Long-Lasting Protection by Live Attenuated Simian Immunodeficiency Virus in Cynomolgus Monkeys: No Detection of Reactivation after Stimulation with a Recall Antigen

Leonardo Sernicola, Franco Corrias, Martin Luther Koanga-Mogtomo, Silvia Baroncelli, Simonetta Di Fabio, Maria Teresa Maggiorella, Roberto Belli, Zuleika Michelini, Iole Macchia, Armando Cesolini, Livia Cioè, Paola Verani, and Fausto Titti

Laboratory of Virology, Istituto Superiore Sanità, Viale Regina Elena, 299-00161 Rome, Italy

Received October 9, 1998; returned to author for revision December 9, 1998; accepted February 9, 1999

The infection of cynomolgus monkeys with an attenuated simian immunodeficiency virus (SIV) (C8) carrying a deletion in the nef gene results in a persistent infection associated with an extremely low viral burden in peripheral blood mononuclear cells. The aim of this study was to determine (1) the breadth of the protection after repeated challenges of monkeys with SIV homologous strains of different pathogenicity, (2) the genotypic stability of the live virus vaccine, (3) whether the protection might depend on cellular resistance to superinfection, and (4) whether immunogenic stimuli such as recall antigens could reactivate the replication of the C8 virus. To address these goals, the monkeys were challenged at 40 weeks after C8 infection with 50 MID50 of cloned SIVmac251, BK28 grown on macaque cells. They were protected as indicated by several criteria, including virus isolation, anamnestic serological responses, and viral diagnostic PCR. At 92 weeks after the first challenge, unfractionated peripheral blood mononuclear cells from protected monkeys were susceptible to the in vitro infection with SIVmac32H, spl. At 143 weeks after C8 infection, the four protected monkeys were rechallenged with 50 MID50 of the pathogenic SIVmac32H, spl grown on macaque cells. Once again, they were protected. The C8 virus remained genotypically stable, and depletion of CD4+ cells was not observed during ~3 years of follow-up. In contrast, it was found that the infection with SIVmac32H, spl induced CD4+ cell depletion in three of three control monkeys. Of importance, stimulation with tetanus toxoid, although capable of inducing specific humoral and T cell proliferative responses, failed to induce a detectable reactivation of C8 virus.

Key Words: live virus vaccine, simian immunodeficiency virus, tetanus toxoid.

INTRODUCTION

In the past decade, several vaccination strategies against the human immunodeficiency virus (HIV) have been evaluated in the simian immunodeficiency virus (SIV) macaque model. Whole inactivated (Almond et al., 1995; Dormont et al., 1995; Stahl-Hennig et al., 1996a; Titti et al., 1993) or subunit (Hu et al., 1992; Israel et al., 1994; Lehner et al., 1996) SIV vaccines have been shown to be partially effective in preventing infection or in reducing viral burden in monkeys challenged with low doses of cell-free or cell-associated homologous or heterologous viruses. A common characteristic of all these vaccine trials in macaques was the capability of inducing humoral and cellular immune responses, which, however, were not always sufficient in obtaining a complete protection or a significant reduction in viral load (Giavedoni et al., 1993; Hulskotte et al., 1995; Mills et al., 1993).

At the present, the vaccination of macaques with live attenuated SIV strains yielded the most consistent protection data in the SIV macaque model (Almond et al., 1995; Cranage et al., 1997; Daniel et al., 1992; Norley et al., 1996; Stahl-Hennig et al., 1996a; Titti et al., 1997; Wyand et al., 1996). More recently, chimeric SIV-containing portion of HIV regions (SHIV) has also been used as a vaccine (Igarashi et al., 1997; Letvin et al., 1995) or as challenge virus (Bogers et al., 1995; Shibata et al., 1997). Despite the proved efficacy of live attenuated SIV vaccines in monkeys, there remain safety concerns regarding the use of a similar approach in humans. Among the safety issuesler is the possible reaction that may occur in patients vaccinated with an attenuated HIV after vaccination against other infectious agents or after exposure to foreign antigens. In fact, a major characteristic of the infection with an attenuated virus is the establishment of a persistent but attenuated infection. It is well known from in vitro studies that cellular activation and production of inflammatory cytokines can reactivate latent viruses (Fauci, 1993; Goletti et al., 1998) and/or rescue defective proviruses (Barillari et al., 1992). In addition, other studies, in both animals and humans, have reported evidence of the association between immune activation or dysregulation and clinical course of the disease in HIV-infected patients, SIV-infected monkeys, and Visna virus-infected sheep (Folks et al., 1997; Fultz et

1 To whom reprint requests should be addressed. Fax: 39-06-49387184. E-mail: titti@virus1.net.iss.it.
To examine the protection elicited by a naturally occurring variant of SIVmac251,32H (C8) carrying an in-frame 12-bp deletion in the nef gene (Rud et al., 1992, 1994), cynomolgus macaques have been infected with the C8 live attenuated virus to (1) evaluate its capability of establishing a protective status against subsequent challenges with SIV strains differing in their pathogenic potential, (2) verify the genotypic stability of the C8 virus before and after viral challenges, (3) test the susceptibility of peripheral blood mononuclear cells (PBMCs) from protected monkeys to the in vitro infection with SIV, with the goal of determining whether a phenomenon of cellular resistance to superinfection could occur, and (4) study the effect of immune stimulation with a recall antigen on reactivation of the vaccine virus.

In this study, we demonstrate that vaccination with live attenuated virus elicits a strong protective status, as demonstrated by the protection against repeated challenges with homologous SIV strains of increasing pathogenicity, which lasts ~3 years. This effect does not seem to be related to a cellular resistance to superinfection. Of importance, we found that the immune stimulation with a common recall antigen [tetanus toxoid (TT)] does not apparently reactivate the replication of the vaccine virus.

RESULTS

Infection of cynomolgus monkeys with the nef-deleted C8 virus results in protection against the first challenge

The outcome of the infection of cynomolgus monkeys with the C8 virus has been reported (Titti et al., 1997). Briefly, each of the four C8-infected animals seroconverted at the fourth week after infection and anti-SIV antibody titers progressively increased (Fig. 1A). C8 virus established a persistent infection characterized by a very low viral load (data not shown). The C8-vaccinated monkeys were protected from the first challenge with 50 MLD50 of SIVmac, BK28 that was inoculated 40 weeks after the primary infection, as indicated by the failure to isolate the challenge virus from PBMCs (Table 1). However, the C8 virus, although no longer isolated from PBMCs, was isolated from lymph node cells of two of three vaccinated and protected monkeys. In any case, SIV provirus showing the genotype of the vaccine virus was detectable in samples of all monkeys by nested DNA PCR. In addition, neither histological modifications of lymph nodes nor CD4+ cell depletion was observed among the same monkeys. In contrast, SIVmac, BK28 was isolated from PBMCs of all monkeys and from lymph node cells of three of four control monkeys. The infection with SIVmac, BK28 induced in control monkeys a CD4+ cell depletion, which was more evident in monkeys 28 and 91 at 62 weeks post-challenge (p.c.). In addition, microhemorrhagic areas and infiltration of lymphocytes to germinal centers were observed at 28 weeks p.c. in three of four control monkeys.

Susceptibility of PBMCs from C8-vaccinated and protected monkeys to in vitro infection with SIV

To determine whether the protection in C8-vaccinated monkeys might depend on a cellular resistance to superinfection, SIVmac32H, spl was used to infect in vitro unFractionated PBMCs (multiplicity of infection, 1 TCID50/cell) from C8-vaccinated and two naive monkeys. Before the in vitro infection, the C8 provirus was undetectable by a single-round PCR in PBMCs of all vaccinated monkeys (data not shown) and was still undetectable after PHA stimulation of the same PBMCs (Fig. 2B). In contrast, after in vitro infection, SIV provirus was detectable by the same method in PHA-stimulated PBMCs of both vaccinated and naive monkeys. In particular, because the 12-bp deletion in the nef region of the C8 virus eliminates one of the two Rsal recognition sites, a nested-PCR diagnostic assay was used to distinguish between the C8 and the full-length nef of SIVmac32H, spl proviruses. Specifically, after in vitro infection, full-length nef DNA could be detected in samples of both vaccinated and control monkeys (Fig. 2C). Although there were individual variations, PBMCs of vaccinated monkeys were productively infected, as indicated by the presence of p27gag antigen in the culture supernatants at levels comparable to those found in the supernatants of PBMCs from control monkeys (Fig. 2A).

The second challenge of C8-vaccinated monkeys with wild-type SIV results in protection from infection

Based on the previous results and to verify the breadth of the protection, the four vaccinated and protected monkeys along with three naive monkeys were rechallenged with 50 MLD50 of the same strain of wild-type SIVmac32H, spl used for in vitro infection at week 103 after the first challenge (143 weeks from the original C8 infection). Control monkeys seroconverted (Fig. 1B) and a peak of p27gag plasma antigen was observed at 2 weeks postinfection (p.i.) (data not shown) paralleled by an increase of the SIV proviral copies at 2 and 8 weeks p.i. (Table 2). After the rechallenge, a moderate increase of anti-SIV antibody titers was observed (Fig. 1A) in vaccinated monkeys that, however, remained virus-isolation negative (Table 2). The level of plasma p27gag antigen was at no time detectable (<50 pg/ml) by commercial p27gag antigen capture assay (data not shown). At the time of the second challenge, the cell-associated viral load was undetectable in two of four monkeys (7 and 46133), whereas it was <10 SIV proviral copies/μg of DNA in monkeys 09 and 33. A moderate increase in the SIV proviral copies was detectable at 2 weeks p.c. in three of four monkeys, being more evident in monkey 09, and, at 13 weeks p.c., in monkey 46133. However, the C8 ge-
nome maintained its original deletion (amino acids 144–147) because no sequences that correspond to the full-length nef DNA genome of the challenge virus were detected in the PBMCs (at 2 weeks p.c.) by both diagnostic PCR assay (Table 2) and sequence analysis of the nef region (data not shown). Accordingly, full-length nef DNA could be detected in samples of control monkeys. The percentage of CD4 cells remained rather stable among vaccinated monkeys, although some variations were observed (Fig. 3). However, a depletion of the CD4 cells was observed among control infected monkeys during the period of observation of 31 weeks (Fig. 3). In particular, disease progression appears to be more pronounced in monkey A91. In fact, the percentage and the number of CD4 cells were 31.75% and 990 cells/mm³, respectively, at the time of infection and decreased to 3.32% and <10 cells/mm³ at 31 weeks p.i.

Stimulation with TT does not result in a detectable C8 virus reactivation

To analyze whether immune activation could reactivate the replication of the C8 virus in protected monkeys, at week 13 after the second challenge, the four vaccinated and the three control animals received a booster immunization with TT (40 IU/dose/monkey), followed by a second booster 3 weeks later. The analysis of the in vitro proliferation before the first dose of TT (13 weeks p.c.) revealed that although PBMCs from all monkeys proliferated in response to PHA (stimulatory index (S.I.),

FIG. 1. Anti-SIV antibody titers in plasma of C8-immunized monkeys before and after the challenges (A) and in plasma of naive monkeys after infection (B). Dashed arrows indicate the time at which the tetanus toxoid treatment was given (at 13 and 16 weeks after the second challenge).
TABLE 1
Summary of the Responses of C8-Vaccinated (7, 09, 33, and 46133) and Naïve Monkeys (22, 28, 91, and 48126) after the First Challenge with SIVmac251/BK28 Virus

<table>
<thead>
<tr>
<th></th>
<th>VC</th>
<th>PCR</th>
<th>Histology</th>
<th>CD4⁺ (cells/mm³)</th>
<th>Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>C8-vaccinated monkey</td>
<td>7</td>
<td>+</td>
<td>N.D.</td>
<td>1570 (1461)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>09</td>
<td>+</td>
<td>N.D.</td>
<td>2470 (2600)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>+</td>
<td>N.D.</td>
<td>1220 (889)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>46133</td>
<td>+</td>
<td>N.D.</td>
<td>1180 (986)</td>
<td>+</td>
</tr>
<tr>
<td>Naïve infected monkey</td>
<td>22</td>
<td>+</td>
<td>+ (BK28)</td>
<td>1210 (1423)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>+</td>
<td>+ (BK28)</td>
<td>650 (1659)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>91</td>
<td>+</td>
<td>+ (BK28)</td>
<td>650 (1080)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48126</td>
<td>+</td>
<td>+ (BK28)</td>
<td>1900 (1450)</td>
<td></td>
</tr>
</tbody>
</table>

a Virus isolation (+, positive; −, negative) from PBMCs cocultured with CEMX174 human cell line.

b Differential PCR analysis to discriminate C8 from SIVmac/BK28, performed on DNA extracted from PBMCs of monkeys by using RSA I restriction enzyme as described in Materials and Methods.

c Lymphnodal biopsy performed at 28 weeks p.c. Single-cell suspension was used for virus isolation and for PCR analysis on DNA extracted from monkey cells.

d Presence of microhemorrhagic areas and infiltration of lymphocytes to germinal centers, as observed with histopathological analysis of the hematoxylin and eosin-stained tissue sections.

e Absolute cell counts at 62 weeks p.c. Numbers in parentheses indicate the number of CD4⁺ cells at the day of the first challenge.

f Monkey 28 died at 78 weeks p.i.

h Monkey 91 died at 109 weeks p.i.

N.D., not done.

≥20.0; data not shown]) a specific proliferation to TT was undetectable (S.I. ≤ 1.0) (Fig. 4). Six weeks after the first TT booster (3 weeks after the second dose), all C8-vaccinated monkeys responded to TT treatment and showed a specific increase of the proliferative response (S.I. > 2.0; range, 2.93–5.98), and an increase in plasma IgG antibody level to TT (fivefold to ninefold increase from the baseline values) (Fig. 4A). Similarly, the specific S.I. was >2 (range, 3.83–7.66) in two of three control infected monkeys with a contemporary increase of anti-TT IgG level (from fivefold to eightfold increase from the baseline values). A poor antibody and proliferative response to TT (S.I. ≤ 1.0) before the first TT booster were instead detectable 6 weeks after the first TT booster in all monkeys (S.I. > 2; range, 0.38–1.39) before TT booster were instead detectable 6 weeks after the first TT booster in all monkeys (S.I. > 2; range, 2.38–4.51) and >10 (range, 11.43–15.05), respectively. To evaluate their capability to respond to a recall antigen, four of these monkeys were boosted twice with TT at 3-week interval. The proliferative responses that were undetectable in these monkeys (S.I. < 2; range, 0.38–1.39) before TT booster were instead detectable 6 weeks after the first TT booster in all monkeys (S.I. > 2; range, 2.83–6.61). These data altogether are not in contrast to those obtained from C8-vaccinated and from two of three SIV-infected monkeys.

**DISCUSSION**

We previously reported that monkeys vaccinated with the C8 virus are protected from the challenge with an homologous cell-free cloned SIVmac, BK28 (Titti et al., 1997). In the same study, we have observed that the protected monkeys had levels of interleukin (IL)-2, interferon-γ, and IL-15 mRNAs higher than those of control monkeys, suggesting that a Th1-like response may be one of the correlates of protection along with other virus-specific immune responses (Heeney et al., 1997). Here we have shown that vaccination with a live at-
attenuated virus establishes and maintains a long-term (3-year) protective status even after a second challenge with the homologous SIVmac32H, spl showing a pathogenic potential higher than that of SIV mac251, BK28 (Naidu et al., 1988), as indicated by the rapid decline in CD4\(^+\) cells in control monkeys. During this period, the C8 virus maintained its original genotype, as indicated by sequence analysis of the nef region in PBMCs of protected monkeys. By using the same live attenuated virus, at the same dose in rhesus macaques, other groups have reported that in some animals the truncated nef open reading frame can revert to a wild-type genotype by restoring or duplicating the nef deleted sequence. Consequently, these animals developed an AIDS-like disease similar to that observed in monkeys inoculated with pathogenic SIV (Dittmer et al., 1995; Whatmore et al., 1995). It remains to be elucidated whether the use of different species of monkeys (rhesus vs cynomolgus), different routes of inoculation, or challenge viruses differing in their pathogenic potential with respect to the SIV mac251 used in our experiment or retroviral recombinations (Wooley et al., 1997) may have any influence on the reversion to the wild-type genotype.

In a previous study (Titti et al., 1993), we demonstrated that a long immunization schedule (~2 years) with a whole inactivated SIV vaccine was insufficient to achieve protection in cynomolgus monkeys. Thus from these as well as from data by others (Norley et al., 1996; Wyand et al., 1996), it seems that the capability of the live attenuated virus to continuously replicate without induction of a severe immune defect (CD4\(^+\) cell depletion) has a central role to mount and maintain a fully protective status. Although the vaccination with a live attenuated SIV represents the most successful result in the past and current vaccine approaches, the mechanisms underlying the protective effect of vaccination are not yet fully understood. As described for murine leukemia viruses (Mitchell et al., 1992) and for HIV (Hart et al., 1990; Taddeo et al., 1993), a nonimmune mechanism such as viral interference to superinfection may play an important role in the establishment of the protection from challenging viruses. This hypothesis is not sustained by the report that monkeys vaccinated with live attenuated HIV-2 become superinfected when challenged with heterologous uncloned SIVsm, even if they were still protected from the clinical signs of the disease for a considerable period of time (Putkonen et al., 1995). Here we show that unfractionated PBMCs from vaccinated and
protected monkeys are susceptible to in vitro infection with SIV, similar to those from uninfected monkeys. Our data support the hypothesis that the protection observed with attenuated SIV is based not on the competition for available target cells but rather on the ability of the live attenuated virus to induce a long-lasting protective immune response or responses adequate to prevent or to control the replication of the challenge virus. In fact, strong immune responses have been reported to be associated with protection established after vaccination with different attenuated viruses in macaques (Clements et al., 1995; Cranage et al., 1997; Gauduin et al., 1998; Johnson et al., 1997; Lohman et al., 1994; Xu et al., 1997), as well as in Friend leukemia murine model (Dittmer et al., 1998).

As mentioned above, the live attenuated virus still represents the most successful vaccine approach to prevent SIV infection. Nevertheless, apart from the contrasting evidence of its pathogenic potential in macaques (Baba et al., 1995; Desrosiers et al., 1998; Wyand et al., 1997), the effects of other factors on the evolution of the attenuated infection have not been fully elucidated. Activation of the immune system is associated with an increased replication of HIV in infected patients. We have previously shown that the treatment of SIV-infected macaques with inactivated whole SIV or with nonspecific antigens, although leading to an increase in the number of circulating T cells, could induce an increase of the cell-associated viral load, probably predisposing the monkeys to an accelerated clinical course of the infection (Titti et al., 1996). However, in this study, the activation of the immune system induced by TT did not cause a detectable reactivation of the C8 virus in vaccinated and protected monkeys. Specifically, TT activated the immune system by inducing an increase of the in vitro proliferative response and of plasma IgG and, at the same time, induced an increase of SIV proviral copies in two of three control monkeys. In monkey A91, as already observed in HIV-infected patients (Oprail et al., 1991), the severity of the immune deficit correlated with the absence of TT response and with detectable variations of the number of SIV proviral copies. Similar findings have been observed after vaccinia virus superinfection of monkeys infected with live attenuated virus but not exposed to any challenge virus (Dittmer et al., 1997). Based on a comparison of the data on the proliferative responses observed in naive monkeys with those derived from vaccinated and control infected monkeys before the TT boosters, it seems that the failure to detect proliferative responses is not dependent on the SIV infection per se.

In conclusion, our results demonstrate that vaccination with genotypically stable live attenuated virus can elicit a strong protection that seems to be independent from a cellular resistance to superinfection.

MATERIALS AND METHODS

Animals

Adult cynomolgus monkeys (Macaca fascicularis) used for this study were housed in single cages within level 3 biosafety facilities according to the European guidelines for nonhuman primate care (EEC, Directive No. 86-609, November 24, 1986). As a part of a routine procedure, all monkeys were vaccinated against tetanus (40 IU; Anatetal, Sclavo, Siena, Italy) at least 4.0 years
before SIV inoculation. Animals were clinically examined, and weight and rectal temperature measurements were made while they were under ketamine hydrochloride anesthesia (10 mg/kg). Blood samples for hematological analysis and immunological and virological assays were obtained the morning before food administration.

**Schedule of treatment and doses of viruses**

The SIV\textsubscript{mac251, 32H, C8} (C8) cell-free virus, carrying a 12-bp deletion in the nef gene (obtained from M. Cranage through MRC AIDS Reagent Project, Potters Bar, UK), was grown in the human C8166 cell line and used to intravenously infect (5 $\times$ 10$^4$ TCID$_{50}$) four adult male monkeys serologically tested negative for SIV and STLV.

At 40 weeks p.i. with the C8 virus, the four infected monkeys, along with four naive monkeys, were intravenously challenged with 50 MID$_{50}$ of SIV\textsubscript{mac, BK28} molecular clone grown in monkey PBMCs (provided by A. M. Aubertine, Institute National de la Santé et de la Recherche Médicale, Strasbourg, France).

One hundred three weeks after the first challenge with the SIV\textsubscript{mac, BK28}, the four C8-infected monkeys, along with three naive monkeys, were intravenously challenged with 50 MID$_{50}$ of monkey-derived SIV\textsubscript{mac32H, spl}. The SIV\textsubscript{mac32H, spl} challenge virus stock was prepared from a spleen homogenate of a rhesus monkey inoculated with SIV\textsubscript{mac251, 32H} (Almond et al., 1994) and titrated in vitro in human T cells and in vivo in rhesus monkeys (Lüke et al., 1996; Stahl-Hennig et al., 1996b) (provided by G. Hunsman, Deutsches Primaten Zentrum, Goettingen, Germany). In this study, the four cynomolgus monkeys inoculated with the C8 virus are referred to as C8-infected or C8-vaccinated monkeys, whereas the naive monkeys inoculated with the SIV\textsubscript{mac32H, spl} are controls.
Lymphocyte subset determinations

Citrated whole blood was used for staining with two-color analysis using the following combinations of monoclonal antibodies: anti-CD20-PE and anti-CD2-FITC (clone L26 and clone MT910; DAKO, Glostrup, Denmark) and anti-CD8-PerCP (Leu-2a; Becton Dickinson Immunocytochemistry System, San Jose, CA) combined with anti-CD4-PE (clone MT310; DAKO). Briefly, 100 μl of citrated whole blood was incubated for 30 min at 4°C with monoclonal antibodies (10 μl each), washed twice with PBS supplemented with 2.5% FCS, resuspended, and fixed in PBS (pH 7.4) containing 1% (w/v) paraformaldehyde. Ten thousand lymphocytes in each sample were analyzed by cytofluorimetry (FACScan; Becton Dickinson Immunocytochemistry System). Isotype-matched murine immunoglobulins conjugated with the different fluorochromes were used as controls.

Detection of anti-SIV antibodies and p27^gag^ antigen in plasma

Because there is a high sequence homology between HIV-2 and SIV, antibody titers to SIV were determined by end point dilution using an HIV-2 ELISA assay (Elavía Ac-Ab-Ak II kit; Diagnostic Pasteur, Paris, France). Samples that showed an optical density (OD) of >0.200 at 1:100 dilution (log_{10} = 2) were scored as positive. SIV p27^{gag} protein measurement was made in untreated plasma by using an antigen capture ELISA assay (SIV Core Antigen Assay, Coulter Immunology, Hialeah, FL) with limits of detection of 50 pg/ml.

Virus isolation and in vitro infection of monkey PBMCs

For virus isolation, Ficoll–Paque (Pharmacia Biotech AB, Uppsala, Sweden)-purified monkey PBMCs (4 × 10^6) were cocultured with 1 × 10^6 human CEMX174 cells (Salter et al., 1985) (obtained from AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) in a 25-cm² tissue culture flask. The cells, reseeded every 3–4 days at 3 × 10^5 cells/ml, were maintained in culture for 30–40 days in RPMI 1640 containing 10% FCS and antibiotics. Samples were scored for syncytia formation, and clarified supernatants were monitored twice a week for the presence of p27^{gag} antigen. Samples found reactive to this assay at least twice were scored as positive.

For the in vitro infection, the virus stock was prepared from the supernatant of CEMX174 cells infected with SIV^{mac32H, spl}. After infection, the cultures were monitored for the presence of the reverse transcriptase activity (RT) as described previously (Rossi et al., 1988). Cell-free supernatants, showing the highest RT activity, were harvested on day 11 and titrated on uninfected C8166 (obtained from AIDS Reagent Project, Programme EVA, Pot-
ters Bar, UK) on 96-well plates by 1:4 end point dilutions. Virus stock was stored in aliquots at −152°C. The infectious dose of the virus stock (2.2 × 10⁶ TCID₅₀/ml) was expressed as 50% tissue culture infectious dose (TCID₅₀/m l) according to the method of Reed and Muench (1938). For the in vitro infection, PBMCs (6.4 × 10⁶) from C8-vaccinated monkeys that were protected after the first challenge along with those from two naive monkeys were resuspended in 2.2 ml of the CEMX174-derived stock of SIVmac32H, spl (multiplicity of infection, 1 TCID₅₀/cell) or with 2.2 ml of a mock supernatant from uninfected CEMX174 in the presence of PHA (0.1 µg/ml v/v) and incubated overnight at 37°C in 5% CO₂. The cells were washed five times with PBS, and after the last wash, clarified supernatants were used to determine the baseline level of p27gag protein (time 0). The cells were finally plated at 1 × 10⁶ cells/ml in a 6-well tissue culture plate in RPMI complemented with 15% FCS, antibiotics, and IL-2 (70 U/ml) (rhIL-2; Pepro Tech, Rocky Hill, NJ) and finally incubated at 37°C in 5% CO₂. At 3, 6, 10, and 13 days after infection the cells were counted and reseeded at 1 × 10⁶ cells/ml in the presence of complete medium, and virus production was monitored by determining the p27gag antigen release in the clarified supernatants. DNA extracted from cell pellets (1 × 10⁶) was analyzed by PCR to detect and to discriminate the SIV genomes, as described below.

Quantification of the SIV proviral copies, differential PCR, and nucleic acid sequencing

DNA was extracted from the whole blood by using a commercially available kit (QIAamp Blood Kit, QIAGEN GmbH, and QIAGEN Inc., Hilden, Germany). To verify the quality of the DNA to be amplified, primers (PCO3, PCO4) specific for a 110-bp sequence of the human β-globin gene were used (Saiki et al., 1985). To determine the relative number of SIV proviral copies present in the experimental samples, a semiquantitative DNA PCR was performed. Briefly, 1 µg of DNA was used to amplify a 496-bp region of the gag gene of SIVmac251 (SG1096N gag, SG1592C gagD; kindly provided by P. Kitchin, AIDS Reagent Project, Programme EVA, Potters Bar, UK) in 100 µl of reaction mixture for a total of 35 cycles, as described previously (Titti et al., 1996). To quantify the viral copy number, an external reference standard curve was prepared for each PCR experiment by using the pCMRII-Δgag plasmid containing a 986-bp region of the nef gene (LS1-nef, LS2-nef, 9335–9691, 356 bp). After an initial denaturation step at 95°C for 4 min, the samples were subjected to 35 cycles each of 1.30 min at 95°C, 1.30 min at 55°C, and 2 min at 72°C. The final product (356 bp) was digested with the RsaI restriction enzyme according to established procedures (Rose et al., 1995), resolved by electrophoresis on 1.5% agarose gel, and transferred onto a filter. Hybridization was performed at 42°C with a 32P-labeled oligonucleotide probe (LS3-nef, 9587–9606). After washing, the filters were exposed to x-ray film for 12–18 h. This analysis allows one to distinguish between undigested nef product (356 bp) of C8 and the digested nef products (172 and 184 bp) of the SIVmac32H, spl-

DNA extracted from PBMCs was amplified by using primers specific for the nef region (LS1–LS2, 356 bp). The amplified products were separated onto a 2% agarose gel and eluted by using the QIAquick gel extraction kit (QIAGEN GmbH and QIAGEN Inc.). Purified fragments were denatured and sequenced by using Sequenase kit (Version 2.0 USB; Amersham, Life Science, Cleveland, OH).

Tetanus toxoid vaccination, in vitro proliferative assay, and detection of anti-tetanus IgG

At 13 and 16 weeks after the second challenge, C8-vaccinated and control monkeys were boosted intramuscularly with TT (40 IU/dose; Anatetal). To determine the proliferative response time after vaccination with TT in the absence of booster doses of antigen, a group of 23 naive monkeys was also included. In addition, four of these monkeys received two intramuscular booster doses of TT at 3-week interval. To evaluate the proliferative responses to TT, Ficoll-purified PBMCs (2 × 10⁵/well) were placed in flat-bottom 96-well microtiter culture plate in a final volume of 200 µl of RPMI complemented with 10% FCS along with (A) no stimulation, (B) PHA 2
μg/ml (v/v), or (C) TT 5 μg/ml (v/v) (Connaught, Ontario, Canada). Three replicate cultures were performed for each stimulation and incubated at 37°C in 5% CO₂ for 5 days. There was no change of the culture medium during the incubation period. Cultures were then pulsed with 0.5 μCi/well of [³H]thymidine (specific activity, 25 Ci/mM; Amersham Life Science, Buckinghamshire) 5 days after antigenic stimulation, harvested 18 h later onto filter paper using a 96-well harvester (Tomtec, Orange, CT), and washed three times with distilled water under pressure. The filters were then dried, the scintillation fluid was added, and the radioactivity was measured with a Betaplate (Betaplate 1205; Wallac, Turku, Finland). The S.I. was calculated by dividing the mean cpm values of stimulated samples by the mean cpm values of non-stimulated controls. An S.I. of >2 was scored as positive.

To evaluate the level of anti-tetanus IgG in the plasma of monkeys either before or after the TT boosters, a commercial ELISA kit for human samples that cross-reacts with monkey IgG was used (Tetanus IgG ELISA; Serion Institut, Wurzburg, Germany).

ACKNOWLEDGMENTS

We are indebted to S. Mochi (Laboratory of Virology, Istituto Superiore di Sanità) for handling the synthesis of primers and probes and to A. Neri (Laboratory of Virology, Istituto Superiore di Sanità) for editorial assistance. We thank F. Varano, R. Iale, N. Verrone, F. Incitti, S. Fazzitta, A. Neri (Laboratory of Virology, Istituto Superiore di Sanità) for discussion and critical review of the manuscript. This work was supported by grants from the Animal Model Development Project and the AIDS Program of the Italian Ministry of Health, Istituto Superiore di Sanità, Rome, Italy.

REFERENCES


ciency virus induces host immunity that correlates with resistance to pathogenic virus challenge. J. Virol. 68, 7021–7029.


laris) against the infection with SIVmac32H. J. Virol. 70, 244–245.


nowgenetic 21, 235–246.


deficiency virus type 1 chimeric virus. J. Virol. 71, 8141–8148.


Taddeo, B., Federico, M., Titti, F., Rossi, G. B., and Verani, P. (1993). Homologous superinfection of both producer and non producer HIV-

infected cells is blocked at a late retrotranscription step. Virology 194, 441–452.


Titti, F., Koanga-Mogtomo, M. L., Borsetti, A., Geraci, A., Sernicola, L.,


