Immunization with recombinant modified vaccinia virus Ankara can modify mucosal simian immunodeficiency virus infection and delay disease progression in macaques

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In the present study, the immunogenicity and protective efficacy of a recombinant vaccinia virus-based simian immunodeficiency virus (SIV) vaccine, given alone or in combination with a protein boost, were investigated. Cynomolgus macaques were immunized intramuscularly with modified vaccinia virus Ankara (MVA) expressing the SIVsm env and gag–pol genes (MVA–SIVsm) at 0 and 3 months (n = 4), at 0, 3 and 8 months (n = 4) or at 0 and 3 months followed by purified native SIVsm gp148 and recombinant SIVmac p27 in immunostimulatory complexes at 8 months (n = 4). One month after the last immunization, the vaccinees, together with four naive control monkeys and four monkeys immunized with wild-type MVA, were challenged intrarectally with 10 MID50 SIVsm. At the time of challenge, antibody titres to SIV Env and lymphocyte proliferation responses to whole viral antigen were highest in vaccinees receiving MVA–SIVsm in combination with protein immunizations. Following rectal challenge, one of these vaccinees was completely protected. A prolonged survival time was observed in two of four monkeys in each of the groups immunized with MVA–SIVsm, in two monkeys given MVA–SIVsm followed by protein and in three of four monkeys given wild-type MVA, compared with naive controls. In conclusion, one monkey given the combined vaccine was protected completely against SIVsm infection. Furthermore, immunization with MVA–SIVsm, as well as wild-type MVA alone, seemed to delay disease progression after mucosal SIV infection in a proportion of the monkeys.

Introduction

An effective prophylactic vaccine is needed to contain the global spread of human immunodeficiency virus type 1 (HIV-1). Such a vaccine will have to mediate both humoral and cellular immune responses, since there is evidence that resistance to HIV is mediated by multiple immune effector mechanisms (Heeney et al., 1999). Furthermore, a successful HIV vaccine will have to protect against mucosal exposure, because the virus is generally transmitted by sexual contact or via perinatal transmission (Piot, 1998).

In the development of an AIDS vaccine, no single immune correlate to protective immunity has been defined (Heeney et al., 1999). In the absence of a single immune response correlate that will predict vaccine efficacy, non-human primate models are important. Macaques infected with simian immunodeficiency viruses (SIVmac, SIVsm and SIVmne) develop a disease similar to AIDS. By using this model, induction of vaccine protection against both infection and disease can be assessed (Bogers et al., 2000).
The best protection against SIV has been elicited by live attenuated vaccines (Putkonen, 1996). However, live attenuated, multiply deleted SIV has been shown to cause AIDS in infant and adult macaques (Baba et al., 1999). Furthermore, prolonged infection with a nef-defective HIV-1 strain that showed characteristics similar to attenuated SIV vaccines caused AIDS in individuals who previously showed no sign of disease progression (Learmont et al., 1999; Rhodes et al., 2000). Thus, attenuated HIV vaccines may not be applicable to man because of issues of safety.

Prime-boost regimens induce both humoral and cellular immune responses and are therefore attractive alternatives. Combinations of virus vectors and proteins, naked DNA and proteins, naked DNA and virus vectors and combinations of different virus vectors have been tested (Excler & Plotkin, 1997; Barnett et al., 1998; Heeney et al., 2000).

Modified vaccinia virus Ankara (MVA) is a highly attenuated poxvirus vector under investigation as a vector for possible HIV vaccine candidates. Although MVA is replication-deficient in human cells, it expresses recombinant proteins at levels equal to that of fully replication-competent vaccinia virus (Sutter & Moss, 1992). Recombinant MVA–SIV has been shown to induce neutralizing antibodies, T-cell proliferative responses and CTL responses in macaques. Furthermore, partial protection has been reported against intravenous homologous SIV challenge (Hirsch et al., 1996; Ourmanov et al., 2000; Seth et al., 2000).

Immune-stimulating complexes (ISCOMs) are lipid particles that comprise the immunostimulatory fractions from Quillaia saponaria (Quil A), cholesterol and phospholipids (Barr et al., 1998). Immunization of HIV envelope glycoproteins incorporated into ISCOMs has been shown to induce antibodies, T-cell proliferation (Verschoor et al., 1999; Nilsson et al., 1995) and CTLs (Heeney et al., 1994). Furthermore, we reported long-term protection against intravenous HIV-2 challenge in macaques induced by immunization with native HIV-2 Env in ISCOMs or Ribi adjuvant (Nilsson et al., 1995).

Early HIV-1 vaccine studies focused on Env as the immunogen. Lately, prime-boost vaccination regimens have been initiated using vaccinia virus vector priming and protein boosts (Mulligan & Weber, 1999). In the SIVmne/Macaus nemestrina model, Polacino et al. (1999b) reported complete protection against intravenous infection with pathogenic, uncloaked SIVmne by vaccinia virus priming and multiple protein-boost immumnizations in Freund’s incomplete adjuvant that included both envelope and core antigens.

In the present study, we evaluated the immunogenicity and protective efficacy against intrarectal SIV challenge of an MVA–SIVm vaccine and a prime–boost regimen in which MVA–SIVm was combined with a single ISCOM-formulated protein boost of gp148 and p27. A stronger humoral and cellular immune response was elicited by the prime–boost regimen than by vaccination with MVA–SIVm alone. After intrarectal challenge, all control monkeys as well as all but one vaccinee given the combined vaccine became infected. Prolonged survival was seen in two of four monkeys in each of the groups immunized with MVA–SIVm, in two of the three infected monkeys given the combined vaccines and in three of four monkeys given MVA wild-type, compared with naive controls.

Methods

Animals. A total of 20 cynomolgus macaques (Macaca fascicularis) were included in the study. The monkeys were seronegative for SIV and simian T-lymphotropic virus and PCR-negative for simian retrovirus type D before entering the study. The guidelines of the Swedish Ethical Committee for Animal Protection were adhered to for housing and animal handling.

Immunogens. The recombinant virus MVA–SIVm co-expresses the gag–pol and env coding sequences of SIVsmmH4 (kindly provided by Bernard Moss, NIH, Bethesda, MD, USA) (Hirsch et al., 1996). gag–pol was placed under the transcriptional control of the natural vaccinia virus early–late promoter P7.5 and env was expressed using a strong synthetic virus vector early–late promoter (Chakrabarti et al., 1997).

For the production of vaccines, MVA–SIVm and non-recombinant MVA were amplified on primary chicken embryo fibroblasts (CEF) and purified by ultracentrifugation through a cushion of 36% sucrose. Purified viruses were reconstituted in PBS and titrated by end-point dilution in CEF to obtain the TCID₅₀ (infectious units), aliquotted and stored at −70 °C.

The MVA–SIVm vaccine preparations were controlled for synthesis of recombinant proteins by immunostaining and Western blot analysis of infected CEF cell preparations by using sera from an SIVm-infected macaque (data not shown).

Native SIVm envelope glycoprotein gp148 purified by affinity chromatography (Gilljam, 1993) and recombinant SIVmac251 p27 (rp27) were used for booster immunizations. The proteins were conjugated to ISCOM matrix (Lövgren & Morein, 1988), consisting of a mixture of low-tox Quillaia saponin fractions QH-A and QH-C (Verschoor et al., 1999), essentially as described by de Vries et al. (1994).

MVA–SIVm recombinant viruses and rp27 were provided by the EU programme EVA/MRC Centralised facility for AIDS Reagents, NIBSC, UK. Purified SIVm gp148 was prepared by the Department of Virology, Swedish Institute for Infectious Disease Control, Solna, Sweden.

Study design. Three groups of four monkeys (n = 12) were inoculated intramuscularly (i.m.) with 5 × 10⁸ p.f.u. each of MVA–SIVm env and gag–pol, one group (A) at 0 and 3 months, another group (B) at 0, 3 and 8 months and a third group (C) at 0 and 3 months followed by 50 µg purified native SIVm gp148 and 50 µg recombinant SIVmac p27 in ISCOMs at 8 months. One month after the last immunization, the vaccinees, together with four naive control monkeys (group D) and four monkeys immunized with wild-type MVA at 0, 3 and 8 months (group E), were challenged intrarectally with 10 MLD₅₀ cell-free, monkey-cell-grown SIVm. On the day of challenge, aliquots of liquid-nitrogen-stored SIVm were thawed, pooled and diluted 1:10. Three ml of the virus dilution was delivered intrarectally atraumatically to each monkey using a paediatric feeding tube (Quesada-Rolander et al., 1996).

The monkeys were monitored for clinical changes and blood samples were collected for immunological and virological studies. The study was completed 27 months after challenge (groups B–E) or 32 months after challenge (group A).
Detection of antibody responses. ELISAs were used to detect antibodies in serum to native SIVsm gp148 as described in detail previously (Nilsson et al., 1995). To detect antibodies to p27, the antigen was changed to rp27 diluted to 0.5 µg/ml for coating. The ELISA titres were defined as the reciprocal of the highest serum dilution that gave an absorbance value more than twice that of individual pre-immunization sera.

The presence of antibodies mediating SIVsm-neutralizing activity was determined in an assay using PHA-stimulated human PBMCs (Zhang et al., 1997) and an SIVsm stock virus that had been grown on monkey PBMCs. Neutralizing-antibody titres were defined as the dilution of serum that resulted in at least 90% reduction in antigen synthesis, compared with control wells that did not contain serum, by using an in-house antigen ELISA (Thorstenson et al., 1991).

SIV Env-specific IgA antibodies in serum and rectal washes were determined in an ELISA using SIVsm gp148 diluted to 1 µg/ml for coating, as described in detail elsewhere (Nilsson et al., 2001). A sample was considered positive if the absorbance was greater than twice that of pre-immunization samples. Rectal washes were collected 9 or 14 days after the last immunization. The rectal mucosa was flushed with 2 ml PBS and aspirates were collected, centrifuged and stored at −70 °C.

T-cell proliferation assay. T-helper cell responses were determined in a standard [3H]thymidine incorporation assay using HIV_SIVmac4448 whole virus antigen (5 µg/ml) to stimulate proliferation as described elsewhere (Nilsson et al., 2001). Stimulation indices (SI) were calculated by dividing the mean c.p.m. of the antigen-stimulated wells by the mean of the unstimulated wells. An SI ≥ 2.0 was considered positive.

CTL assay. The cytolytic activity of T lymphocytes was described as described previously (Mäkitäri et al., 2000; Andersson et al., 1996). Target cells were an autologous herpesvirus papio-transformed B lymphoblastoid cell line (B-LCL) infected with recombinant vaccinia virus expressing SIVmac Gag/Pol or Env products or with vaccinia virus only as control. One part 31Cr-labelled target cells was mixed with 40 parts unlabelled, MVA-infected target cells (cold targets) to reduce vaccinia virus-specific cytolysis. For generation of effector cells, monkey PBMCs were stimulated with concanavalin A (Sigma) and cultured for 14–21 days in medium containing IL-2 (Amersham). An effector-to-target cell ratio of 100:1 was used. Specific lysis (%) was calculated by the following formula: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). A specific chromium release of > 4% was considered positive for SIV Gag/Pol CTL (Nilsson et al., 2001). The criterion for a positive Env CTL was based on results with or without CD8+ T-cell depletion. Thus, Env CTL was considered positive when the difference in chromium release was > 10%. However, the trend for each animal was always considered and a single positive value was not accepted unless confirmed on another occasion.

CD8+ T lymphocytes were enriched or depleted by using immunomagnetic beads (Dynabeads; Dynal) according to the manufacturer’s instructions.

Assessment of virus infection after challenge. Virus re-isolation was performed essentially as described previously (Nilsson et al., 1995) using 2 × 10^6 macaque PBMCs and 1.8 × 10^7 human PHA-stimulated PBMCs for co-cultivation. In order to detect SIV proviral DNA in lymphocytes, a nested PCR and primers specific for the long terminal repeat (LTR) and gag genes were used (Walther et al., 1998). SIV RNA levels in plasma were assessed by using a highly sensitive quantitative competitive (QC) RT–PCR assay with a detection limit of 40 RNA equivalents/ml plasma (ten Haaf et al., 1998).

Lymphocyte subset analysis. Immunized monkeys were monitored for changes in their T-lymphocyte subsets by flow cytometry analysis, as described previously (Mäkitäri et al., 2000). Briefly, EDTA-treated whole blood was stained with fluorescein-conjugated anti-CD8 and phycoerythrin-conjugated anti-CD4 antibodies. After lysis of the red blood cells and fixation, quantification of cell-surface immunofluorescence was done in a FACScan flow cytometer and analysis was performed using cellquest software (Becton-Dickinson Immunocytometry Systems).

Statistical analysis. Statistical calculations were made using the Mann–Whitney U-test for non-parametric observations.

Results

Immune responses to MVA–SIVsm recombinants and protein boosts

Antibody responses to SIV envelope glycoprotein (gp148) and SIV Gag (p27), T-cell proliferative responses to whole virus lysate and induction of SIV-specific CTLs were studied over time to compare the immune responses generated in macaques by administering two MVA–SIVsm immunizations, three MVA–SIVsm immunizations or two MVA–SIVsm immunizations in combination with a protein boost.

All vaccinees had undetectable anti-Env antibody levels after the first MVA–SIVsm immunization. One month after the second MVA–SIVsm immunization, low anti-Env antibody titres (range 100–500) were detected in 9 of 12 vaccinees (Fig. 1a–c). Furthermore, 14 days after the third MVA–SIVsm immunization, given to monkeys in group B, a 25-fold increase in anti-Env antibody titre was seen in all vaccinees (Fig. 1b). A larger, more than 600-fold increase in anti-Env antibody titre was detected in all prime–boosted vaccinees (group C) 14 days after the Env and Gag protein boost (Fig. 1c).

After the first MVA–SIVsm immunization, antibody titres to recombinant p27 were either low (range 100–500), as observed in D11, D17, D18 and D19, or remained undetectable. After the second MVA–SIVsm immunization, all vaccinees except D1, D2 and D16 had detectable anti-Gag antibodies, but the titres remained low. Neither the third MVA–SIVsm immunization of monkeys in group B nor the protein immunization of monkeys in group C boosted the anti-Gag antibody responses in the vaccinees (data not shown).

Sera collected 9 or 14 days after the last immunization were analysed for anti-SIV gp148 IgA antibodies. Borderline levels of anti-Env IgA antibodies were seen in 3 of 4 monkeys immunized twice with MVA–SIVsm. Higher levels were seen in monkeys immunized three times with MVA–SIVsm and even higher levels were seen in prime–boosted vaccinees. In parallel, anti-Env IgA antibodies were also detected in rectal washes from all monkeys immunized twice with MVA–SIVsm, in 2 of 4 monkeys immunized three times with MVA–SIVsm and in 2 of 4 prime–boosted vaccinees (Table 1).

Two weeks after the second MVA–SIVsm immunization, virus-specific T-cell proliferative responses to whole HIV-2 lysate were detected in 9 of 12 vaccinees (SI range 3–24). Two weeks after the third MVA–SIVsm immunization, two mon-
keys, D9 and D10, exhibited increased proliferative responses (SI range 15–20). Strong T-cell proliferative responses were detected in 3 of 4 vaccinees (D17, D18 and D19) 14 days after the Env and Gag protein boost (SI range 48–98) (Fig. 2).

SIV-specific CTLs were not detected in any of the vaccinees after two MVA–SIVsm immunizations, nor were cytotoxic activities detected in the vaccinees after three MVA–SIVsm immunizations. After two immunizations with MVA–SIVsm and a protein boost in ISCOMs, only monkey D17 exhibited SIV Gag/Pol- and Env-specific CTL responses, respectively of 11 and 16% lysis. These CTLs were of the CD8 phenotype, as determined by cell-depletion experiments (data not shown). No CTL analysis was performed on monkey D20 since we could not establish a B-LCL for this monkey.

Fig. 1. Anti-SIV gp148 responses in individual macaques as determined by ELISA. (a) Monkeys immunized twice with MVA–SIVsm. (b) Monkeys immunized three times with MVA–SIVsm. (c) Monkeys immunized twice with MVA–SIVsm and boosted with gp148 and rp27 in ISCOMs. (d) Naive control monkeys included at the time of challenge. (e) Control monkeys immunized three times with wild-type MVA. Open arrows indicate times of MVA–SIVsm immunizations, the plain arrow indicates the time of protein boost and the filled arrows indicate the time of intrarectal SIVsm challenge.
Table 1. SIV-specific immune responses at the time of challenge

Positive reactions are given in bold. The definitions of a positive reaction for the various assays are described in Methods.

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Anti-SIV IgG ELISA</th>
<th>Anti-SIV IgA ELISA*</th>
<th>Neutralizing-antibody titre</th>
<th>CTL against SIV Gag/Pol (% lysis)</th>
<th>T-cell proliferation with virus lysate (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum rp27 (titre)</td>
<td>Serum gp148 (titre)</td>
<td>Rectal wash (absorbance)</td>
<td></td>
<td></td>
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<tr>
<td>Two immunizations with MVA–SIV</td>
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<tr>
<td>D1</td>
<td>&lt; 100</td>
<td>&lt; 100</td>
<td>0.055</td>
<td>0.686</td>
<td>&lt; 40</td>
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<tr>
<td>D2</td>
<td>&lt; 100</td>
<td>&lt; 100</td>
<td>0.025</td>
<td>0.652</td>
<td>&lt; 40</td>
</tr>
<tr>
<td>D3</td>
<td>100</td>
<td>100</td>
<td>0.096</td>
<td>1.732</td>
<td>&lt; 40</td>
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<tr>
<td>D16</td>
<td>&lt; 100</td>
<td>100</td>
<td>0.088</td>
<td>1.056</td>
<td>&lt; 40</td>
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<tr>
<td>Three immunizations with MVA–SIV</td>
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<tr>
<td>D8</td>
<td>500</td>
<td>2500</td>
<td>0.196</td>
<td>0.021</td>
<td>&lt; 40</td>
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<tr>
<td>D9</td>
<td>100</td>
<td>2500</td>
<td>0.440</td>
<td>0.038</td>
<td>&lt; 40</td>
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<tr>
<td>D10</td>
<td>100</td>
<td>2500</td>
<td>0.334</td>
<td>0.721</td>
<td>&lt; 40</td>
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<tr>
<td>D11</td>
<td>100–500</td>
<td>2500</td>
<td>0.078</td>
<td>0.711</td>
<td>&lt; 40</td>
</tr>
<tr>
<td>Two immunizations with MVA–SIV plus protein boost</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>D17</td>
<td>500</td>
<td>62500</td>
<td>1.478</td>
<td>0.785</td>
<td>80</td>
</tr>
<tr>
<td>D18</td>
<td>500</td>
<td>62500</td>
<td>0.173</td>
<td>0.021</td>
<td>160</td>
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<tr>
<td>D19</td>
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<td>12500</td>
<td>0.821</td>
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<tr>
<td>D20</td>
<td>100</td>
<td>2500</td>
<td>1.418</td>
<td>1.365</td>
<td>40</td>
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</table>

* Rectal wash and serum collected 14 or 21 days before challenge and analysed in an anti-SIV gp148 IgA ELISA.
† Proliferative responses determined 14 days before challenge.

Immune responses at the time of challenge

The immune responses at the time of challenge are summarized in Table 1. At this time-point, 1 month after the last immunization, we confirmed the trend seen shortly after the final immunization, such that, when anti-Env antibodies exhibiting binding or neutralizing activity and T-cell proliferative responses were studied, clear differences were seen between the three groups of immunized monkeys. Monkeys given two MVA–SIVsm immunizations followed by a protein boost had strong to moderately strong immune responses, monkeys given three immunizations with MVA–SIVsm had weak immune responses and monkeys given two immunizations with MVA–SIVsm had weak immune responses or levels of immune responses that were below the detection limit. Furthermore, titres of antibodies to SIV p27 and SIV Gag/Pol-specific CTLs were unchanged and remained low or undetectable.

Outcome of intrarectal SIVsm challenge

One month after the last immunization, the vaccinees (groups A–D) and four naive controls were challenged intrarectally with 10 MID₅₀ SIVsm. One monkey, D19, immunized twice with MVA–SIVsm and boosted with gp148 and p27 in ISCOMs, was repeatedly virus isolation-negative, lacked proviral DNA in PBMC and exhibited a reduction in circulating antibodies (data not shown and Fig. 1). No virus RNA could be detected in plasma after challenge by using a highly sensitive QC RT–PCR assay (Fig. 3). The monkey was considered to be protected completely. All the other vaccinees, as well as the control monkeys, became infected. Varying degrees of virus isolation positivity over time and varying levels of virus RNA in plasma were displayed (Fig. 3). Virus load data obtained 3 months after challenge were compared because viraemia levels at this time-point had previously been suggested to be predictive of disease outcome. ten Haaft et al. (1998) reported that a virus load that remained below a threshold of 10⁴ RNA equivalents/ml plasma indicated a non-pathogenic course of infection. Monkeys D9 and D11, both immunized three times with MVA–SIVsm, displayed a reduced frequency of positive virus isolation and a low virus copy number. Monkey D2, immunized twice with MVA–SIVsm, displayed an even lower frequency of virus isolation positivity and a plasma RNA load of 40 copies/ml plasma. Another monkey in this group (D3) yielded virus at 15 of 20 time-points and had a plasma copy number of 5 × 10³ 3 months after challenge. Also, three of four monkeys immunized three times with wild-type MVA displayed a reduced frequency of positive virus isolation (D5, D6 and D7) and their plasma RNA copy numbers 3 months after challenge ranged between 10³ and 5 × 10⁵. The remaining vaccinees, two monkeys immunized twice with MVA–SIVsm (group A), two monkeys immunized three times with MVA–SIVsm (group B), three
prime–boosted monkeys (group C) and one monkey immunized three times with wild-type MVA (group D), were generally virus isolation-positive on every occasion tested and most yielded virus copy numbers of more than $5 \times 10^4$ copies/ml plasma 3 months after challenge. Even higher virus copy numbers ($> 10^5$) were detected in three of four of the naive control monkeys (D22, D23, D24). The other naive control monkey (D21) exhibited a lower virus copy number, with $10^3$ viral RNA copies/ml plasma at this time-point. The virus load at 2 weeks after challenge was significantly lower in the SIV vaccinees compared with the naive and wild-type MVA-vaccinated controls ($P = 0.0008$; Mann–Whitney U-test). However, no statistically significant difference was seen when comparing the virus loads at 3 months.

**Humoral immune responses after intrarectal SIVsm challenge**

Three of four monkeys immunized twice with MVA–SIVsm (D1, D3, D16) showed strong anamnestic anti-SIV Env antibody responses (Fig. 1). Monkeys D1 and D3 exhibited respective neutralizing antibody titres of 640 and 160 already 14 days after challenge. The fourth monkey (D2) lacked neutralizing antibodies and showed a comparatively slow increase in circulating antibodies.

All vaccinees immunized three times with MVA–SIVsm showed anamnestic antibody responses to SIV Env, as determined by ELISA. However, only monkeys D9 and D10 yielded antibodies exhibiting neutralizing activity (titre of 40) 14 days after challenge. Among the monkeys immunized twice with MVA–SIVsm and boosted with protein, the protected monkey (D19) showed a steady drop in anti-SIV Env antibodies (Fig. 1). Monkeys D17 and D18 maintained a constant anti-Env antibody titre of 62,500 until 9 months after challenge, when a 5-fold increase in antibody titre was seen (data not shown). Fourteen days after challenge, monkey D18 exhibited high neutralizing-antibody responses, yielding a titre of 640. The fourth monkey in this group (D20) exhibited a strong anamnestic antibody response of binding antibodies (Fig. 1). However, only a 2-fold increase in neutralizing-antibody titre was detected.

All MVA wild-type controls had seroconverted by 1 month after challenge (Fig. 1). Anti-SIV Env antibodies detected by ELISA did not reach levels comparable to those in the other vaccinees until 2 or 3 months after infection. However, SIVsm-neutralizing antibody titres in monkeys D6 and D7 were similar to those seen in the MVA–SIVsm-vaccinated monkeys by 1 month after infection (titre range 80–640).

**Cell-mediated immune responses after intrarectal SIVsm challenge**

A strong T-cell proliferative response to HIV-2SBL6669 was observed in monkey D19, the monkey that was protected from SIV infection, for more than 8 months after challenge. In contrast, all infected vaccinees lost lymphoproliferative responses after challenge. Furthermore, monkeys D5 and D7, which had received wild-type MVA vaccination, displayed strong T-cell proliferative responses 4 months after challenge (Fig. 2).

SIV Env-specific CTLs were regularly detected during the first year of follow-up in two vaccinees. Cytolysis ranged between 11–17 and 14–23% for two prime–boosted monkeys (D17 and D18). In one monkey immunized three times with MVA–SIVsm (D10), cytolytic activities of 26, 16 and 16% were detected at 3, 9 and 12 months after challenge. Monkey
Influence of MVA on mucosal SIV infection

D16, immunized twice with MVA–SIVsm, had detectable SIV Gag/Pol-specific cytotoxic activities of 15 and 16% at 1 and 2 months after challenge. None of the other seven SIV vaccinees tested showed any cytolytic activity at any of the five time-points between 1 and 12 months after challenge. Furthermore, none of the wild-type MVA-immunized monkeys developed detectable CTLs in peripheral blood (data not shown).

Clinical outcome of intrarectal SIVsm challenge

The monkeys were monitored for changes in CD4+ T-cell counts, haematology, body weight and signs of disease over a period of more than 2 years after challenge. Fig. 4 summarizes the CD4+ T-cell counts and survival time in the vaccinees and control monkeys.
Monkey D19, immunized with MVA–SIVsm in combination with a protein boost, maintained normal CD4⁺ cell counts and developed no evidence of disease. We concluded that this monkey was indeed protected against infection and disease.

Two infected monkeys, immunized two (D3) or three (D9) times with MVA–SIVsm, showed little change in their CD4⁺ T-cell counts and they remained healthy during the study period. Yet another monkey (D11), immunized three times with MVA–SIVsm, showed a moderate decline in CD4⁺ T cells and it also stayed healthy during the follow-up. Surprisingly, three of four wild-type MVA-immunized monkeys (D5, D6 and D7) remained healthy, with normal CD4⁺ T-cell counts. Moreover, although the fourth wild-type MVA-
immunized monkey (D4) showed a progressive CD4+ T-cell depletion after challenge and, by 11 months, had lost most of its CD4+ T cells, it was not euthanized until 15 months later, when it started to lose weight.

The other three monkeys immunized twice with MVA–SIVsm developed clinical signs of disease within 32 months after challenge. Monkey D17 started a slow but steady decline in CD4+ T cells, which dropped rapidly after 26 months of infection. It was euthanized 4 months later. Monkey D16 was euthanized 21 months after challenge, together with monkey D2, which, despite an initially low level of plasma virus RNA, showed a severe depletion of CD4+ T cells beginning 1 year after challenge.

Two monkeys, D8 and D10, that had received MVA–SIVsm three times, showed progressive declines in CD4+ T cells and D10 was euthanized 16 months after challenge. Although D8, at that time-point, had an equally low CD4+ T-cell count, it was not euthanized until 10 months later, when it had started to lose weight.

Among the monkeys immunized with MVA–SIVsm in combination with a protein boost, another two vaccinees (D17 and D18) showed moderate changes in CD4+ T cells during the first 16 months of follow-up. D17 was euthanized 10 months later because of clinical signs of disease. Monkey D18 remained clinically healthy during the follow-up period. The last monkey in the group (D20) lost CD4+ T cells rapidly and was euthanized 10 months after challenge.

The rate of disease progression in monkey D20 was similar to that observed in the SIVsm-infected control monkeys. All these controls exhibited rapid CD4+ T-cell losses. A near total depletion was already observed after 6 months of infection in monkey D24, which was euthanized 6 months later. The other controls were euthanized within 18 months of infection.

The survival time of the monkeys immunized with wild-type MVA was prolonged significantly compared with that of 26 naive historic controls challenged intrarectally with SIVsm ($P = 0.043$, Mann–Whitney U-test).

**Discussion**

In this study, we investigated the immunogenicity and protective efficacy of MVA–SIVsm vaccines expressing SIVsm Env and Gag/Pol, given alone or in a prime–boost regimen in which MVA–SIVsm was combined with an ISCOM-formulated protein boost of SIV gp148 and SIV p27. Following each immunization with MVA–SIVsm, an increase in antibodies to SIV Env was noted, although antibodies to SIV p27 remained low or undetectable. These findings support the notion that the vaccines elicited strong antibody responses against both envelope and core antigens. Protection against intrarectal challenge was reported after priming with recombinant vaccinia virus expressing SIVmne gp160 and multiple immunizations with gp160 in incomplete Freund’s adjuvant (Polacino et al., 1999a). Moreover, after four immunizations with another attenuated pox vector, NYVAC, expressing SIVmne gag and pol genes, partial protection was demonstrated against intrarectal SIVmac251 challenge (Benson et al., 1998). Thus, the present study supports and extends observed for the MVA vaccine used in this study. We could detect only low levels of SIV-specific CTLs. This is in contrast to other MVA–SIVsm vaccine studies, where SIV-specific CTLs were detected readily by either in vitro peptide stimulation (Seth et al., 1998) or tetramer staining (Seth et al., 1998, 2000). This discrepancy may be explained by the lower sensitivity of classical bulk cytotoxic T-cell assays and the possible induction of high background lysis, a result of the method used for stimulation, despite cold-target inhibition.

By combining the MVA–SIVsm vaccine with a protein boost, strong T-helper cell responses and high neutralizing-antibody titres were induced. The T-cell proliferative responses detected were similar to those reported in human trials after two immunizations with ALVAC–HIV-1 (vCP125) and boosting with rgp160 in alum or Freund’s incomplete adjuvant (Pialoux et al., 1995). Both T-helper cell responses and neutralizing-antibody responses were stronger than those we reported previously after giving two immunizations with ALVAC expressing HIV-2 Env, Gag and Pol and a boost of native HIV-2 gp125 in QS21 adjuvant (Andersson et al., 1996). The stronger neutralizing-antibody response could be attributed in part to the use of a more sensitive assay, using virus grown on monkey PBMCs. The T-cell proliferative responses also exceeded those seen after immunizations with either SIV Env formulated in ISCOMs or DNA priming and boosting with rgp120 in ISCOMs (Verschoor et al., 1999).

A potential HIV vaccine candidate will have to protect against mucosal challenge. We therefore challenged monkeys intrarectally with the homologous SIVsm. In order to ensure that all monkeys were exposed to the same virus dose, 3 ml pooled and diluted virus was delivered intrarectally to each monkey. After challenge, all naive control monkeys, as well as all but one of the vaccinees given the MVA–SIV prime–protein boost, became infected. Control of viraeemia was seen in two of four monkeys in each of the groups immunized with MVA–SIVsm, in two of the three infected monkeys given the combined vaccines and in three of four monkeys given wild-type MVA. Previous studies have shown partial protection against AIDS after intravenous challenge with a highly related SIVsm virus strain after MVA–SIVsmH-4 vaccination with (Hirsch et al., 1996) or without (Ourmanov et al., 2000) boosting with whole inactivated SIV. In M. nemestrina, Polacino et al. (1999b) reported complete protection against intravenous infection with pathogenic, uncloned SIVmne by vaccinia virus prime–protein boost immunizations including both envelope and core antigens. Protection against intrarectal challenge was reported after priming with recombinant vaccinia virus expressing SIVmne envelope gp160 and multiple immunizations with gp160 in incomplete Freund’s adjuvant (Polacino et al., 1999a). Moreover, after four immunizations with another attenuated pox vector, NYVAC, expressing SIVmne gag and pol genes, partial protection was demonstrated against intrarectal SIVmac251 challenge (Benson et al., 1998). Thus, the present study supports and extends
these findings to show that MVA–SIVsm in combination with a single protein boost in ISCOMs may prevent infection or delay immunosuppression and disease caused by mucosal SIV infection.

Following the intrarectal SIVsm challenge, given 1 month after the final immunization, three of four monkeys immunized with $5 \times 10^{5}$ p.f.u. wild-type MVA (no SIV gene) exhibited control of viraemia and remained clinically healthy. All wild-type MVA-immunized monkeys initially exhibited high virus loads. By 3 months after challenge, viraemia had dropped to a plasma virus copy number of between $10^4$ and $5 \times 10^3$ in three of the monkeys (D5, D6 and D7). These three monkeys also exhibited low virus-isolation frequencies and they maintained normal CD4+ T-cell levels during the follow-up. Hanke et al. (1999) have reported protection in a wild-type MVA-immunized rhesus macaque challenged intrarectally with SIVmac2 months after the last MVA immunization. However, when wild-type MVA vaccinees were challenged intrarectally 3 months after the last immunization, no protective effect was seen (Nilsson et al., 2001). Although not fully proven, these data suggest that MVA may induce a short-lived, non-specific antiviral activity. In the present study, we found that the control of viraemia and maintenance of CD4+ T cells was associated with persistent T-cell proliferative responses (Fig. 2). Blanchard et al. (1998) have reported that MVA is a potent inducer of IFN type I. Furthermore, it has been shown that, unlike other vaccinia virus strains, MVA does not express soluble receptors for IFN-γ, IFN-α/β, tumour necrosis factor and CC chemokines (Antoine et al., 1998), antiviral factors that could potentially contribute to the observed effect. Importantly, our findings stress the need for longer waiting periods after the final MVA immunization in order to allow investigations of virus-specific immune correlates of protective immunity in MVA vaccine studies. Therefore, we did not attempt to define such correlates in our study, despite the reduced viraemia seen in some of the vaccinees that received MVA–SIVsm two (group A) or three times (group B).

A third group (C) of monkeys was given two immunizations with MVA–SIVsm at 0 and 3 months and a boost of gp148 and p27 in ISCOMs at 8 months, i.e. challenged 6 months after the last MVA immunization. At this time-point, the non-specific antiviral activity of MVA should not have influenced the challenge experiment. It is interesting to note the high neutralizing-antibody titres and levels of T-cell proliferative response seen in the prime–boosted vaccinees that displayed control of infection. One vaccinee (D19) was repeatedly virus isolation-negative and proviral DNA-negative in PBMCs and no virus RNA was detected after challenge by using a highly sensitive QC RT–PCR assay (ten Haaf et al., 1998). Furthermore, D19 lacked an anamnestic antibody response in serum and retained normal CD4+ T-cell levels during more than 2 years of follow-up. The monkey was considered completely protected. At the time of challenge, monkey D19 had a high T-cell proliferative response but showed neither detectable SIV-specific CTLs nor neutralizing antibodies. After SIVsm challenge, the high T-cell proliferative responses were maintained for more than 8 months, although levels of circulating antibodies slowly waned. In addition, two other monkeys (D17 and D18) in this group showed signs of controlled infection, as judged by the virus load at 6 months after challenge and a delayed decrease in CD4+ T cells. Monkey D18 had very high neutralizing-antibody titres and levels of T-cell proliferative response at the time of challenge. Monkey D17 also had a high neutralizing-antibody titre and T-cell proliferative responses. It also had a detectable SIV Gag/Pol-specific CTL response. In contrast, the fourth monkey (D20) in this group exhibited weaker immune responses at the time of challenge and rapidly lost its CD4+ T cells. The presence of potent T cells and antibodies exhibiting virus neutralization at the time of challenge may have contributed to the balance established between the virus and the host.

We conclude that the MVA–SIVsm prime–protein boost protocol elicited a stronger immune response than MVA–SIVsm alone. Furthermore, one MVA-primed protein-boosted monkey was protected completely and another two monkeys in this group showed controlled viraemia. Immunization with MVA–SIVsm as well as wild-type MVA seemed to delay disease progression in a proportion of the monkeys.

Currently, clinical vaccine trials are being planned or are ongoing in which a live recombinant vector such as MVA (D. L. Birx, personal communication) or ALVAC (Excler & Plotkin, 1997) is combined with a subunit boost (gp120). Our findings bring hope that an MVA prime–protein boost vaccine strategy may induce immune responses potent enough to establish complete protection against mucosal HIV-1 infection or induce control of viraemia and delay disease progression.

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