The appearance of escape variants \textit{in vivo} does not account for the failure of recombinant envelope vaccines to protect against simian immunodeficiency virus

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The presence or evolution of immune escape variants has been proposed to account for the failure of recombinant envelope vaccines to protect macaques against challenge with simian immunodeficiency virus (SIVmac). To address this issue, two groups of three cynomolgus macaques were immunized with recombinant SIV Env vaccines using two different vaccine schedules. One group of macaques received four injections of recombinant SIV gp120 in SAF-1 containing threonyl muramyl dipeptide as adjuvant. A second group were primed twice with recombinant vaccinia virus expressing SIV gp160 and then boosted twice with recombinant SIV gp120. Both vaccine schedules elicited neutralizing antibodies to Env. However, on the day of challenge, titres of anti-Env antibodies measured by ELISA were higher in macaques primed with recombinant vaccinia virus. Following intravenous challenge with 10 monkey infectious doses of the SIVmac J5M challenge stock, five of the six immunized macaques and all four naive controls became infected. The virus burdens in PBMC of macaques that were primed with recombinant vaccinia virus were lower than those of naive controls, as determined by virus titration and quantitative DNA PCR. Sequence analysis was performed on SIV env amplified from the blood of immunized and naive infected macaques. No variation of SIV env sequence was observed, even in macaques with a reduced virus load, suggesting that the appearance of immune escape variants does not account for the incomplete protection observed. In addition, this study indicates that the measurement of serum neutralizing antibodies may not provide a useful correlate for protection elicited by recombinant envelope vaccines.

Introduction

Much effort has been invested in developing potential AIDS vaccines based on recombinant human immunodeficiency virus type 1 (HIV-1) Env, despite concerns that the genetic heterogeneity of the envelope of HIV-1 will overwhelm any immunity induced. The infection of macaques with simian immunodeficiency virus (SIV) is a valuable model for the evaluation of AIDS vaccine strategies (Stott & Almond, 1995). Hu \textit{et al.} (1992) found that priming cynomolgus macaques (\textit{Macaca fascicularis}) with live vaccinia virus that expressed Env from SIVmne (isolated from \textit{Macaca nemestrina}), followed by boosting with baculovirus-derived SIV gp160, conferred complete protection against homologous challenge with a matched clone of SIVmne. However, similar vaccine strategies have conferred only partial protection in rhesus and cynomolgus macaques against challenge with a number of different isolates of SIVmac. In spite of the generation of strong serological and cellular immune responses to the virus, these vaccines have seldom achieved more than a reduction in virus loads (Ahmad \textit{et al.}, 1994; Daniel \textit{et al.}, 1994; Giavedoni \textit{et al.}, 1993; Israel \textit{et al.}, 1994).

It is possible that the failure of vaccines based on monotypic envelope is due to the rapid evolution and selection of escape variants in the immunized host following challenge. The
genetic evolution of SIVmac in macaques is very similar to that of HIV-1. Viruses expressing distinct env sequences appear readily, even when the initial infection is with virus prepared from infectious molecular clones (Burns & Desrosiers, 1991; Johnson et al., 1991). There is evidence that the rate of mutation and in vivo selection is accelerated in macaques that have been immunized with Env-based vaccines prior to challenge with uncloned virus stocks (Almond et al., 1993; Polacino et al., 1999). Thus, the simian model is well suited to determine whether monotypic envelope vaccines drive this process of mutation and selection in macaques following challenge with cloned virus stocks.

To address this hypothesis, six macaques were immunized with recombinant SIV Env (gp120) with or without priming with recombinant vaccinia virus expressing SIV Env. After challenge with SIV derived from an infectious molecular clone, sequences of env genes from recovered viruses were analysed for evidence of immune selection.

### Methods

**Animals.** The study group comprised ten juvenile cynomolgus macaques that were purpose-bred in Europe. The animals were housed and maintained in accordance with Home Office guidelines for the care and maintenance of primates. Animals were sedated with Ketamine before vaccination or venepuncture and were examined clinically while under anaesthesia.

**Recombinant vaccines.** The construction of vaccinia virus expressing recombinant (r) gp160 derived from the SIVmac molecular clone BK28 has been described previously (Horth et al., 1991). Recombinant gp120, derived from the molecular clone BK28, was produced in Drosophila cells and purified by using lentil lectin. For immunization, it was mixed with the Syntex emulsion SAF-1 containing threonyl muramyl dipeptide as described previously (Mills et al., 1992).

**Serological responses.** Antibodies that bound SIV Env were determined by ELISA with recombinant SIV gp140 (Repligen Corporation) as antigen, as described previously (Stott et al., 1990).

The capacity of the serum to neutralize a stock of SIVmac J5 in vitro was determined as described previously (Kent et al., 1994). It is presented as the reciprocal dilution of heat-inactivated serum capable of inhibiting mean SIV p27 antigen production by 75% following infection of C8166 cells.

**Proliferative T-cell responses.** Proliferation of PBMC following stimulation with recombinant SIV gp120 was determined as described previously (Jones et al., 1992).

**Virus recovery from PBMC and the assessment of virus load.** Virus re-isolation from the blood of macaques was performed by co-culture of 5 × 10⁶ PBMC with 10⁶ C8166 cells. The presence of CPE was confirmed by an antigen-capture assay specific for SIV Gag protein (Rose et al., 1995). To determine the virus load, tenfold dilutions of PBMC (10⁶ to 10⁴) from each macaque were co-cultivated in duplicate, in 24-well plates, with 10⁶ C8166 cells.

**Diagnostic PCR and quantification of the SIV provirus burden.** The extraction of DNA from the blood of macaques and the diagnostic amplification of the SIV gag gene have been described previously (Rose et al., 1995). The SIV provirus load was determined by combining end-point dilution of DNA with PCR (Slade et al., 1995). The number of SIV genomes per 10⁶ cells was calculated on the basis that 6.6 µg total DNA is equivalent to 10⁶ cells.

**Nucleotide sequencing of SIV env.** The amplification of the entire gp120 region of SIV env by PCR and the direct determination of the nucleotide sequence of the PCR product has been described previously (Almond et al., 1992).

**Experimental design.** Group A: three cynomolgus macaques (K79, K89 and K90) received four subcutaneous immunizations of 85 µg purified rgp120 protein with adjuvant on weeks 0, 4, 8 and 104. Group B: on weeks 0 and 4, three cynomolgus macaques (K83, K84 and K94) were inoculated intradermally with 2 × 10⁶ p.f.u. of the recombinant vaccinia virus expressing the SIV Env (gp160). At weeks 8 and 104, these macaques were boosted by subcutaneous injection of 85 µg purified rgp120 formulated with adjuvant.

Two weeks after the final boost (week 106), all six immunized macaques, as well as four naive controls (group C: M77-M80), were challenged by intravenous inoculation with 10 monkey infectious doses (MID₅₀) of the 3/92 stock of the SIV molecular clone J5 prepared on simian cells (also known as JSM; Rud et al., 1994). The animals were sacrificed between 8 and 10 weeks after challenge.

### Results

**Immune responses.**

All macaques in groups A and B responded to immunization by making antibodies to recombinant Env (Fig. 1). The three animals primed with recombinant vaccinia virus seroconverted by 4 weeks post-immunization (p.i.). Antibodies were detected by ELISA in all immunized macaques from 8 weeks p.i. Between the third and fourth immunizations, plasma antibody titres declined from a peak at week 10, with half-lives between...
Env in the three macaques of group B (mean log response to the fourth dose was seen in macaque K79. Levels equivalent to or greater than those at week 10. No the fourth vaccination, titres rose in five of the six macaques to challenge (data not shown).

By ELISA plateaued at between 4–15 weeks post-challenge. The log titres of antibodies in the two groups of immunized macaques were not significantly different. Control macaques the titres of antibodies in the two groups of immunized

The vaccines and schedules used to immunize macaques in groups A and B are indicated. The end-point titres of serum neutralizing antibodies (Neut. Ab.) against SIV and anti-SIV Env antibodies determined by ELISA in plasma collected on the day of challenge are shown for each macaque. In addition, the virus burdens are presented, determined by virus titration of the PBMC and by quantitative DNA PCR of template extracted from the blood of each macaque 2 weeks after challenge. Animals were challenged in week 106 p.i. No done; Co-cult., co-culture; VV, vaccinia virus.

Table 1. A comparison between the serological anti-SIV responses determined in immunized macaques and naive control macaques on the day of challenge and the virus burden in PBMC recovered 2 weeks post-challenge

<table>
<thead>
<tr>
<th>Animal</th>
<th>Vaccine</th>
<th>Immunization schedule (weeks)</th>
<th>Antibody titre on day of challenge</th>
<th>Virus burden in blood at 2 weeks p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>K79</td>
<td>rgp120</td>
<td>0, 4, 8, 104</td>
<td>Anti-Env ELISA (log10 end-point titre)</td>
<td>Neut. Ab. (reciprocal titre)</td>
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<tr>
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</tr>
<tr>
<td>K89</td>
<td></td>
<td></td>
<td>2.1</td>
<td>125</td>
</tr>
<tr>
<td>K90</td>
<td></td>
<td></td>
<td>3.3</td>
<td>125</td>
</tr>
<tr>
<td>K83</td>
<td>VV-Env + rgp120</td>
<td>0, 4 (VV-Env), 8, 104 (rgp120)</td>
<td>4.3</td>
<td>1000</td>
</tr>
<tr>
<td>K84</td>
<td></td>
<td></td>
<td>3.8</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>K94</td>
<td></td>
<td></td>
<td>4.5</td>
<td>125</td>
</tr>
<tr>
<td>M77</td>
<td>None</td>
<td></td>
<td>&lt; 2.0</td>
<td>ND</td>
</tr>
<tr>
<td>M78</td>
<td></td>
<td></td>
<td>&lt; 2.0</td>
<td>ND</td>
</tr>
<tr>
<td>M79</td>
<td></td>
<td></td>
<td>&lt; 2.0</td>
<td>ND</td>
</tr>
<tr>
<td>M80</td>
<td></td>
<td></td>
<td>&lt; 2.0</td>
<td>ND</td>
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</tbody>
</table>

15.7 and 31.25 weeks (mean 24.8 weeks). However, following the fourth vaccination, titres rose in five of the six macaques to levels equivalent to or greater than those at week 10. No response to the fourth dose was seen in macaque K79.

On the day of challenge, the titres of ELISA antibodies to Env in the three macaques of group B (mean log₁₀ ELISA ± SD 4.2 ± 0.36) were significantly higher (P < 0.05; Student’s t-test) than the titres in macaques of group A (mean log₁₀ ELISA ± SD 2.7 ± 0.6; Table 1). Neutralizing antibodies were detected in all immunized macaques except K84 on the day of challenge (Table 1). However, there was no correlation between the titres of neutralizing antibodies and the titres of ELISA antibodies.

By 4 weeks after challenge (week 110), five of the six macaques showed an anamnestic antibody response (Fig. 1), the exception being K84, where the titres remained stable and then declined slightly. At 8 weeks post-challenge (week 114), the titres of antibodies in the two groups of immunized macaques were not significantly different. Control macaques M77–M80 seroconverted between weeks 2 and 4 post-challenge. The log₁₀ titres of anti-Env antibodies as measured by ELISA plateaued at between 4.0 and 5.0 from week 4 post-challenge (data not shown).

T-cell proliferative responses were assessed in selected macaques from each group throughout the course of immunization. Fourteen days after the third immunization, a significant specific proliferative response [stimulation index (SI) 2.0] to SIV Env was detectable in all three macaques tested (K89, SI = 5.2; K90, SI = 2.3; K94, SI = 9.4). On the day of challenge, proliferative responses to SIV Env were assessed for all six immunized macaques. A specific response was detected only in macaque K84 (SI = 2.4).

Detection of virus after challenge

Nine of the ten macaques became infected after challenge with SIVmac J5M, as determined by co-cultivation of PBMC (Table 2). Virus was recovered from every sample taken from control macaques at 2, 4 and 8 weeks post-challenge. The recovery of virus from immunized macaques was more sporadic. Virus was not recovered from K83 and K90 at 2 weeks post-challenge or from K89, K90 and K94 at 8 weeks post-challenge. Virus was never reisolated from the PBMC of macaque K84.

After challenge, the presence of SIV DNA was detected by nested PCR in the blood of nine of the ten macaques (Table 2). In the nine positive macaques, SIV DNA was amplified from all blood samples tested. It was not possible to amplify SIV-specific DNA from macaque K84.
Table 2. Detection of SIV in the blood of immunized and control macaques after challenge with SIVmac J5M

SIV was detected by virus co-cultivation (Co-cult.) or diagnostic PCR specific for SIV gag. The results of each co-culture of $5 \times 10^8$ PBMC with $10^6$ C8166 indicator cells and each diagnostic PCR containing DNA extracted from 75 µl blood are scored as virus-positive (+), virus-negative (−) or assay not performed (ND).

<table>
<thead>
<tr>
<th>Animal</th>
<th>Co-cult.</th>
<th>PCR</th>
<th>Co-cult.</th>
<th>PCR</th>
<th>Co-cult.</th>
<th>PCR</th>
<th>Co-cult.</th>
<th>PCR</th>
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<tbody>
<tr>
<td>Group A</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>K79</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>K89</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>K90</td>
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<td>+</td>
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<td>−</td>
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<td>−</td>
<td>−</td>
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<td>M78</td>
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<td>−</td>
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<td>+</td>
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<td>ND</td>
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<tr>
<td>M80</td>
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<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
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</table>

Virus burden by co-culture and DNA PCR

The virus load in PBMC was determined at 2 weeks post-challenge (Table 1). In all naive controls (M77-M80) and two of the immunized macaques (K79 and K89), the virus load was greater than 1000 infected cells per $10^6$ cells cultured. For macaque K94, the virus load was 100-fold lower. No virus was co-cultured from $2 \times 10^6$ PBMC of macaques K84, K89 or K90, although virus was recovered from $5 \times 10^6$ PBMC of K89 and K90.

The SIV DNA burden in the blood at 2 weeks p.i. was determined by a combination of end-point dilution and PCR (Table 1). The provirus burden in the control macaques (M77-M80) ranged between 9900 and 210 000 copies per $10^6$ cells. The provirus burden in the blood of immunized macaque K79 fell within this range. In all other immunized macaques, the provirus burdens were lower (range $<15-2400$ copies per $10^6$ cells). For macaques in group B, which had been primed with recombinant vaccinia virus, the provirus burdens were significantly lower than in controls ($P < 0.05$ by Student’s $t$-test).

Relationship between virus burden and antibody response

There was no significant correlation between ELISA antibody titres at challenge and reduction in virus load at challenge (Table 1) (Spearman’s rank correlation coefficient; $r = −0.30; P = 0.57$). Although a group A macaque, K90, with the highest neutralizing antibody titre, had the lowest virus load, this was certainly not the case in group B, where macaque K84 had no detectable neutralizing activity but was apparently protected from detectable infection.

Sequence of SIV env

The gp120 region of SIV env was amplified by nested PCR from DNA extracted from the blood of immunized macaques K79, K89, K90, K83 and K94 and control macaques M77, M78 and M80 at 2 weeks post-challenge. The nucleotide sequence of each PCR product was determined from twelve amino acids upstream of the V1 region to fourteen amino acids from the gp120:TM protein cleavage site. The predicted amino acid sequences obtained from nucleotide sequencing are shown in Fig. 2. Changes in the nucleotide sequence that did not alter the predicted amino acid sequence were detected in two macaques, K90 and K94 (Fig. 2). Only two macaques, immunized macaque K90 and control macaque M80, exhibited any differences. In control M80, the amino acid difference was at amino acid position 424, in the V4 region (equivalent to amino acid 427 of BK28 Env), where a nucleotide sequence ambiguity changed the histidine residue to a mixture of histidine and tyrosine residues. In immunized macaque K90, the change was at amino acid 323, in the V3 loop equivalent (amino acid 326 of BK28 Env), changing the predicted amino acid from isoleucine to serine. This identical change was observed in the predicted sequence of two independent PCR products amplified from the same DNA template. However, sequence analysis across this V3 equivalent region of PCR product amplified from the blood
Env sequence in immunized, SIV-infected macaques

Fig. 2. A comparison of the amino acid sequences from V1 to V5 of SIV Env predicted from the PCR product amplified from the blood from immunized (K79, K89, K90, K83 and K94) and naive control (M77, M78 and M80) macaques 2 weeks post-challenge with 10 MID50 of the J5M challenge stock. The predominant amino acid sequences obtained by direct sequence analysis of each PCR product are compared with that of the molecular clone J5, shown in single letter code. A dash indicates that the predicted amino acid is identical to J5; sequence variation is given in single letter code; ambiguities are denoted by a subscript and superscript letter at a single amino acid position. Non-coding changes are indicated by 1 (GGA → GGG) and 2 (AGG → AGA). Variable regions V1-V5 are identified on the diagram. By way of comparison, the sequence of the equivalent region of Env derived from the SIV molecular clone BK28, used to prepare the recombinant vaccines, is presented above the sequence of J5.

of K90 at 4 weeks p.i. found that the only detectable sequence was identical to that of the infecting virus, J5.

Discussion

In this study, we were able to compare two vaccine strategies, assess the role of neutralizing antibodies in reducing virus load and determine the effect of immune pressure on the sequence of recovered virus. The combination of recombinant vaccinia virus priming followed by boosting with purified Env protein (group B) elicited higher titres of antibody to Env on the day of challenge when compared with immunization with protein alone, as determined by ELISA. However, consistent with previous reports (Ahmad et al., 1994; Giavedoni et al., 1993; Hu et al., 1991), neutralizing antibody titres were not enhanced by the vaccinia virus priming. This combined
immunization strategy conferred partial protection against challenge with SIVmac JSV. The virus loads in the blood of macaques in this group were significantly lower than in controls, as determined by both virus co-culture and quantitative DNA PCR. Indeed, for one macaque from group B (K84), no evidence for infection was obtained by co-culture or DNA PCR and virus challenge failed to elicit an anamnestic antibody response. Since this virus challenge (10 MID<sub>50</sub> SIV) has been used in a number of studies at NIBSC and has infected 39 of 40 naive controls, this suggests that K84 was protected against challenge with a potentially infectious dose of SIV.

This study failed to correlate antibodies with protective immunity in the macaque. Titres of ELISA antibody to Env were higher in macaques receiving the combined immunization protocol of vaccinia virus priming and boosting with recombinant Env in adjuvant than in animals receiving purified Env protein in adjuvant alone. Previous studies have produced contradictory evidence for a role for antibody in vaccine protection against SIV (Almond et al., 1997; Gardner et al., 1995; Haigwood et al., 1996; Kent et al., 1994; Putkonen et al., 1991). It is widely believed that neutralizing antibodies are a useful immune correlate of protection by lentivirus vaccines (Clements et al., 1995; Wyand et al., 1996). In this study, however, neutralizing antibody titres on the day of challenge did not correlate with the ability to control virus load after challenge. Indeed, for macaque K84, which did not become infected after challenge, no neutralizing activity was detected in the serum at the time of virus challenge. Others have suggested that cellular immune responses are more beneficial in conferring protection and preventing progression to disease (Clerici & Shearer, 1994; Gallimore et al., 1995), although the evidence is inconclusive (Hulskotte et al., 1995). Only limited analysis of cellular immunity was performed in this study, but differences were not detected between T-cell proliferative responses of the two groups of immunized macaques. However, cytotoxic T-cell responses were more likely to have developed in group B than in group A. It is intriguing that K84, which did not become infected, was the only macaque that had a significant proliferative T-cell response on the day of challenge. Although inconclusive, the contrast between the lack of neutralizing antibody and the stronger proliferative responses would reinforce the view that T-cell rather than B-cell responses are in general more beneficial.

In spite of intensive research by a number of groups, recombinant envelope vaccines have seldom conferred protection against detectable infection with SIVmac (Ahmad et al., 1994; Daniel et al., 1994; Giavedoni et al., 1993; Israel et al., 1994). In this study, we addressed whether immune pressure resulted in the rapid selection, within 2 weeks, of variants capable of side-stepping vaccine protection. The analysis of SIV env sequences amplified from the blood of immunized and control macaques failed to detect any group-specific sequence differences, suggesting that immune escape does not account for the vaccine failure. Indeed, it was surprising that the predominant env sequence was so stable in all groups of macaques. It is possible that the results obtained by direct sequence analysis of the PCR products did not reflect the true situation in the blood, due to selective sampling from macaques with a low provirus burden. Similar problems have skewed the results of previous studies with a similar design (Slade et al., 1995). However, this is unlikely to be the case in this study, since quantification prior to sequencing ensured that every PCR used for sequence analysis contained at least 10 copies of SIV template. Furthermore, the sequence changes detected were obtained reproducibly from independent PCR products derived from the same DNA template. Alternatively, this result may have arisen because the sequence of the challenge virus SIVmac JSV represented an immune escape variant compared with the immunogens, based on the clone SIVmac BK28. This suggestion is also unlikely, since both neutralizing antibody and proliferative T-cell responses were assessed by using virus or antigens derived from the challenge virus. Significant immunological cross-reaction was observed between the vaccine and the challenge virus. Furthermore, by definition, a vaccine escape variant would not be expected to be susceptible to the effects of immunization, whereas a significant decrease in virus load was observed in macaques from group B.

The results of this study provide a valuable comparison for recent reports of participants in phase I/II clinical vaccine trials who have become infected during or following immunization with HIV-1 Env (Berman et al., 1997; Connor et al., 1998). So far, the infected vaccine volunteers studied in these reports have been recipients of subunit HIV-1 Env vaccines only and frequently had significant neutralizing antibody titres in serum before they became infected. These observations, that immunization with Env subunit vaccines has not protected recipients from infection, are very similar to those obtained from the macaques in group A that received recombinant SIV gp120 only. Nevertheless, the results from the macaques in group B confirm the observations of Hu et al. (1992), that a vaccine combining a poxvirus-based vaccine to prime and a recombinant HIV-1 gp120 protein boost can be more successful in the generation of effective protection. Nevertheless, the sequence analysis in this simian study suggests that we should be cautious in the interpretation of similar sequencing studies in humans. In the report from Connor et al. (1998), the authors concluded that the failure to detect any skewing of env sequence recovered from the infected vaccinees indicated that there was no vaccine effect. Our simian study clearly demonstrates that, even in cases where a vaccine effect (i.e. reduction in virus load) is observed, this need not necessarily result in the more rapid appearance of genetic variants of the virus.

This study indicates that the rapid appearance of immune escape variants does not account for the failure of recombinant Env vaccines to confer complete protection. The sequence stability of the env gene sequence in the blood for at least 2
weeks after challenge will renew hopes that improvements in the immunization regime of recombinant envelope vaccines may enhance their efficacy. It is possible that evaluating vaccine efficacy by intravenous challenge with SIV is too severe. This may account for the greater success reported by Lehner et al. (1996), who reported that immunization with recombinant Gag and Env proteins targeted to specific lymph nodes draining the gut elicited protection against rectal challenge with SIVmac.

This study did not address whether the partial protection achieved in macaques in group B would improve the clinical progression following infection. Reports indicate that individuals who control the initial infection most effectively and have the lowest ‘set-point’ viraemia progress to disease most slowly (Haigwood et al., 1996; Hirsch et al., 1996; Mellors et al., 1996; Watson et al., 1997). However, a clear relationship between the circulating virus load 2 weeks post-challenge and the ‘set-point’ viraemia at 6–12 weeks post-challenge has yet to be identified.

This study did not identify a reason for the failure of recombinant envelope vaccines to confer more than partial protection. Nevertheless, the results provide encouragement for the continued development and evaluation of monotypic recombinant vaccines against immunodeficiency viruses. If the failure of the vaccines to confer potent protection cannot be ascribed to the rapid mutation and selection of immune escape variants, then further work to evaluate alternative immunization regimens may prove fruitful in the quest for a safe, effective AIDS vaccine.

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References


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