objective (Chapter 5). However, the qualitative and quantitative requirements to induce these responses appear much more complex than originally envisaged. The complex oligomeric structure of Env, which changes during the different stages of virus entry, Env variability, the possible interference by infectivity enhancing mechanisms, the masking of potentially important Env epitopes from the immune system, and the difficulties encountered in inducing high avidity VN antibodies, may all have been major obstacles in the development of a vaccine based on whole recombinant Env. Therefore, the rational design of a successful candidate HIV-1 vaccine will probably benefit from further studies on the ability of specific antigenic sites of Env to induce broadly neutralizing antibodies against primary isolates. Vaccination with selected epitopes recognized by such broadly neutralizing antibodies may perhaps have a better chance to induce protection than immunization with whole Env. Similarly, the different results obtained by vaccines targeting the cellular response (Chapter 3 and[125,161]), indicate that subtle differences in epitope or protein recognition may have large implications for vaccine outcome. In this respect, the association between Rev- and Tat-specific CTL responses and long term survival in HIV-1 infected humans and SIV-infected macaques may be relevant[150,151]. Currently experiments are ongoing to evaluate the protective potential of vaccine-induced SIV Rev- and Tat-specific CTL against SIV challenge.

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NEDERLANDSE SAMENVATTING

AIDS

HIV-1 (human immunodeficienly virus type 1) werd zo'n 15 jaar geleden ontdekt. Sindsdien heeft het virus zich over de wereld verspreidt met een alarmerende snelheid. Geschat wordt dat in het jaar 2000 tussen de 30 en 40 miljoen mensen met HIV-1 geïnfecteerd zullen zijn. De infectie met HIV-1 gaat aanvankelijk vaak ongemerkt voorbij, maar kan ook gepaard gaan met griep-achtige verschijnselen. Daarna volgt een fase zonder ziekteverschijnselen die kan variëren van 3 tot meer dan 12 jaar. In deze fase ondermijnt het virus echter wel het immuunsysteem, hetgeen uiteindelijk leidt tot een compleet falen, waarna de eerste verschijnselen van AIDS (acquired immunodeficiency syndrome) zich manifesteren. AIDS wordt onder andere gekenmerkt door het feit dat allerlei bacteriën, schimmels en virussen die onder normale omstandigheden geen ziekte veroorzaken nu wel tot ziekte en uiteindelijk de dood kunnen leiden. Ook kunnen zich bepaalde vormen van kanker ontwikkelen. Op dit moment, hebben wereldwijd al meer dan 6 miljoen mensen het AIDS-stadium bereikt. Recente successen met de behandeling van HIV-1 geïnfecteerde mensen hebben een zeker optimisme gewekt over een mogelijke totale genezing. Echter, het grootschalig gebruik van deze middelen in ontwikkelingslanden, waar meer dan 90% van de HIV-1 geïnfecteerde mensen leven is erg problematisch, onder andere door de hoge kosten, het ingewikkelde doseringsschema en de ernstige bijwerkingen van deze middelen. De ontwikkeling van een effectief vaccin, dat bij voorkeur de infectie kan voorkomen, wordt daarom algemeen gezien als de enige manier om de verspreiding van HIV-1 te kunnen controleren.

HIV-1 en de specifieke afweer

HIV-1 is een bolvormig virus met een doorsnede van ongeveer 0,0001 mm (zie hoofdstuk 1, Figuur 3). In het virus partikel bevinden zich een aantal virus eiwitten (bv. het Gag en Nef eiwit) en het genetisch materiaal, het RNA. Het virus partikel wordt omgeven door een membraan, waarin zich de envelope-eiwitten bevinden. Het virus gebruikt de envelope-eiwitten om aan een cel te binden, en deze vervolgens binnen te dringen. Door het afweersysteem van de gastheer worden onder andere antistoffen gemaakt. Sommige antistoffen kunnen door bijvoorbeeld binding aan de envelope-eiwitten van het virus, infectie van een cel verhinderen. Ze worden daarom virus-neutralizerende antistoffen genoemd. Naast antistoffen heeft het immuunsysteem nog andere middelen om een virus-infectie te bestrijden. In dit opzicht zijn waarschijnlijk vooral virus specifieke T cellen, waaronder de cytotoxische T cellen (CTLs) erg belangrijk. Wanneer een cel geïnfecteerd word met een virus, gaat die cel virus-eiwitten maken. Stukjes van deze eiwitten komen aan de buitenkant van de geïnfecteerde cel. Deze stukjes worden herkend door CTLs, die vervolgens de virus-geïnfecteerde cel kunnen doden.

Het onderzoek

Doelstelling. De studies beschreven in dit proefschrift zijn erop gericht meer inzicht te krijgen in het infectie verloop van een HIV-1 infectie en om uiteindelijk een vaccin tegen HIV-1 in mensen te kunnen ontwikkelen.

Model systeem. De in dit proefschrift beschreven studies gebruiken infectie van apen met SIV of SHIV als model voor HIV-1 infectie bij de mens. SIV (simian immunodeficiency virus) is het ape-equivalent van HIV. SIV infecteert ook cellen van het immuunsysteem en veroorzaakt een vergelijkbaar infectie-verloop in bepaalde apesoorten, de makaken, als HIV-1 in mensen. De periode tussen het moment van SIV infectie tot de ontwikkeling van AIDS duurt meestal maar 1 tot 4 jaar. Met behulp van recombinant DNA technieken zijn nu ook hybride virussen gemaakt, SHIVs, die in feite een kruising tussen SIV en HIV zijn.

Het onderzoek. De vaccinstudies beschreven in dit proefschrift hebben als achterliggende gedachte dat een vaccin dat zowel humorale (in het bijzonder virus neutralizerende antistoffen) als cellulaire (in het bijzonder CTLs) immuniteit kan opwekken, de meeste kans van slagen heeft om bescherming te bieden tegen een HIV/SIV infectie. Het envelope-eiwit is eigenlijk het enige virus eiwit dat een effectief doelwit van zowel de humorale als de cellulaire immuunrespons kan zijn. Daarom is het beschermende effect van een kandidaat vaccin gebaseerd op dit envelope-eiwit als eerste bestudeerd.

Met behulp van recombinant DNA technieken is het nucleïnezuur behorend bij het envelope-eiwit uit het bloed van SIV geïnfecteerde apen geïsoleerd. Vervolgens is dit gebruikt om het envelope-eiwit te produceren (hoofdstuk 2). Om zowel de kwantiteit als de kwaliteit van de immuunrespons te verhogen, zijn de envelope-eiwitten vervolgens ingebouwd in zgn. iscoms (immune stimulating complexes).

Immunisatie van apen met het envelope-eiwit in iscoms leidde zowel tot het aanmaken van virus-neutralizerende antistoffen als envelope-specifieke CTLs. Helaas bleek dit niet voldoende om infectie van de apen met SIV te voorkomen (hoofdstuk 3.1). Om de opwekking van virus specifieke CTLs te stimuleren, werden nog twee andere SIV eiwitten of delen daarvan, Gag en Nef, aan het vaccin preparaat toegevoegd. Ook dit bleek niet voldoende om de apen te beschermen tegen SIV infectie .

In bepaalde opzichten blijkt het SIV envelope-eiwit te verschillen van het HIV-1 envelope-eiwit. Daarom zijn ook apen met verschillende HIV-1 envelope-eiwitten geïmmuniseerd (hoofdstuk 3.2). Vervolgens werden deze apen blootgesteld aan infectie met SHIV. In dit virus is het genetisch materiaal coderend voor o.a. het envelope-eiwit van SIV vervangen door het corresponderende genetisch materiaal van HIV-1. Vaccinatie van apen met de HIV-1 envelope-iscoms bleek infectie niet te kunnen

voorkomen, maar in dit experiment werden wel aanwijzingen gevonden voor een verlaging van de graad van infectie. De hoeveelheid circulerend virus vlak na infectie lijkt een voorspellende waarde te hebben voor de lengte van de periode tot wanneer zich AIDS ontwikkelt. Een door vaccinatie veroorzaakte verlaging van de hoeveelheid virus, kan daardoor wellicht toch een positieve invloed op het uiteindelijke ziekteverloop hebben.

In het meest ideale geval zal vaccinatie tegen een virus, infectie door dit virus geheel verhinderen. Als 'second best', wordt niet de infectie maar wel de ontwikkeling van ziekte voorkomen. In dit licht lijkt wederom de al eerder genoemde cellulaire immuunrespons, met name CTLs, van belang, aangezien CTLs een eenmaal geïnfecteerde cel kunnen opruimen.

Om inzicht te krijgen in de rol van CTLs op het infectie verloop, werd de samenhang tussen de hoeveelheid virus en de hoogte van de CTL respons bestudeerd in SIV geïnfecteerde apen (hoofdstuk 4). In het algemeen bleek de CTL respons een direkte afspiegeling van de hoeveelheid virus in het bloed of in het onderzochte orgaan; bij aanwezigheid van veel virus bleek ook de CTL respons hoog, en vice versa. Het is echter nog niet duidelijk of er een causaal verband bestaat tussen de hoeveelheid CTLs en de mate waarin de virus infectie gecontroleerd wordt. Wel suggereert het gevonden verband dat wanneer het immuunsysteem niet constant aan virus eiwitten blootgesteld wordt, het aantal virus specifieke CTLs zal afnemen. Dit kan van belang zijn voor de effectiviteit van een toekomstig vaccin, omdat doorgaans bij vaccinatie het lichaam slechts voor een relatief beperkte periode aan virus eiwitten wordt blootgesteld.

Conclusie

De studies beschreven dit proefschrift, evenals de studies uitgevoerd door andere onderzoeksgroepen, geven gezamenlijk aan dat aan zeer strenge eisen betreffende zowel de kwaliteit als de kwantiteit van de opgewekte immuunrespons voldaan moet worden om bescherming tegen HIV/SIV infectie of de hieraan gerelateerde ziekte te bewerkstelligen (hoofdstuk 5). Voor het efficient opwekken van breed-neutralizerende antistoffen, is waarschijnlijk meer fundamenteel onderzoek nodig naar de struktuur van het envelope-eiwit en de invloed ervan op de immuunrespons. Tegelijkertijd lijken subtiele verschillen in herkenning van virus eiwitten door CTLs belangrijk te zijn voor hun eventuele beschermende effect. In dit opzicht is het van belang dat de aanwezigheid van CTLs gericht tegen twee kleine HIV-1 eiwitten, Rev en Tat, gecorreleerd is met een langere overlevingsduur van HIV-1 geïnfecteerde mensen. Op dit moment is daarom een vervolg-experiment op de studies in dit proefschrift gestart, dat het beschermende effect van Rev- en Tat- specifieke CTLs tegen SIV infectie bestudeerd.

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Ellen

CURRICULUM VITAE

De auteur van dit proefschrift werd of 24 september 1968 geboren te Tubbergen. Aan het Twents Carmellyceum te Oldenzaal werd in 1986 het VWO diploma behaald. In aansluiting hierop werd gestart met de studie Medische Biologie aan de Rijksuniversiteit Utrecht. De bijvak stage werd uitgevoerd op het Hubrecht Laboratorium der KNAW te Utrecht (Nederlands Instituut voor Ontwikkelingsbiologie, dr. W. Kruijer en dr. J. Schoorlemmer). De hoofdvak stage vond plaats op de afdeling Virologie van de faculteit Diergeneeskunde van de Rijksuniversiteit Utrecht (prof. dr. P.J.M. Rottier en dr. E. Verschoor). Het doctoraalexamen werd behaald in april 1991. In mei 1991 werd gestart met het hier beschreven promotie-onderzoek, allereerst als wetenschappelijk medewerker van het Laboratorium voor Immunobiologie van het Rijksinstituut voor Volksgezondheid en Milieuhygiëne te Bilthoven, en vanaf september 1994 als wetenschappelijk medewerker van de afdeling Virologie van de Erasmus Universiteit Rotterdam, in beide gevallen onder leiding van prof. dr. A.D.M.E. Osterhaus. Vanaf 15 september 1997 is zij werkzaam als Medical Writer binnen de afdeling Clinical Trial Operations van N.V. Organon te Oss.

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