Repeated exposure of rhesus macaques to low doses of simian immunodeficiency virus (SIV) did not protect them against the consequences of a high-dose SIV challenge

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As part of an in vivo titration study of the macaque simian immunodeficiency virus (SIVmac) strain 251/spl, macaques were inoculated intravenously with various dilutions of this infectious SIVmac. Seven animals received dilutions from $10^{-3}$ to $10^{-6}$ of SIVmac$_{251}$/spl. Two monkeys infected with the $10^{-3}$ dilution of SIVmac exhibited a productive infection as indicated by seroconversion, detection of genomic RNA and proviral DNA and positive virus isolation. These animals showed a cytotoxic T cell (CTL) response against different SIVmac proteins without any measurable T cell proliferation. The five macaques receiving higher virus dilutions did not seroconvert and were negative for both viral RNA and for infectious virus, although proviral DNA was detected in their peripheral blood mononuclear cells. In contrast to the animals receiving the $10^{-5}$ virus dilution, these five silently infected monkeys developed an SIV-specific proliferative T cell response but SIV-specific CTL could not be observed. The SIV-specific T cell proliferation of the silently infected animals could be boosted by a second low-dose exposure with a $10^{-4}$ or $10^{-5}$ dilution of SIVmac$_{251}$/spl. The virological status of the animals was not changed following this second virus inoculation. Four months later these macaques were challenged intravenously with 2 ml of a $10^{-4}$ dilution of SIVmac$_{251}$/spl containing 10 monkey ID$_{50}$. After this challenge all SIV-pre-exposed animals and three naive controls became productively infected. In addition, all infected animals developed typical signs of an immunodeficiency within 6 months after infection. These observations indicate that macaques infected silently by a low-dose exposure to infectious virus generated a virus-specific cellular immune response. However, SIV-specific T cell proliferation alone could not protect the monkeys against an intravenous challenge with SIVmac and the subsequent development of AIDS-like symptoms.

Introduction

Rhesus macaques develop an AIDS-like disease after infection with simian immunodeficiency virus (SIV) (Baskerville et al., 1990, Letvin & King, 1990). This SIV–macaque model is most reliable and widely used for AIDS pathogenesis and vaccine studies. Several research groups have successfully prevented the experimental SIV infection of macaques with candidate vaccines (Desrosiers et al., 1989; Murphey-Corb et al., 1989; Stahl-Hennig et al., 1992). However, any clear correlation between the induction of humoral or cell-mediated immunity (CMI) and protection against an experimental infection is still lacking. Therefore, vaccine experiments have to be carried out in which one arm of the immune response becomes activated whereas the other remains inactive. Antibody transfer experiments have been conducted to evaluate the role of humoral immunity for protection against human immunodeficiency virus 1 (HIV-1) or SIV infection but contradictory results have been reported (Emini et al., 1992; Putkonen et al., 1991; Kent et al., 1994, Coulibaly et al., in press). Important information about cellular immune mechanisms involved in protection should arise from studies of individuals exposed to HIV without subsequent seroconversion. Such uninfected individuals include people at high risk of HIV infection (for review see Clerici & Shearer, 1993) and people silently infected for a longer period of time (Ranki et al., 1987; Imagawa et al., 1989; Vaira et al., 1990). As demonstrated lately, many of the HIV-exposed
but uninfected individuals have developed HIV-specific CMI despite their lack of seroconversion (Clerici et al., 1992, 1994a). In these studies the HIV-specific CMI seems to induce protection against an HIV infection. Likewise, in persons silently infected with HIV the CMI might efficiently control virus replication (Imagawa et al., 1989). However, in the latter study the cellular immune response has not yet been investigated.

To induce such an immune response and perhaps protection in an animal model, we have repeatedly exposed rhesus monkeys to low doses of defined SIV strains and later challenged them with higher doses of SIV.

The results of such experiments should shed light on the mechanism of protective immunity against HIV or SIV and could be instrumental for designing new vaccine formulations and immunization protocols to prevent HIV infection in man.

**Methods**

*Inoculation of rhesus macaques with SIV stocks.* Eleven rhesus macaques (*Macaca mulatta*; Mm) from the breeding colony of the DPZ were used for the experiment. Animal housing, handling of the macaques, blood sampling and clinical monitoring have been described elsewhere (Stahl-Hennig et al., 1990a). As a part of an in *vitro* titration experiment seven animals were inoculated intravenously with various dilutions of the infectious virus stock SIVmac251/sp1 prepared from the spleen of an SIVmac251/32H-infected monkey (Lüke et al., 1994). Four different virus dilutions ranging from $10^{-3}$ to $10^{-6}$ were used for this first SIVmac251/sp1 inoculation (Table 1). In addition, one more rhesus macaque (Mm1775) was included in our study. This naive control animal was inoculated with 2 ml of a $10^{-4}$ dilution of SIVmac251/32H corresponding to 10 monkey IDs0 (Cranage et al., 1990). Unexpectedly, in contrast to seven other macaques inoculated with the same virus dose Mm1775 was the only one which became positive for proviral DNA, but exhibited neither viraemia nor seroconversion during the whole observation period (Coulibaly et al., in press).

At 24 weeks after the first inoculation, five animals were again exposed to either the $10^{-4}$ or $10^{-5}$ dilution of the SIVmac251/sp1 stock (Table 1). At the same time the animal Mm1775 was inoculated with 1 ml of a $10^{-4}$ dilution of SIVmac251/sp1.

Twelve weeks after the second SIV inoculation all six animals were challenged along with three naive control animals with 2 ml of the $10^{-4}$ dilution of the SIVmac251/32H stock containing 10 monkey IDs0.

**Recovery of replicating virus and proviral sequences.** Virus isolation from monkey peripheral blood mononuclear cells (PBMC) was performed as reported previously (Stahl-Hennig et al., 1992) with slight modifications. Briefly, $3 	imes 10^6$ unstimulated PBMC were separated on Ficoll gradients as described (Stahl-Hennig et al., 1990a) and simultaneously cocultivated with $3 	imes 10^6$ C81-66 cells (Salahuddin et al., 1983) and divided every 3–4 days in a ratio of 1:3. Virus replication became evident by syncytia formation and was confirmed by the detection of viral antigen using an HIV antigen capture assay (Innogenetics, Zwijndrecht, Belgium; formerly Organon Teknika). Cultures were considered virus-negative and discarded if after 4 weeks neither syncytia nor supernatant antigen were detectable.

To determine the cell-associated virus load, PBMC were diluted twofold from $1.25 	imes 10^4$ to 122 cells. Each of the respective dilutions was simultaneously seeded with C81-66 cells at a density of $3 	imes 10^5$ in 24-well plates using one well per dilution. Cultures were divided as described above. The lowest dilution leading to virus replication within 14–16 days after initiation of the cultures was regarded as the endpoint. The virus load was expressed as the number of infectious cells per $1 	imes 10^6$ PBMC.

SIV-specific antibodies appearing either after SIV exposure or SIV challenge were investigated by radioimmunoprecipitation assay (RIPA) and Western blotting as described earlier (Stahl-Hennig et al., 1990a). Pelleted SIVmac251/32H was used for blotting and SIVmac251/32H-infected C81–66 cells were radiolabelled with 1 mCi $[^{35}S]$methionine and cysteine, disrupted with detergent and used as RIPA antigen.

For polymerase chain reaction (PCR), template DNA was prepared from fresh PBMC. About 1 μg of DNA was added to each PCR mixture containing 1.5 mM-Mg$^{2+}$, 0.1% Triton X-100, 200 nmol of each deoxynucleoside triphosphate, 50 pmol of each oligonucleotide primer and 1 U Taq polymerase (Technomara, Fernwald, Germany). DNA was amplified by a nested reaction. The outer SIVmac-specific primer pair comprised nucleotides 1095–1119 (5' TTAGGCATACGACCGGCGGAAAGA 3') and 1592–1569 (5' ATAGGGGGTGCAG-CCC 3').

**Table 1. Inoculation schedule of the rhesus macaques**

<table>
<thead>
<tr>
<th>Animal</th>
<th>First inoculation</th>
<th>Second inoculation</th>
<th>Challenge</th>
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<tbody>
<tr>
<td></td>
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<td>Strain</td>
<td>Dose†</td>
</tr>
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<td></td>
</tr>
<tr>
<td>1623</td>
<td>0.5 x $10^{-3}$ spl</td>
<td></td>
<td></td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td></td>
</tr>
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<td></td>
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<tr>
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<td></td>
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<tr>
<td>1752</td>
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</tbody>
</table>

* The first inoculation was considered to be time zero, the second inoculation took place 24 weeks later and the challenge at 36 weeks after the initial inoculation.
† Dose of the indicated dilution in ml.
§ SIVmac251/32H was prepared from the spleen of an SIVmac251/32H-infected monkey.
§ SIVmac251/32H.
Low-dose exposure of SIV to macaques

CCTCTGAGACAG 3') and the inner primer pair nucleotides 1122–1140 (5'-AAGTACATGTGTCAAGACATG-3') and 1541–1558 (5'-CCTGGC-ACTACTCTCTGC-3'). The first PCR round for detection of SIVmac239-specific gag sequences had the following cycle profile: denaturation at 92 °C for 30 s, annealing at 55 °C for 30 s, and extension for 30 s at 72 °C. For the second round 2 μl from the first round was amplified using the profile of the first cycle. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. The sensitivity of this PCR ranges between 1 and 5 copies of proviral DNA (H. Petry, unpublished data).

RNA for PCR was prepared from viral particles pelleted from 2 ml of plasma at 100000 g for 2 h at 4 °C. The genomic RNA was extracted with phenol and chloroform, precipitated with ethanol and treated with 20 U RNAase-free DNAseI (Boehringer Mannheim). The RNA was transcribed with the MMLV reverse transcriptase (USB) by incubation with 200 U reverse transcriptase, in the presence of 1 mM-MgCl2, 200 mM-dNTPs, 50 mM-Tris-HCl pH 8.6 and 10 U RNAase inhibitor. As primer for the cDNA, the antisense oligonucleotide of the outer primer pair described above was used. The cDNA was directly PCR amplified.

Cellular immune response. To evaluate the cellular immune response of the SIV-exposed animals, virus-specific T cell proliferation and cytotoxic T lymphocyte (CTL) response of the monkeys were investigated.

An antigen-specific T cell proliferation assay was performed repeatedly after virus inoculation as previously described (Voss et al., 1993). Briefly, PBMC obtained as described above were seeded in microtitre plates (1 x 10^5 cells/well) in 100 μl cell growth medium supplemented with 1% human AB serum. Cells were stimulated with 50 U per ml recombinant human interleukin-2 (IL-2) and kept for an additional 4 days.

Effector cells were obtained from whole blood as described for the CTL response of the monkeys. The expression of HLA antigen class I was determined using a monoclonal antibody and flow cytometry.

A statistical comparison of SI from silently infected, acutely infected, and control animals was conducted with a twofold variance analysis (F(A)= 23.61, P > 0.001, dF = 2;8) and a subsequent LSD test. The CTL response of the SIV-exposed macaques was determined with a commercial CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega). The assay measures levels of lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis (Korzeniewski & Callewaert, 1983; Decker & Lohmann-Matthes, 1988).

Results

SIV inoculation induced two different types of infection in rhesus macaques in a dose-dependent fashion

The virological status of the SIV-exposed animals was determined using several independent parameters indicative of an SIV infection. After the first SIV inoculation, proviral DNA could be detected by PCR within 2 weeks in the PBMC of all monkeys, independent of the infecting virus dose (Table 2). In contrast, at that time and during the whole observation period thereafter, virus could only be reisolated from the two animals that received the highest virus dose (Mm1513 and Mm1623).

However, seroconversion, viral RNA and urinary neo-pterin increase were detected in these two animals indicating a persistent acute infection. The four monkeys exposed to lower doses of SIVmac239/sv1 and the monkey Mm1775 remained seronegative (Fig.1), their neo-pterin concentrations were not elevated and viral RNA could not be detected for 24 weeks (Table 2). Thus, these animals were silently infected.

Twenty-four weeks after the first SIV inoculation the six silently infected animals were again exposed to low doses of infectious SIV (Table 1). Again, no virus could be reisolated from these animals after this second low-dose inoculation. All six animals remained silently
infected, as indicated by repeated negative results for virus reisolation, urinary neopterin increase and seroconversion (data not shown).

**Silently infected macaques developed an SIV-specific proliferative T cell reactivity but no CTL response**

To evaluate the cellular immune response of the SIV-exposed macaques we investigated antigen-specific T cell proliferation and the development of specific CTL. Interestingly, the two acutely infected animals (Mm1513 and Mm1623) had a suppressed T cell proliferation when tested against inactivated SIV antigen (Table 3) and SIV rgp140 during the investigation period. In contrast, the virus-specific T cell proliferation of the silently infected macaques was significantly higher than that of the acutely infected animals ($P < 0.002$) and that of naive controls ($P < 0.001$). No significant difference was observed between acutely infected macaques and naive controls (LSD-test). The T cell response of the silently infected animals was detectable within 12 weeks after virus exposure in all six animals but gradually disappeared thereafter (Table 3). At the time of the second SIV exposure (24 weeks after the first inoculation) none of the silently infected macaques exhibited a measurable SIV-specific T cell proliferation. Nevertheless, following the second virus exposure the virus-specific T cell proliferation reappeared in five out of six animals (Table 3).

The T cell proliferation of the silently infected macaques after stimulation with whole inactivated SIV was very similar to that obtained with SIV rgp140. Animals which responded to whole inactivated SIV antigen were also reactive after stimulation with SIV rgp140 (data not shown). Therefore, virus specificity of the proliferating cells is likely. During the whole observation period three naive control animals remained persistently negative for SIV-specific T cell proliferation (Table 3).

The SIV-specific CTL response of all animals was investigated at several time points after the respective SIV exposures. Interestingly, in spite of the T cell
Table 3. SIV-specific cellular immune response (SI) of SIV-exposed rhesus macaques

<table>
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<tr>
<th>Animal</th>
<th>0</th>
<th>4</th>
<th>12</th>
<th>14</th>
<th>20</th>
<th>24</th>
<th>30</th>
<th>36</th>
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<td>0.8</td>
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<td>1.1</td>
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</tr>
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</tr>
<tr>
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<td>3.9*</td>
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<tr>
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<td>1.5</td>
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<tr>
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<td>2.8</td>
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</tr>
<tr>
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<td>1.6</td>
<td>1.4</td>
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<td>2.8</td>
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<tr>
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<tr>
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<td>ND</td>
<td>ND</td>
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<td>1.0</td>
<td>0.7</td>
<td>1.1</td>
<td>ND</td>
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</table>

* Positive SI (≥ 2) are printed in bold. The mean c.p.m. of triplicate control cultures ranged between 752 and 4162.
† Effector target ratio (E:T) was 10:1.
ND, Not determined.

reactivity of all silently infected macaques, no SIV-specific CTL were found in these animals at any time after the low-dose SIV exposure. In contrast, the acutely infected animal Mm1513 repeatedly showed CTL activity against the Gag and Env proteins of SIV and the animal Mm1623 against Pol (Table 3).

Silently infected macaques became acutely infected after a high-dose SIV challenge

Twelve weeks after the second low-dose virus exposure the six silently infected macaques together with three naive control animals were challenged with a high-dose of SIVmac251/329 (Table 1). All silently infected animals were SIV-seronegative but DNA-positive by PCR at the time of challenge (data not shown). Acute infection was demonstrated by virus reisolation and positive results from genomic RNA-PCR in all monkeys by two weeks after challenge. Cell-associated viraemia persisted over the whole observation period of 24 weeks. Nevertheless, the control animal Mm1752 did not seroconvert during the observation period (Fig. 1). In one SIV-pre-exposed animal (Mm1679) seroconversion was detected 24 weeks after challenge for the first time (data not shown), also virus could be reisolated from the PBMC of this animal by 2 weeks after challenge. In addition, these two animals showed clearly elevated urinary neopterin levels 12 weeks after acute infection (Fig. 2). All other animals seroconverted within 8 weeks after challenge as shown by RIPA (Fig. 1). However, the antibody response of Mm1641 was weak and could be detected for the first time by Western blotting analysis at week 16 post-challenge (data not shown). The cell-associated virus load determined at 8, 16, 20 and 24 weeks post-infection did not discriminate between the control and SIV-pre-exposed monkeys. In all animals, between 128 and 4096 infectious cells per 10⁶ PBMC were detected independent of their immunological status before challenge. Interestingly, the SIV-specific T cell proliferation detectable in the silently infected macaques at the time of challenge was abrogated within 2 weeks after the acute SIV infection and did not reappear later (data not shown).

The only difference between the silently infected animals and the control animals after challenge was observed with respect to the initial urinary neopterin profiles. The urinary neopterin values rose earlier in the naive controls than in most of the SIV-pre-exposed monkeys. The neopterin peak in the naive control animals was found between days 11 and 15, which was also observed in other naive animals infected with SIVmac251/329 (Stahl-Hennig et al., 1992; C. Stahl-Hennig, unpublished data). In contrast, in five out of six SIV-pre-exposed macaques the neopterin peak was delayed for 5 days and appeared between 16 and 20 days post-infection (Fig. 2).

SIV-pre-exposed macaques developed AIDS-like disease after acute SIV infection

The SIV pre-exposure did not seem to be beneficial with respect to the development of an immunodeficiency. Like the control monkeys, the SIV-pre-exposed monkeys showed a constant drop in their CD4⁺/CD8⁺ cell ratios from a mean of 1.3 to a mean of 0.56 at 24 weeks after challenge. Twenty-six weeks after challenge two monkeys had to be euthanized, one because of untreatable diarrhoea induced by an opportunistic infection with Giardia lamblia (Mm1710), the other because of rapidly
developing blindness (Mm1641). At that time these two animals showed a suppressed T cell response against SIV antigen and the mitogen PHA. In addition, the animal with the highest urinary neopterin level (Mm1679) also developed a T cell unresponsiveness against SIV and PHA (data not shown). One of the control monkeys (Mm1752) had to be sacrificed at 12 weeks post-challenge owing to signs of a central nervous system disorder and diarrhoea. This monkey had not developed any SIV-specific antibodies as tested both by RIPA (Fig. 1) and Western blotting (data not shown) and its neopterin values remained highly elevated after the initial peak with a steep increase shortly before death (Fig. 2). Another monkey from the SIV-pre-exposed group showed severe wasting (Mm1679) and one of the two remaining control monkeys (Mm1687) had developed severe enlargement of all peripheral lymph nodes. All the other macaques (one control and three SIV-pre-exposed animals) remained clinically healthy and without any immunological disorders during the observation period.

**Discussion**

In the present study rhesus macaques were exposed intravenously to low doses of infectious SIV. This virus exposure resulted in a silent infection of the monkeys. No evidence for viral replication could be found in these animals but proviral DNA was detectable in their PBMC. Furthermore, they remained seronegative for at least 36 weeks after the first virus exposure.

Such silent infections have previously been described in humans at high risk of HIV infection (Ranki *et al.*,...
1987; Imagawa et al., 1989; Vaira et al., 1990). Although, some of these individuals seroconverted after a long latent period, it is not known whether some people harbour the virus but never seroconvert (Imagawa et al., 1989). However, silently infected individuals remained clinically healthy with stable CD4 counts while their HIV infection was in a latent state (Imagawa et al., 1989; Ranki et al., 1987). Obviously, viral replication was efficiently suppressed in these individuals, perhaps through the induction of virus-specific CMI. However, this has not yet been demonstrated. In our experiment, all silently infected macaques remained clinically healthy and developed an SIV-specific, proliferative T cell response. In earlier experiments of our group such proliferating T cells were characterized as CD4+ and depended on MHC class II DR antigen presentation (Voss et al., 1992b, 1993). In general, the T cell response of the silently infected macaques was weaker than that of macaques immunized with adjuvant-formulated, disrupted SIV (Voss et al., 1992b, 1993). After a low-dose SIV exposure, specifically proliferating T cells may have been induced by an initial low-level SIV replication or just by an exposure to SIV antigens. However, the presence of proviral DNA favours the first explanation. On the other hand, the inability to find genomic RNA in plasma and specific CTL in concanavalin A-stimulated bulk cultures indicates that virus replication was suppressed. This virological status was not changed by a second low-dose SIV exposure of the animals but their SIV-specific T cell response could be boosted.

A similar virus-specific T cell proliferation has been described in man (Clerici et al., 1992, 1993, 1994a) and monkeys (Dittmer et al., 1994a) exposed to HIV without subsequent infection. From the data obtained in man it was speculated that the induction of a stable CMI might protect against HIV infection (Clerici & Shearer, 1993; Salk et al., 1993). We have investigated in silently infected macaques whether CMI, seemingly controlling virus replication and its pathogenic consequences in these animals, was able to protect them against a high-dose SIV challenge. At the time of the intravenous challenge, only one (Mm1679) of six animals did not show a detectable T cell proliferation. Unfortunately, in spite of this SIV-specific T cell activity all animals became acutely infected after challenge. Moreover, the animal Mm1775, which remained silently infected after the first inoculation with 2 ml of a 10⁻⁴ dilution of SIVmac251/239, became acutely infected after a second inoculation with the same virus dose. Supporting our previous findings, acutely SIV-infected macaques lose their specific T cell proliferation, indicating an impaired T cell function due to virus replication (Voss et al., 1993; Dittmer et al., 1994b).

Recently, Salvato et al. (1994) and Clerici et al. (1994b) have described that some macaques, which generated a virus-specific T cell response after exposure to low doses of SIV, were protected against a rectal challenge with homologous SIV. These results are in contrast to our findings. An explanation for these differences might be the different inoculation route used for challenge of the SIV-primed animals. Thus, the CMI of the mucosae seems to play a critical role in protection against HIV or SIV transmission. Consequently, the induction of a mucosal immunity by candidate vaccines against HIV might be a very important aspect in future vaccine development (Elrodige et al., 1993).

Infection of macaques with immunodeficiency viruses which are apathogenic for the animals can modulate disease development after infection with a pathogenic virus (Putkonen et al., 1990; Petry et al., 1994). We have therefore investigated the clinical course after acute SIV infection of the formerly silently infected macaques. Six months after challenge no differences were found between SIV-exposed and naive control animals in the development of AIDS-like symptoms, except a delay of the initial urinary neopterin peak in five of the SIV-pre-exposed monkeys. Such a delay was also observed in macaques infected with the slowly replicating HIV-2ben (Stahl-Hennig et al., 1990b; C. Stahl-Hennig, unpublished data). The SIV replication in the SIV-pre-exposed monkeys was probably reduced early after the acute infection. However, by 8 weeks after infection the cell-associated virus load was similarly high in both the control and the SIV-pre-exposed macaques. By 26 weeks after challenge two of the SIV-pre-exposed animals and one control had to be sacrificed owing to AIDS-like symptoms. Thus, pre-exposing macaques to SIV did not ameliorate the clinical course of acute SIV infection.

In conclusion, silent infection of rhesus macaques was induced by low doses of infectious SIVmac. Such animals generated virus-specific T cell proliferation but viral replication was suppressed as indicated by the absence of SIV-specific antibodies. However, no virus-specific CTL were observed and the monkeys were not protected against the consequences of a high-dose SIVmac challenge. These results contradict hypotheses based on retrospective observations made in selected HIV-exposed humans. The SIV–macaque model allows controlled and detailed studies on immunological mechanisms potentially involved in protection against immunodeficiency virus infection.

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