Protective efficacy of a multicomponent vector vaccine in cynomolgus monkeys after intrarectal simian immunodeficiency virus challenge

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We investigated the protective efficacy of a systemic triple vector (DNA/rSFV/rMVA)-based vaccine against mucosal challenge with pathogenic simian immunodeficiency virus (SIV) in cynomolgus monkeys. Animals were immunized at week 0 with DNA (intradermally), at weeks 8 and 16 with recombinant Semliki Forest virus (rSFV, subcutaneously) and finally, at week 24, with recombinant modified vaccinia virus Ankara strain (rMVA, intramuscularly). Both DNA and recombinant viral vectors expressed a wide range of SIV proteins (Gag, Pol, Tat, Rev, Env and Nef). This immunization strategy elicited cell-mediated rather than humoral responses that were especially increased following the last boost. Upon intrarectal challenge with pathogenic SIVmac251, three of the four vaccinated monkeys dramatically abrogated virus load to undetectable levels up to 41 weeks after challenge. A major contribution to this vaccine effect appeared to be the T-cell-mediated immune response to vaccine antigens (Gag, Rev, Tat, Nef) seen in the early phase of infection in three of the four vaccinated monkeys. Indeed, the frequency of T-cells producing antigen-induced IFN-γ mirrored virus clearance in the vaccinated and protected monkeys. These results, reminiscent of the efficacy of live attenuated virus vaccines, suggest that vaccination with a combination of many viral antigens can induce a robust and stable vaccine-induced immunity able to abrogate virus replication.

INTRODUCTION

According to recent estimates, more than 70 million people have so far been infected with human immunodeficiency virus (HIV), 28 million of whom have died as a result. The effect of HIV infection on individuals and communities is socially and economically devastating. Although the development of new antiviral drugs in recent years has had a dramatically beneficial effect on the health of those HIV-infected individuals with access, it has had little impact on the global epidemic. There therefore remains an urgent need to develop both prophylactic and therapeutic HIV/AIDS vaccines. Infection of various macaque species with simian immunodeficiency virus (SIV) has provided one of the best animal models for AIDS vaccine development (Johnson, 1996; Stott & Almond, 1995). For example, in this model, live attenuated virus vaccines have been routinely shown to protect monkeys against single or repeated challenge with homologous or heterologous pathogenic virus strains (Almond et al., 1995; Cranage et al., 1997; Heeney et al., 1994; Sernicola et al., 1999; Titti et al., 1997). However, the subsequent demonstration that some neonates and adult monkeys develop AIDS as a result of infection with attenuated SIV renders this strategy unsuitable for use in

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humans (Baba et al., 1999; Norley et al., 1996). To avoid such safety issues and assuming that prolonged expression of antigens would enhance vaccine efficacy, approaches such as the use of ‘naked’ DNA and highly attenuated virus vectors have been developed and tested in the non-human primate model. For example, vectors expressing structural and regulatory proteins alone or in partial combination have been proposed as AIDS vaccine components (Amara et al., 2001; Barouch et al., 2001; Hel et al., 2002a; Osterhaus et al., 1999; Robinson, 2002; Robinson et al., 1999; Shiver et al., 2002; Warren, 2002). In general, these approaches have induced immune responses (predominantly T-cell) able, to some extent, to contain virus replication and to delay clinical progression in macaques. These results are encouraging since they demonstrate that containment of virus replication and control of disease progression is an achievable goal. However, the degree of vaccine efficacy remains poor compared to the full protection mediated by live attenuated virus vaccines.

It therefore seemed likely that a vaccine based on structural and regulatory viral antigens would elicit appropriate and broad humoral and cell-mediated protective immune responses in an outbred simian or human population (Mooij & Heeney, 2001). Hypothetically, responses to a wide range of viral antigens would minimize the likelihood of immunological escape and therefore favour an effective long-lasting control of virus replication in vaccinees (Barouch et al., 2002). Immunization of rhesus monkeys with a combination of nearly all SIV antigens in a DNA priming and recombinant MVA (rMVA) boost regimen reduced virus replication, but was unable to prevent disease progression (Horton et al., 2002). In the cynomolgus model, a multiprotein approach including priming with recombinant Semliki Forest virus (rSFV) and boosting with rMVA, elicited T-cell immune responses but failed to control SIV replication (Nilsson et al., 2002). We previously demonstrated that a homologous prime–boost immunization regimen using rMVA expressing both structural (Gag-Pol, Env) and regulatory (Rev, Tat, Nef) proteins of SIV significantly controlled the challenge virus replication in two out of four vaccinated cynomolgus monkeys (Negri et al., 2001). This outcome, however, did not match the expected protective efficacy.

Taken together these results prompted us to investigate whether an improved schedule of immunization, different routes of antigen delivery and the use of a new generation of recombinant vectors could induce a more effective control of challenge-virus replication (Heeney et al., 2000). In addition, since HIV infection is transmitted predominantly via the mucosal route we investigated the effects of a systemic vaccination against mucosal challenge. As part of a multi-centre project funded by the European Union (European Sponsored Network for AIDS Vaccine Evaluation in Primates, ENVEP), we evaluated the protective efficacy of a polyvalent DNA/rSFV/rMVA vaccine expressing different SIV proteins (Gag, Pol, Tat, Rev, Nef and Env) in cynomolgus monkeys. The immunogenicity was assessed by evaluation of anti-SIV antibodies in plasma and of T-cell responses (lymphocyte proliferation and frequency of IFN-γ-spot forming cells) measured in peripheral blood mononuclear cells (PBMC) following stimulation with whole inactivated SIV and 20- or 15-mer peptide pools of SIV-Gag, -Rev, -Tat and -Nef. The protective efficacy was determined by quantitative analyses of viral RNA in plasma and of provirus in PBMC.

**METHODS**

**Animals.** Male cynomolgus macaques (Macaca fascicularis) from the Mauritius breeding colony were matched for source and for comparable age and weight, and maintained in accordance with European guidelines for non-human primate care (EEC Directive No. 86-609, 24 November 1986). All monkeys were negative for infection with SIV, type D simian 1, 2, 5 (SRV) and simian T-cell leukaemia (STLV-I) viruses, simian herpes B virus, cytomegalovirus, Ebola and Marburg viruses. Animals were examined clinically and weight and rectal temperature were measured while under ketamine hydrochloride anaesthesia (10 mg kg⁻¹, intramuscularly). Blood samples for haematological analysis and for immunological and virological assays were taken in the morning prior to feeding.

**Vaccine trial.** Single plasmid DNAs (pDNA) expressing SIV-Gag (EVA2023.1, pTH.UbgagPK), -Pol (EVA2023.2, pTH.UbpolPK), -Tat (EVA2023.3, pTH.tat), -Rev (EVA2023.4, pTH.rev), -Nef (EVA2023.5, pTH.nef), -Env (EVA2023.6, pTH1 empty; 600 μg) was inoculated intradermally in two contralateral sites of the abdomen near the inguinal lymph node draining region. Recombinant Semliki Forest virus (rSFV) (Tubulekas et al., 1997) constructs, expressing the same SIV proteins (EVA2108.2, SFV-SIVmacJ5 TMGag; EVA2108.3, SFV-SIVmacJ5 Pol; EVA2108.6, SFV-SIVmacJ5 Tat; EVA2108.5, SFV-SIVmacJ5 Rev; EVA2108.4, SFV-SIVmacJ5 Nef; EVA2108.1, SFV-SIVmacJ5 Env), were resuspended in pyrogen-free saline and inoculated subcutaneously as a mixture at weeks 8 and 16 (10¹⁵ infectious unit per 0.1 ml) from the same sites used for the pDNAs. The final boost was carried out by intramuscular inoculation in both biceps of a mixture, in pyrogen-free saline, of rMVAs (10⁸ TCID₅₀ for each antigen) at the same sites used for the pDNAs. The boost was carried out by intramuscular inoculation in both biceps of a mixture, in pyrogen-free saline, of rMVAs (10¹⁵ TCID₅₀ for each antigen) expressing SIV-Gag-Pol (EVA2107.2, MVA-SIVmacJ5-gag-pol), -Tat (EVA2107.5, MVA-SIVmacJ5-tat), -Rev (EVA2107.4, MVA-SIVmacJ5-rev), -Nef (EVA2107.3, MVA-SIVmacJ5-nef) and -Env (EVA2107.1, MVA-SIVmacJ5-env) proteins. Four cynomolgus monkeys were included as controls and, according to the same schedule, received empty DNAs (EVA2023.6, pTH1 empty; 600 μg), recombinant SFV expressing LacZ protein (SFV-LacZ; 6 × 10⁸ infectious units) and non-recombinant MVA (5 × 10⁸ TCID₅₀). Vectors were provided by EU Programme EVA Centralised Facility for AIDS Reagents (NIBSC, Potters Bar, UK).

**Humoral responses.** Anti-SIV antibody titres were determined by end-point dilution of plasma in a whole HIV-2 virus ELISA (Elavia II; Pasteur). Neutralizing (NT) antibodies were measured as described (Negri et al., 2001). Briefly, serial dilutions of SIVmac251 were incubated with a 1:100 dilution of plasma before addition of C8166 cells. After 7 days incubation, wells were tested for SIVmac251 p27 using an antigen-capture ELISA and the virus titres (TCID₅₀) were determined. The yield reduction for each sample was calculated as the titre of virus in the absence of plasma divided by the titre in the presence of plasma.
Lymphocyte proliferation assay. PBMC from citrated blood were purified by density-gradient centrifugation on Ficoll-Hypaque. The cells were resuspended in complete RPMI medium supplemented with 10% fetal calf serum (FCS), counted and seeded at 2 x 10^5 cells per well in triplicate, in 96-well microtitre plates for 5 days, in the absence or presence of (i) 3.5 µg AT-2 inactivated SIVmac239 ml^{-1} (corresponding to 0-1 µg p27^{env} ml^{-1} ) (CFAR 1018.1, SIVmac239/ SUP-T1-CCR5 CL.30) (Rossio et al., 1998), (ii) concentrated and chemically inactivated SUP-T1 cell supernatant (microvesicles) at 3-5 µg ml^{-1}, as negative control (CFAR 1018.2) or (iii) 20-mer peptide pools corresponding to the Gag, Tat, Rev and Nef proteins of SIVmac5 (ADPT14, SIV-Gag; EVA7057, SIV-Tat; EVA7058, SIV-Rev; EVA777, SIV-Nef) at a concentration of 2 µg PHA ml^{-1} for each peptide. As a control for proliferation, 2 µg PHA ml^{-1} (HA16, Murex Biotech) and 5 µg ml^{-1} of the recall antigen tetanus toxoid (Wyeth Ayerst Laboratories) were included in each assay. At day 5, the cultures were pulsed for 16–18 h with 1 µCi per well of [3H]thymidine (Amersham Bioscience) and incorporated radioactivity was measured in a β-counter (Perkin-Elmer) after harvesting of cellular DNA. The stimulation index (SI) was calculated by dividing the cultures were pulsed for 16–18 h with 1 µCi per well of [3H]thymidine (Amersham Bioscience) and incorporated radioactivity was measured in a β-counter (Perkin-Elmer) after harvesting of cellular DNA. The stimulation index (SI) was calculated by dividing the counts per minute of the cultures treated with the recall antigen by the counts per minute of the cultures treated with medium alone.

ELISPOT assay for IFN-γ. The IFN-γ ELISPOT assay was performed using commercially available reagents (Mabtech). Ficoll-Hypaque-purified PBMC, resuspended in RPMI supplemented with 10% FCS, were seeded (2 x 10^5 cells, 100 µl per well) in duplicate wells of a 96-well microtitre plate (Millipore) which had been previously coated with a monoclonal antibody against monkey IFN-γ. The following stimuli, diluted in RPMI with 10% FCS, were then added (100 µl per well): medium alone; 2 µg PHA ml^{-1}; 3.5 µg AT-2 inactivated SIVmac239 ml^{-1} (corresponding to 0-1 µg p27^{env} ml^{-1} ); 3.5 µg SUP1T1 microvesicles ml^{-1} as negative control; pools of 15-mer peptides at a final concentration of 2 µg each peptide ml^{-1}; SIVmac251/32H-Gag (EVA7066), -Tat (EVA7069), -Rev (EVA7068) and SIVmac5/Nef (EVA7067). After incubation overnight at 37°C, cells were removed and biotinylated, anti-IFN-γ detector antibody was added to the wells followed by the addition of streptavidin–alkaline phosphatase and finally, chromogenic substrate. After development, spots were counted using an ELISPOT reader (Amplimated Bioline). The background was calculated as twice the mean number of IFN-γ spot forming cells per 10^5 cells (IFN-γ-SFC/10^5) in non-stimulated or microvesicle-stimulated samples. Samples yielding IFN-γ-SFC/10^5 higher than 20 after the subtraction of the background were scored as positive.

SIVmac251 virus stock and intrarectal challenge. The challenge virus stock was prepared in rhesus macaque PBMC and titrated intrarectally in rhesus macaques using a 10-fold dilution from 10^{-2} to 10^{-4} and 4 rhesus macaques for each dilution, apart from the 10^{-4} dilution for which 3 monkeys were used. The number of infected animals in each group was 4, 4, 2 and 0 respectively, giving an intrarectal titre in rhesus monkeys of 10^8 MIDs ml^{-1}. In general, the titre of the virus in the two species of monkeys is not identical. According to our experience when crossing between rhesus and cynomolagus monkeys approximately 10-fold higher virus doses are needed to infect all cynomolagus as compared to rhesus macaques (ten Haaf et al., 2001). In this study we challenged intrarectally with 3 ml of a 1:6 dilution of the stock virus (500 rhesus MIDs) corresponding to approximately 50 MIDs in cynomolgus monkeys.

Fasted monkeys were sedated by ketamine injection and laid on their stomachs with the pelvic region slightly elevated. A feeding tube was inserted 4 cm into the rectum and 3 ml of virus diluted 1:6 in sterile, pyrogen-free saline containing 2% inactivated FCS was slowly delivered. After inoculum the feeding tube was slowly withdrawn and the monkeys were held in the same position for 10 min. The monkeys were then returned to their cages and observed for 4 h for the presence of faeces.

Infection of monkey PBMC with SIVmac251. For the in vitro infection, Ficoll-purified monkey PBMC taken before immunization were resuspended in RPMI supplemented with 15% FCS and antibiotics, stimulated for 48 h with 2 µg PHA ml^{-1} and then cultured for an additional 3 days at 37°C with 5% CO2 in RPMI supplemented with 15% FCS, antibiotics and recombinant human IL2 (40 U ml^{-1}) (rhIL2, Becton Dickinson). PBMC were counted and incubated overnight at 37°C with 5% CO2 with cell-free supernatant of CEMX174-derived stock of SIVmac251 at different m.o.i.s (1 and 0-1 TCID50 per cell) or with mock supernatant from uninfected CEMX174. The cells were washed five times with PBS, resuspended and finally plated in duplicate at 1 x 10^5 cells ml^{-1} in a 48-well tissue culture plate in RPMI supplemented with 15% FCS, antibiotics and rhIL2 (40 U ml^{-1}). At 3, 6 and 10 days after infection, virus production was monitored by determining p27^{env} antigen release in clarified supernatants using an ELISA assay (Innotest; Innogenetics).

Determination of virus loads. Plasma was separated from whole blood by centrifugation, clarified at 3000 r.p.m. for 30 min at 4°C and stored at −80°C. Virus loads in guanidine isothiocyanate-treated plasma were determined by quantitative competitive (QC) RT-PCR assay with a threshold limit for detection of 50 RNA Eq ml^{-1} (ten Haaf et al., 1998). To determine the cell-associated virus load, DNA was extracted from 400 µl whole citrated-blood using the QIAmp DNA blood Mini kit (Qiagen) according to the manufacturer’s instructions. SIV proviral copies were determined using TaqMan real-time PCR. Probe and primers to specifically amplify a region of 71 bp within the gag region of SIVmac251 (GI:334657) were designed using Primer Express software (Applied Biosystems). The Taqman probe, 5'-TGTCACCTGCACTAGGCGGAG-3' (nt 1472-1496) is labelled at the 5' end with a reporter fluorochrome FAM (6-carboxyfluorescein) and at the 3' end with the quencher dye TAMRA (6-carboxytetramethylrhodamine). The primers used were: 5'-ACCCAGTGATCCAAATATGCGTAACT-3' (sense; nt 1444-1470) and 5'-TCAATTTCACCCAGGTTAATGTG-3' (anti-sense; nt 1521-1497). Samples were analysed in triplicate by PCR in a total volume of 25 µl of a mixture containing 400 ng DNA, 12.5 µl PCR master mix (Applied Biosystems), 900 nM each primer and 180 nM probe. Thermal cycling conditions were: 2 min at 50°C (to allow uracil N-glycosylase digestion which prevents cross-over contamination), 10 min at 95°C (to allow thermal activation of the AmpliTaq Gold) and 45 cycles of 15 s at 95°C and 1 min at 60°C. Amplification, data acquisition and analysis were performed using the ABI Prism 7700 sequence detection system (Applied Biosystems).

Statistical analysis. Statistical analyses (ANOVA and Bonferroni adjusted test) were made after log transformation of the data to improve normality.

RESULTS

Humoral and cell-mediated immune responses to vaccination

In this study we used a total of 12 cynomolagus monkeys. Four were primed intradermally with DNA (week 0), boosted twice subcutaneously with rSFV (weeks 8 and 16) and finally boosted intramuscularly with rMVAs at week...
Monkeys remained untreated (naïve) (Table 1).

All monkeys were challenged intrarectally with approximately 50 MID₅₀ of cynomolgus SIVmac251.

<table>
<thead>
<tr>
<th>Monkeys</th>
<th>Treatment and schedule</th>
<th>Week 0</th>
<th>Week 8</th>
<th>Week 16</th>
<th>Week 24</th>
<th>Week 32</th>
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<tbody>
<tr>
<td>Vaccinated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>988, 966, 965, 957</td>
<td>pDNA-SIV⁺</td>
<td>rSFV-SIV⁺</td>
<td>rSFV-SIV⁺</td>
<td>rMVA-SIV⁺</td>
<td></td>
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<tr>
<td></td>
<td>100 µg/each gene i.d.</td>
<td>10⁸ IU/each gene s.c.</td>
<td>10⁸ IU/each gene s.c.</td>
<td>10⁸ IU/each gene i.m.</td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>967, 954, 997, 764</td>
<td>pDNA</td>
<td>SFV-LacZ</td>
<td>SFV-LacZ</td>
<td>MVA</td>
<td></td>
<td></td>
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<tr>
<td>Naïve</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>964, 955, 984, 983</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Challenge</td>
</tr>
</tbody>
</table>

*The inocula consisted of a mixture of each antigen divided equally between two separate inoculation sites.

24. Four monkeys received empty plasmids and non-recombinant viral vectors (control) and an additional four monkeys remained untreated (naïve) (Table 1).

Shortly after the second inoculum with rSFV, anti-whole SIV antibodies were detected and remained at low titres (1 : 50, 1 : 100) until the day of challenge in the plasma of two (988, 965) of the four vaccinated monkeys. Monkey 966 seroconverted after the final boost with rMVA whereas monkey 957 did not (data not shown). This immunization regimen failed to generate neutralizing antibodies in vaccinated animals (data not shown).

Proliferative activities in vaccinated monkeys were measured by in vitro stimulation of PBMC with vaccine antigens (whole inactivated SIV-AT2 and 20-mer peptide pools of SIV-Gag, -Tat, -Rev and -Nef). Proliferative responses to SIV-AT2, already evident in three of the four vaccinated monkeys shortly after the first immunization with rSFV, sharply increased in all vaccinated monkeys after the last boost with rMVA at week 24 (Fig. 1a) and persisted until the day of challenge (week 32). A similar time-course was observed for Gag- and Tat-specific proliferative responses (Fig. 1b, c). Two weeks after the first immunization with rSFV, T-cell proliferative responses to Gag and Tat peptides were detectable in four of four and in three of four vaccinated monkeys, respectively. After the last immunization with rMVA, PBMC from all vaccinated animals responded to Gag antigen (SI range 18–28), remaining stable in vaccinated monkeys 965 and 966 but disappearing in monkey 988 by week 32 (monkey 957 was not tested) (Fig. 1b). Similarly, specific proliferative responses to the Tat peptide pool were observed at week 26 in two out of four vaccinated monkeys and by the time of challenge three out of four animals showed SI values ranging between 7 and 26 (Fig. 1c). However, no responses to the Rev and Nef peptide pools were detected. All control monkeys (immunized with empty vectors) had SI values <3 (data not shown).

The ELISPOT assay was used to measure the frequency of IFN-γ-SFC/10⁶ upon in vitro stimulation with whole inactivated SIV-AT2 and 15-mer peptide pools of SIV-Gag, -Tat, -Rev and -Nef. In contrast to the proliferative responses described above, a strong antigen-specific IFN-γ release was detected only after the final rMVA boost (Table 2). In particular, the three vaccinated monkeys tested showed high frequencies of IFN-γ-SFC/10⁶ in response to SIV-AT2 (range 222–605 SFC/10⁶). At week 32 (time of challenge) the number of IFN-γ-SFC was similar for all vaccinated monkeys (range 112–750 SFC/10⁶). Similarly, responses to 15-mer peptide pools of SIV-Nef developed only after the immunization with rMVA in two out of four vaccinated monkeys (966, 965) although at a lower level compared with that observed upon stimulation with SIV-AT2 (Table 2). It is noteworthy that monkey 966 was unique among the vaccinated monkeys with its broad but sporadic response to all vaccine antigens tested (Table 2).

**Vaccine impact on intrarectal challenge with SIVmac251**

Eight weeks after the final immunization, the vaccinated, control and naïve monkeys were challenged with approximately 50 MID₅₀ of SIVmac251 administrated via the intrarectal route.

All naïve and control monkeys became infected. Control monkey 954, although seroconverted, was never viraemic and on only one occasion gave a weakly positive proviral DNA reading. Due to a technical problem (a mixture of faeces and inoculum solution was observed under the cage immediately after the intrarectal challenge), this monkey did not receive a challenge dose comparable to that of other control monkeys. Nevertheless, to determine whether the transient infection was dependent on cellular resistance to infection, SIVmac251 was used to infect in vitro unfractonated PBMC, taken before immunization, from monkeys 954, 966, 997 and 984. At 6 days after infection the level of p27⁶⁸ was detected in the supernatant of PBMC of monkey 954 (m.o.i. of 0.1 TCID₅₀ per cell) was similar to that of other monkeys (162 and 119 ± 63 pg per 10⁶ cells, respectively).
The plasma viral RNA concentrations in control (Fig. 2b) and naïve (Fig. 2c) monkeys peaked at 2 weeks post-infection (range $4.6 \times 10^5$–$1.3 \times 10^6$ and $1.4-6.1 \times 10^5$ RNA Eq ml$^{-1}$, respectively), declining thereafter. Similarly, the proviral load in control (Fig. 2e) and naïve (Fig. 2f) monkeys peaked 2 weeks after the challenge (range 116–754 and 168–517 SIV proviral copies (µg DNA)$^{-1}$, respectively). Subsequently, the virus loads were variable but remained persistently detectable up to 41 weeks post-challenge. One control monkey (967) was euthanized, although not for AIDS symptoms, after week 35.

In contrast to the control and naïve macaques, three out of the four vaccinated monkeys were either fully protected against SIV challenge or exhibited a dramatic reduction in both the magnitude and period of viraemia (Fig. 2a). Monkey 966 was never viraemic and monkeys 965 and 957, at week 2 post-challenge, had peak levels of $1.6 \times 10^2$ and $2.2 \times 10^3$ RNA Eq ml$^{-1}$ respectively. After this time-point the viraemia in monkeys 965 and 957 rapidly dropped to (and remained) below the detection limit of the assay. Moreover, at 2 weeks post-challenge, the proviral load in the PBMC of these three vaccinated monkeys ranged from undetectable to 12 copies (µg DNA)$^{-1}$ (Fig. 2a) and remained at the same level [$<10$ proviral copies (µg DNA)$^{-1}$] at the subsequent time-points. The fourth vaccinated monkey (988) developed a persistent infection as demonstrated by the plasma RNA and proviral copies that by week 4 became almost indistinguishable from those of the controls and naïve infected monkeys.

ANOVA and the Bonferroni adjusted test revealed that the four vaccinated monkeys at week 2 had levels of SIV RNA and proviral DNA significantly lower than those of control (for RNA, $P=0.034$; for DNA, $P=0.003$) and naïve monkeys (for RNA, $P=0.027$; for DNA, $P=0.004$). In the four vaccinated monkeys virus loads had diverged drastically by 4 weeks post-challenge. Monkey 988 exhibited virus loads similar to those of the control and naïve groups and vaccinated monkeys 966, 965 and 957, becoming plasma viraemia negative and maintaining a very low load [$<10$ proviral copies (µg DNA)$^{-1}$] between 4 and 41 weeks post-challenge. The virus loads of the immunized monkeys at week 4 and 12 were significantly lower than those of control (for RNA $P=0.001–0.001$; for DNA $P=0.000–0.030$, respectively) and of naïve monkeys (for RNA $P=0.001–0.042$; for DNA $P=0.005–0.023$, respectively).

**Immune responses after challenge**

The abrogation of challenge-virus replication in vaccinated monkeys 966, 965 and 957 coincided with the burst of antiviral T-cell responses (Table 2). Two weeks post-challenge all vaccinees showed a marked increase in the frequency of IFN-$
\gamma$-SFC upon stimulation with SIV-AT2 (range 580–4820 SFC/10$^6$). Moreover, at the same time-point, only the three vaccinated and protected monkeys increased the frequencies of IFN-$
\gamma$-SFC responses upon stimulation with SIV-Gag, -Tat, -Rev and -Nef peptide pools. These differences were found to be statistically significant when compared to the frequencies of control and naïve monkeys (Table 2). These responses slowly declined but were still detectable at week 27. In contrast to the other three vaccinated monkeys, macaque 988 (which did not control the challenge virus) mounted a moderate response to SIV-Nef but failed to generate specific responses to SIV-Gag, -Tat and -Rev antigens at 2 weeks post-challenge. Compared to the vigorous recall of T-cell

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**Fig. 1.** Time-course analyses of proliferative responses in vaccinated monkeys (○, 988; □, 966; Δ, 965; O, 957) to (a) whole SIV-AT2, and 20-mer peptide pools of (b) SIV-Gag and (c) SIV-Tat. The proliferative responses are expressed as stimulation indices (SI). The arrows indicate the time at which the vaccine antigens were given.
**Table 2.** Frequency of IFN-γ-SFC/10^6 cells in vaccinated, control and naïve monkeys at the end of the vaccination and after the challenge, upon stimulation with whole SIV-AT2 inactivated virus, and with 15-mer peptide pools SIV-Gag, -Tat, -Rev and -Nef proteins of SIVmac251/32/J5

Week −6 corresponds to week 26 of the vaccination period (2 weeks after the last boost with rMVA). 0, day of challenge. wpc, weeks post-challenge. ND, not done. P values indicate the significant differences in IFN-γ-SFC/10^6 at 2 weeks post-challenge obtained by comparing the vaccinated and protected monkeys (966, 965 and 957) to control and naïve infected monkeys, respectively.

<table>
<thead>
<tr>
<th>Monkeys</th>
<th>SIV-AT2</th>
<th>Gag</th>
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<th>Rev</th>
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<tr>
<td></td>
<td>Weeks</td>
<td>−6</td>
<td>0</td>
<td>2wpc</td>
<td>27wpc</td>
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<td>988</td>
<td>222</td>
<td>312</td>
<td>610</td>
<td>32</td>
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<tr>
<td>966</td>
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<td>605</td>
<td>750</td>
<td>580</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>957</td>
<td>ND</td>
<td>112</td>
<td>1740</td>
<td>40</td>
<td>ND</td>
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**versus controls**

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<tr>
<th></th>
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<th>P=0.007</th>
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<tr>
<td>Vaccinated</td>
<td>vs. naive</td>
<td>P=0.002</td>
<td>P=0.005</td>
<td>P=0.007</td>
<td>P=0.004</td>
</tr>
<tr>
<td>Control</td>
<td>967</td>
<td>&lt;20</td>
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| Naive | 964 | <20 | <20 | 90 | 157 | <20 | <20 | <20 | 122 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 110 | <20 | <20 | <20 | 185 |
| 955 | <20 | <20 | <20 | <20 | 230 | <20 | <20 | <20 | 157 | <20 | <20 | <20 | <20 | <20 | 275 | <20 | <20 | <20 | 87 |
| 984 | <20 | <20 | 40 | 222 | <20 | <20 | <20 | 110 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 172 | <20 | <20 | <20 | 282 |
| 983 | <20 | <20 | 60 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 40 | <20 | <20 |
responses induced by the challenge virus in three of the four vaccinated monkeys, the control and naïve monkeys mounted a modest response against SIV-AT2 and exhibited low or undetectable levels of IFN-γ-SFC/10^6 to peptide pools (Table 2).

We also analysed vaccinated monkeys for the presence of virus-specific T-helper responses and observed a temporary decrease rather than an anamnestic increase in the response to SIV-AT2 (Fig. 3a), to SIV-Gag (Fig. 3b) and SIV–Tat (Fig. 3c) peptide pools. Only vaccinated monkey 966 maintained Gag-specific responses up to week 35 after the challenge. With the exception of naïve animal 955, which showed an early response at 2 weeks post-challenge, T-cell proliferative responses to SIV-AT2 appeared in control (Fig. 3d) and naïve (Fig. 3g) monkeys by week 4 after the challenge. In contrast, the responses to SIV-Gag were moderate and sporadic among the naïve monkeys (Fig. 3h) and practically absent in control monkeys (Fig. 3e). Furthermore, a response to SIV-Tat was not detected in either the control or the naïve monkeys (Fig. 3f, i). Like the vaccinees, both control- and naïve-infected monkeys failed to develop detectable proliferative responses to SIV–Rev and SIV–Nef peptide pools (data not shown).

An anamnestic humoral response following challenge was not seen in the vaccinated animals. A moderate increase in antibodies to whole SIV was measured in three of the four vaccinated and protected monkeys (966, 965 and 957) at 4 weeks post-challenge (Fig. 4a). Of interest in the plasma of the vaccinated and protected monkey 957, the anti-SIV antibodies dropped to an undetectable level by week 35 after the challenge, accounting for the absence of a chronic SIV replication. Only in the vaccinated and productively infected monkey, 988, did the antibody titres reach those observed for the control and naïve monkeys (1:51 200) (Fig. 4b, c). It is interesting that monkey 954, which for technical reasons (see Methods) received a lower dose of virus, seroconverted later at week 8.

**DISCUSSION**

In this study we report that a systemic polyvalent DNA/SFV/MVA vaccine abrogated virus replication in three of the four vaccinated monkeys following mucosal challenge with SIVmac251, apparently by inducing robust T-cell immune responses. The pattern of virus replication in these three monkeys was dramatically altered. Each of these animals yielded low copy numbers of plasma virus RNA and/or provirus DNA during the acute phase, demonstrating that infection had indeed occurred. However, at 2 weeks post-challenge the levels of viral RNA in plasma were low to undetectable and by week 4 all monkeys had become and remained plasma viraemia negative. Moreover, even proviral DNA was only sporadically detected in the PBMC of the three vaccinated and protected monkeys. This result is more promising than the partial control of virus replication observed in other similar studies.
Previous studies had confirmed the importance of a T-cell-mediated immune response (Hel et al., 2002b; Jin et al., 1999; Schmitz et al., 1999) and had suggested, although with contrasting evidences (Johnson et al., 2003; Rasmussen et al., 2002; Robinson et al., 1999), a role for neutralizing antibodies in controlling virus replication. In our study, antibody responses to SIV were extremely low and neutralizing antibodies in particular were lacking during the period of vaccination. In addition, a humoral anamnestic response in particular were lacking during the period of vaccination. In addition, a humoral anamnestic response was not seen in any of the vaccinees following challenge. It therefore seems that neutralizing antibodies did not appear to be associated with the protective effect seen in this study. In contrast, pre-challenge ELISPOT and proliferative analyses demonstrated impressive and specific T-cell responses to SIV-AT2 antigen in all vaccinated monkeys. Although we were not able to address the immunological correlates during the vaccination, it is noteworthy that early after challenge (week 2) only immunized animals that had specific anamnestic responses to all vaccine antigens tested (Gag, Rev, Tat, Nef) were able to contain SIV infection. The challenge infection therefore appeared to have expanded the already existing pools of memory T-cells. After the challenge, vaccinated and infected monkey 988, which failed to contain the infection, demonstrated a T-cell response to whole SIV but not to all vaccine antigens. It is therefore likely that the control of viraemia observed in this study was due to the induction of T-cell immune responses by the DNA/SFV/MVA prime-boost regimen facilitating secondary immune responses, that first contained and later abrogated the spread of replicating virus. This pattern of immune reactivity could explain the different outcome of the challenge, highlighting the importance of a wide spectrum of responses against many SIV vaccine antigens. As shown by the protection achieved using live attenuated virus vaccines, our results support the concept that an effective

Fig. 3. Post-challenge proliferative responses of vaccinated (○, 988; □, 966; △, 965; ○, 957), control (○, 967; □, 954; △, 997; ○, 764) and naïve monkeys (○, 964; □, 955; △, 984; ○, 983) upon stimulation with (a, d, g) whole inactivated SIV-AT2, 20-mer peptide pools of (b, e, h) SIV-Gag and (c, f, i) SIV-Tat. The proliferative responses are expressed as stimulation index (SI). *Control monkey 967 was euthanized soon after week 35, although not for AIDS symptoms.
vaccine should contain and elicit immune T-cell responses to both structural and regulatory viral antigens (Mooij & Heeney, 2001).

After the challenge with pathogenic SIV, vector-immunized monkey 954 (plasma viraemia negative and proviral DNA occasionally detectable) controlled virus replication. The innate antiviral responses potentially raised by vector immunization do not seem to play a role in the control of virus replication, since all vector controls had levels of viraemia at least as high as those of naïve monkeys. As far as a genetic predisposition to control virus replication is concerned, we observed that the PBMC of monkey 954, when infected in vitro, were equally susceptible to virus infection as were the PBMC of other monkeys. Thus, any genetic restriction factor of virus replication at the level of virus entry or post-entry (Peng et al., 2002; Pal et al., 2002) was ruled out. Conversely, it is very likely that the limited infection observed in monkey 954 was due to the very low amount of virus taken up by the rectal mucosa, as a consequence of the incomplete delivery of virus inoculum. Nevertheless, this limited dose of infectious virus elicited an immune response profile (seroconversion, proliferative responses limited to SIV-AT2 and IFN-γ ELISPOT responses to Gag and Rev peptides) that could be responsible for the control of virus replication in this monkey, as described (Trivedi et al., 1996; McChesney et al., 1998; Makitalo et al., 2000).

In the experimental animal model the combination of vector antigens, the schedule of immunization, the route of antigen delivery and of challenge can all greatly influence the degree of the protection achieved (Lehner et al., 1999). In this respect, the triple vector-based DNA/SFV/MVA systemic immunization targeting the iliac lymph nodes could have generated antiviral responses in those lymph nodes draining the genital and rectal tracts, as has been suggested previously (Baig et al., 2002; Colmenero et al., 2001; Klavinskis et al., 1996; Mitchell et al., 1998; Stevceva et al., 2002). The presence of antiviral responses at mucosal sites could have played an important role in the containment or in the abrogation of virus spread. Despite the small sample size analysed and the failure to detect consistent pre-challenge immune correlates, our results are of importance in the development of a multiprotein and triple vector-based vaccine approach able to establish complete protection against mucosal HIV infection.

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