Evaluation of CD8+ T-cell and antibody responses following transient increased viraemia in rhesus macaques infected with live, attenuated simian immunodeficiency virus

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In vivo depletion of CD8+ T cells results in an increase in viral load in macaques chronically infected with simian immunodeficiency virus (SIVmac239Δnef). Here, the cellular and humoral immune responses associated with this transient period of enhanced viraemia in macaques infected with SIVmac239Δnef were characterized. Fourteen days after in vivo CD8+ T-cell depletion, two of six macaques experienced a 1–2 log10 increase in anti-gp130 and p27 antibody titres and a three- to fivefold increase in gamma interferon-secreting SIV-specific CD8+ T cells. Three other macaques had modest or no increase in anti-gp130 antibodies and significantly lower titres of anti-p27 antibodies, with minimal induction of functional CD8+ T cells. Four of the five CD8-depleted macaques experienced an increase in neutralizing antibody titres to SIVmac239. Induction of SIV-specific immune responses was associated with increases in CD8+ T-cell proliferation and fluctuations in the levels of signal-joint T-cell receptor excision circles in peripheral blood cells. Five months after CD8+ T-cell depletion, only the two high-responding macaques were protected from intravenous challenge with pathogenic SIV, whilst the remaining animals were unable to control replication of the challenge virus. Together, these findings suggest that a transient period of enhanced antigenaemia during chronic SIV infection may serve to augment virus-specific immunity in some, but not all, macaques. These findings have relevance for induction of human immunodeficiency virus (HIV)-specific immune responses during prophylactic and therapeutic vaccination and for immunological evaluation of structured treatment interruptions in patients chronically infected with HIV-1.

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Supplementary methods and a figure showing thymic output and peripheral CD8+ T-cell proliferation following in vivo CD8+ T-cell depletion are available in JGV Online.
INTRODUCTION

Induction of effective antiviral immune responses that control replication of human immunodeficiency virus type 1 (HIV-1) is a prerequisite for any candidate prophylactic or therapeutic vaccine. However, to date, the relative contribution of cellular and humoral immune responses in this process is unclear (Pantaleo & Koup, 2004). In macaque models, definitive evidence from in vivo depletion studies indicates that CD8⁺ T lymphocytes play a critical role in suppressing virus replication during both the acute (Madden et al., 2004; Schmitz et al., 1999) and chronic (Jin et al., 1999; Rasmussen et al., 2002; Schmitz et al., 1999) phases of infection with either pathogenic simian immunodeficiency virus (SIV) or simian–human immunodeficiency virus (Mackay et al., 2004), and these cells contribute to sustaining a low viral load in conjunction with antiretroviral therapy (Lifson et al., 2004). A similar approach involving in vivo depletion of B cells has demonstrated that humoral immune responses may help to control viremia during the immediate post-acute phase of infection, whilst their effect on viremia during acute infection may be minimal (Schmitz et al., 2003). In macaques chronically infected with the live, attenuated strain SIVmac239Δnef, we have shown that in vivo CD8⁺ T-cell depletion results in a 1–2 log₁₀ increase in plasma viral load (Metzner et al., 2000). In this model, ablation of CD8⁺ T cells by administration of the anti-CD8 mAb OKT8F leads to a transient increase in plasma viremia. Viraemia increases over a period of 8–10 days, after which the levels of SIV RNA decline concurrently with the return of the peripheral CD8⁺ T-cell population (Metzner et al., 2000). In macaques with otherwise low viral loads and normal CD4⁺ T-cell counts, this short period of enhanced viremia provides a unique opportunity to examine changes in virus-specific immunity that may occur in response to a temporary increase in levels of endogenous viral antigens. This parallels, in certain aspects, the autologous antigenemia incurred as a result of structured treatment interruption (STI) in chronically HIV-infected patients on long-term highly active antiretroviral therapy (Trkola et al., 2004). In this study, we characterized both humoral and cellular responses in SIVmac239Δnef-infected macaques in the period immediately following in vivo CD8⁺ T-cell depletion. We found evidence that SIV-specific immune responses can be boosted in vivo following endogenous antigenic challenge, but with marked variation in the magnitude and kinetics of these responses among macaques. Only those macaques mounting a rapid and high anti-SIV immune response involving SIV-specific anti-gp130 and p27 antibodies and functional CD8⁺ T cells were protected from subsequent challenge with pathogenic SIV.

METHODS

Rhesus macaques. Six adult rhesus macaques (Macaca mulatta) were infected with SIVmac239Δnef as part of a larger vaccine study (Connor et al., 1998). Three of these animals (1496, 1502 and 1506) were selected for further evaluation based on evidence that they were protected successfully against an intravenous challenge with pathogenic SIVmac251 (Connor et al., 1998). During 2 years follow-up, SIVmac251 was not detected in any of the protected animals by nested DNA PCR of peripheral blood mononuclear cells (PBMCs) (Connor et al., 1998). Moreover, no evidence of SIVmac251 was found in lymph-node biopsies or multiple, serial PBMC samples by using a real-time PCR assay with a sensitivity of 50 RNA copies ml⁻¹ (Metzner et al., 2000). Three additional macaques (1518, 1520 and 1522) were also immunized with SIVmac239Δnef as part of the original vaccine study, but were never challenged with SIVmac251 (Fig. 1).

CD8⁺ T-cell depletion experiments were performed by using the anti-CD8 mAb OKT8F, as described previously (Metzner et al., 2000). One of six animals (1506) received equivalent dosing of an isotype-matched control antibody, P1.17, in place of OKT8F (Fig. 1). The baseline characteristics of the six macaques prior to CD8⁺ T-cell depletion have been published previously (Metzner et al., 2000) and include plasma viral loads of 0-2 × 10⁻³–6.1 × 10⁶ RNA copies ml⁻¹. All macaques were clinically healthy at the time that these studies were initiated, with CD4⁺ T-cell counts in the normal range. All animal protocols were approved by the International Animal Care and Use Committee at the Tulane Regional Primate Research Center.

Intravenous challenge with pathogenic SIV. Approximately 5 months after in vivo CD8⁺ T-cell depletion (day 160), the macaques were challenged intravenously with uncloned SIVmac055 (kindly provided by Koen van Rompay, California Regional Primate

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**Fig. 1.** Schematic representation of the experimental protocol. Six adult rhesus macaques were immunized with SIVmac239Δnef as part of a larger vaccine study (Connor et al., 1998). Three of these animals (1496, 1502 and 1506) were subsequently challenged intravenously with SIVmac251. In vivo depletion of CD8⁺ T cells and intravenous challenge with SIVmac055 were performed on the macaques as indicated.
This virus was originally isolated by co-culture of PBMCs from an SIVmac251-infected infant rhesus macaque receiving prolonged treatment with 9-[2-(R)-[phosphonomethoxy]propyl] adenine (PMPA, tenofovir). This virus demonstrates a fivefold-reduced in vitro susceptibility to PMPA and a distinct reverse transcriptase (RT) genotype with mutations at positions K65R, N69T, R82K, A158S and in infection (K. J. Metzner & R. I. Connor, unpublished data). Based on this result, the same inoculum was used to challenge the CD8-depleted macaques in the present study intravenously. Plasma viral loads were monitored after challenge by using a differential real-time PCR assay to discriminate between nef-deleted and wild-type SIV RNA as described previously (Metzner et al., 2000).

ELISPOT assay for detection of gamma interferon (IFN-γ). The ELISPOT assay used for detection of IFN-γ secretion by CD8+ T cells was modified from that described by Larsson et al. (1999). In brief, 2 × 10^6 macaque PBMCs were added to the wells of a microtiter plate (U-Cytech) coated with a mAb specific for rhesus IFN-γ. PBMCs had previously been infected with recombinant vaccinia virus (rVV) expressing either SIVmac251 Env (vAbT253), Gag (vAbT252), Pol (vAbT258), Nef (vAbT306) or a control (TkT64) (Therion Biologics) at an m.o.i. of 2. The plates were incubated for 5 h at 37 °C, after which the cells were removed by washing and the wells filled with an appropriate dilution of a biotinylated detector antibody. Subsequently, a goat anti-detector antibody was added, followed by the addition of a chromogenic substrate. After colour development, spots were visualized by light microscopy and adjusted to spot-forming cells (SFCs) per 10^6 input PBMCs.

ELISA detection of antibodies to SIV Env and Gag proteins. Antibodies to SIV gp130 and p27 in macaque plasma samples were detected by standard ELISA methods as described previously (Connor et al., 1998). Either a sheep polyclonal antibody against the C terminus of SIV gp130 (D7369; International Enzymes) and recombinant gp130, or a p27–glutathione (GSH) pull-down fusion protein was used for antibody capture. Plasma samples were preclarified for platelets and titrated threefold (starting at 1:100) before the addition of goat anti-human IgG–alkaline phosphatase conjugate (Accurate). Plates were developed using the AMPAK amplification system (Dako) and the A405 was determined.

Serum neutralization assay. Neutralizing antibodies in macaque sera were assessed in CEMx174 cell assays described previously (Montefiori et al., 1996). Briefly, 50 μl cell-free virus (5000 TCID50) was added to multiple dilutions of test serum in 100 μl growth medium in triplicate wells of 96-well microtitre plates and incubated for 1 h at 37 °C. Cells (7.5 × 10^4) in 100 μl growth medium were added and incubated until extensive syncytium formation and nearly complete cell killing were evident microscopically in virus-control wells. Viable cells were stained with Finter’s neutral red in poly-1-lysine-coated plates as described previously (Montefiori et al., 1988). Percentage protection from virus-induced cell killing was determined by calculating the difference in absorption (A490) between test wells (cells + serum sample + virus) and virus-control wells (cells + virus), dividing this result by the difference in absorption between cell-control wells (cells only) and virus-control wells and multiplying by 100. Neutralizing antibody titres are expressed as the reciprocal of the serum dilution required to protect 50% of cells from virus-induced killing. This cut-off corresponds to an approximate 90% reduction in p24 antigen synthesis. Virus stocks for neutralization assays were produced in either human (SIVmac239/ nef-open) or rhesus (SIVmac055) PBMCs.

Cloning and sequencing of SIV pol and nef genes. Genomic DNA or cDNA generated by RT-PCR was used as a template to amplify sequences within the SIV pol and nef genes. PCRs containing 1× PCR buffer, 0.5 mM dNTPs, 0.4 μM each of the primer pairs pol 2873/pol 3695 (see below) or nef 9034/nef 9904 with 2.5 U HotStarTag DNA polymerase (Qiagen) in a final volume of 50 μl were amplified for 40 cycles (94 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min) and the PCR products were analysed on 1% agarose gels. When necessary, nested PCR was performed by using 5 μl of the first-round PCR product diluted 1:100 and the primer pairs pol 2904/pol 3654 or nef 9049/nef 9894 under the conditions described above. Amplicons were purified with a QIAquick PCR purification kit (Qiagen) and sequenced directly. Primer sequences were as follows: pol 2873, 5′-GTAAAGTCACCCCTAAAGCAGG-3′; pol 2904, 5′-GACCCAAAATGTGAGCCTGCC-3′; pol 3654, 5′-GCTGCCCATACTCTCCTAC-3′; pol 3695, 5′-CCICTAAATTAACCTACAGAGTGTTCG-3′; nef 9034, 5′-AGGRTTGGAAGTCCCTCAGG-3′; nef 9049, 5′-CTCAGGACTGACTGACTACCTACC-3′; nef 9894, 5′-TCCCCTTTGGGAAATGCTCCGGC-3′; and nef 9904, 5′-CCCCCTAAACATCCCCCTGGTGG-3′.

Amplification of an approximately 7 kb fragment spanning the SIV genome from pol to nef was performed by using genomic DNA from rhesus macaques 1496, 1506 and 1522, with first-round primers (pol 2873/nef 9904), second-round primers (pol 2904/nef 9894) and the Expand Long Template PCR System (Roche Molecular Biochemicals). Amplicons were gel-purified and cloned into pCR-XL-TOPO by using a TOPO XL PCR Cloning kit (Invitrogen). Four to eight clones from each transformation were further analysed by sequencing of the RT region of pol and PCR-amplifying the nef gene and analysing the resulting products on 1% agarose gels.

RESULTS

Effect of in vivo CD8+ T-cell depletion on SIV-specific cellular immunity

CD8+ T-cell responses to SIV Gag, Pol, Env and Nef proteins were assessed by measuring IFN-γ secretion in ELISPOT assays using rVV vectors expressing SIV proteins (Moretto et al., 2000). Macaque PBMC samples were assayed at baseline (day −1) prior to CD8+ T-cell depletion and on days 7 and 14 afterwards. SIV-specific IFN-γ responses were highest for cells recognizing rVV-expressed SIV Gag antigens, whilst the responses to SIV Env, Pol and Nef were negligible (Fig. 2a). In ELISPOT assays, the number of SFCs per 10^5 PBMCs initially decreased from baseline in five of six macaques in response to in vivo CD8 depletion. This result is consistent with a previous report demonstrating that CD8+ T lymphocytes constitute the primary population of PBMCs secreting IFN-γ in response to rVV-expressing HIV antigens in vitro (Haslett et al., 2000). The control macaque, 1506, received an isotype-matched antibody (mAb P1.17) in place of OKT8 and showed no change in the number of SFCs over baseline (Fig. 2a). Over the next 2 weeks, two macaques (1502 and 1518) experienced a rapid increase above baseline in the
Fig. 2. Temporal analyses of SIV-specific immune responses and plasma viral load. Rhesus macaques immunized with SIVmac239Δ nef were depleted of CD8+ T cells in vivo by administration of mAb OKT8F on week 0 (black arrowheads). A control macaque (1506) received equivalent dosing of an isotype-matched control antibody, P1.17 (grey arrowhead). After a 5-month interval, all macaques were challenged intravenously with pathogenic SIVmac055 (large grey arrows). (a) CD8+ T cells secreting IFN-γ as enumerated in ELISPOT assays. Data are expressed as the number of spot-forming cells (SFCs) per 10^6 PBMCs against rVV expressing either SIV Env (●), Gag (□), Pol (○) or Nef (△). (b) Midpoint antibody titres to SIV gp130 (●) and p27 (□) antigens. (c) SIV viral load in plasma determined by real-time PCR: SIVmac239Δ nef (●); SIVmac251/055 (wild-type nef) (□).
number of SIV Gag-specific SFCs. A third macaque (1522) also showed an increase in SFCs; however, the levels on day 14 did not exceed the baseline measurement. Two remaining CD8-depleted macaques (1496 and 1520) and the control animal (1506) had little or no change over baseline in the number of SFCs during the same 2 week period.

**Antibody responses to SIV gp130 and p27 antigens**

Binding antibody titres to SIV gp130 and p27 antigens increased rapidly in two macaques (1502 and 1518) in the first 2 weeks after *in vivo* CD8 depletion (Fig. 2b). By day 14, titres to both gp130 and p27 had increased by 1–2 log10 in these macaques, whilst two additional macaques (1496 and 1520) had more modest increases in antibody titres. By day 56, comparable titres to both gp130 and p27 were observed for the two high-responding macaques (1502 and 1518), whilst antibody titres to p27 remained approximately 1 log10 lower than those to gp130 in the other animals (Fig. 2b). Little or no change in antibody titres to either SIV gp130 or p27 was observed for macaque 1522, which had high baseline titres, or for the control animal (1506).

Neutralizing antibody titres were measured against SIVmac239 on days −1 or 0 and subsequently on days 9 or 14 after CD8+ T-cell depletion (Table 1). Three of six macaques (1502, 1506 and 1522) had detectable neutralizing antibodies to SIVmac239 at baseline. The highest baseline titre was measured for macaque 1522, which also had high baseline titres of gp130- and p27-binding antibodies. Three other macaques (1496, 1518 and 1520) had no detectable neutralizing antibodies against SIVmac239 at baseline. Of the five macaques depleted of CD8+ T cells *in vivo*, neutralizing antibody titres increased above baseline values for four of the five (1496, 1502, 1518 and 1520) on days 9 or 14 after administration of mAb OKT8F. Only macaque 1522 experienced a decline in neutralizing antibody titres during this period from a relatively high baseline value (Table 1).

**Source of SIV-specific immune cells**

To investigate the source of SIV-specific immune cells, real-time PCR was used to assess the number of recent thymic emigrants based on quantification of signal-joint T-cell receptor excision circles (sjTRECs) in genomic DNA from PBMC samples (Chakrabarti *et al.*, 2000; Douek *et al.*, 1998; Zhang *et al.*, 1999). sjTREC copy numbers were calculated per 10^6 genomic equivalents of the single-copy CCR5 gene (Kostrikis *et al.*, 1998). Longitudinal variation was seen in the levels of sjTRECs in the majority of macaques, suggesting perturbation of the turnover of naïve T cells. However, we found no correlation between the levels of sjTRECs and recovery of the peripheral CD8+ T-cell population, suggesting that other factors must contribute to the CD8+ T-cell expansion (see supplementary material in JGV Online).

Because concomitant cell division can affect the level of sjTRECs (Chakrabarti *et al.*, 2000; Hazenberg *et al.*, 2000), we also examined the fraction of proliferating CD8+ T cells in the peripheral blood by measuring the number of cells positive for the Ki-67 marker. Ki-67 is detected specifically on proliferating cells and is present on actively dividing cells from macaques (Chakrabarti *et al.*, 2000). We found that 3–9–5–9% of CD3+CD8+ T cells stained positive for Ki-67 at baseline (see Supplementary Figure in JGV Online). All macaques given OKT8F experienced a decline in Ki-67+CD8+ T cells to undetectable levels following *in vivo* depletion of the CD8+ T-cell population. The percentage of Ki-67+CD8+ T cells then increased from days 10 to 14, coincident with the return of CD8+ T cells to the periphery, suggesting that cell proliferation in the periphery or redistribution from lymph nodes may contribute to expansion of the CD8+ T-cell population. The weakest proliferative

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**Table 1. Neutralizing antibody responses to SIVmac239 and SIVmac055**

<table>
<thead>
<tr>
<th>Macaque</th>
<th>Time after <em>in vivo</em> CD8+ T-cell depletion (days)</th>
<th>Neutralizing antibody titres*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1496</td>
<td>0</td>
<td>SIVmac239†</td>
</tr>
<tr>
<td>9</td>
<td>&lt;20</td>
<td>131</td>
</tr>
<tr>
<td>1502</td>
<td>−1</td>
<td>32</td>
</tr>
<tr>
<td>14</td>
<td>&lt;20</td>
<td>38</td>
</tr>
<tr>
<td>1506</td>
<td>−1</td>
<td>29</td>
</tr>
<tr>
<td>1518</td>
<td>−1</td>
<td>&lt;20</td>
</tr>
<tr>
<td>1520</td>
<td>−1</td>
<td>&lt;20</td>
</tr>
<tr>
<td>1522</td>
<td>−1</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

*Neutralizing antibodies in macaque sera were assessed in CEMx174 cell assays as described previously (Montefiori *et al.*, 1996). Neutralizing antibody titres are expressed as the reciprocal of the serum dilution required to protect 50% of cells from virus-induced killing.
†Virus stocks for neutralization assays were produced in either human PBMCs (SIVmac239/nef-open) or rhesus PBMCs (SIVmac055).
‡Macaque challenged on day 160 after *in vivo* CD8+ T-cell depletion by intravenous inoculation with 10^8 TCID50 SIVmac055.*

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response was observed for macaque 1520, which also showed a poor response in ELISPOT assays measuring functional SIV-specific T cells (Fig. 2a). No significant changes in the Ki-67+ CD8+ T-cell population were observed in the control macaque (1506).

**Outcome following intravenous challenge with pathogenic SIV**

To determine whether the SIV-specific immune responses measured following CD8+ T-cell depletion were associated with resistance to pathogenic SIV infection, all six macaques were challenged approximately 5 months later by intravenous inoculation of 10⁴ TCID₅₀ SIVmac055 (Van Rompay et al., 1996, 1999). The outcome following challenge was monitored by using a differential real-time PCR assay to distinguish between nef-deleted SIVmac239Δnef and wild-type SIVmac055. The initial assay was developed to discriminate between SIVmac239Δnef and SIVmac251 (Metzner et al., 2000); however, primer-binding sequences within the nef genes of SIVmac251 and SIVmac055 contain only a single mutation that does not influence the rate of amplification (data not shown). By using this assay, wild-type SIV RNA was detected in the plasma of four macaques following challenge with SIVmac055 (1496, 1506, 1520 and 1522) (Fig. 2c). The levels of plasma viraemia increased rapidly in three of the four unprotected animals, reaching 10⁴–10⁶ RNA copies ml⁻¹ within 6 months after challenge. Two of these animals (1496 and 1522) subsequently died of simian AIDS by 14 months. One other unprotected macaque (1520) had detectable wild-type SIV; however, the levels of plasma viraemia remained low (<10³ RNA copies ml⁻¹), indicating some control over virus replication. Two remaining macaques (1502 and 1518) had consistently undetectable wild-type SIV RNA and stable CD4+ T-cell counts, suggesting that these animals were protected from infection with pathogenic SIVmac055.

Both protected macaques experienced a strong anamnestic antibody response to SIV gp130 and p27 antigens following challenge (Fig. 2b); however, neutralizing antibody titres to SIVmac239Δnef and SIVmac055 remained below the cut-off value (<20) 8 days after challenge (day 168, Table 1). In the unprotected macaques, antibody responses to SIV gp130 and p27 antigens increased minimally or not at all after challenge, with titres against p27 remaining significantly lower than those against gp130. Neutralizing antibody titres to both the vaccine and challenge viruses did not increase above background in any of the unprotected macaques in the first week after challenge. One unprotected animal (1496) had detectable neutralizing antibodies against SIVmac055 on the day of challenge that subsequently declined to low levels (day 168, Table 1).

To determine the origins of the replicating virus in the unprotected macaques, SIV nef genes were analysed by PCR and sequenced to discriminate wild-type from nef-deleted SIV. The results of these analyses were compared with those obtained by differential real-time PCR and confirmed the presence of wild-type SIV nef in sequential samples from all four unprotected macaques (Table 2). In two of these animals (1520 and 1522), the origin of wild-type nef was presumed to be SIVmac055, based on their prior history of exposure to SIVmac055, but not SIVmac251. Wild-type SIV nef detected in the two other unprotected macaques (1496 and 1506) may have its origins in either SIVmac055 or in re-emergence of SIVmac251 from an earlier challenge (Connor et al., 1998). However, during more than 2 years follow-up after SIVmac251 challenge, including a period of enhanced viraemia during in vivo CD8 depletion, we found no evidence of SIVmac251 infection in either lymph nodes or

**Table 2. Detection of SIVmac239 and SIVmac055 following intravenous challenge**

<table>
<thead>
<tr>
<th>Macaque</th>
<th>Gene</th>
<th>Time after challenge with SIVmac055 (days)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-14</td>
</tr>
<tr>
<td>1496</td>
<td>pol</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>nef</td>
<td>Δnef/055</td>
</tr>
<tr>
<td>1502</td>
<td>pol</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>nef</td>
<td>Δnef</td>
</tr>
<tr>
<td>1506</td>
<td>pol</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>nef</td>
<td>Δnef</td>
</tr>
<tr>
<td>1518</td>
<td>pol</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>nef</td>
<td>Δnef</td>
</tr>
<tr>
<td>1520</td>
<td>pol</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>nef</td>
<td>Δnef</td>
</tr>
<tr>
<td>1522</td>
<td>pol</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>nef</td>
<td>Δnef</td>
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</table>

*Cloned 7 kb fragment.
PBMCs from these animals (Metzner et al., 2000), leading us to favour the hypothesis that wild-type nef detected in the unprotected animals was derived from SIVmac055.

When compared with SIVmac251 and SIVmac239, the RT region of SIVmac055 pol contains five coding (K65R, N69T, R82K, A158S and S211N) (Van Rompay et al., 1996) and five non-coding mutations that can be used as identifying markers. Moreover, four silent mutations are present in the pol sequence of SIVmac239 that are not found in either SIVmac251 or SIVmac055 (Regier & Desrosiers, 1990). We repeatedly found pol and deleted-nef sequences consistent with SIVmac239Δnef in the two macaques that were protected from challenge with SIVmac055 (1502 and 1518), indicating persistence of the vaccine virus (Table 2). In contrast, pol sequences characteristic of SIVmac055 were found in one macaque with low levels of wild-type SIV replication (1520). Interestingly, pol sequences consistent with SIVmac239, but with a full-length nef gene, were found in all three unprotected macaques with high levels of SIV replication (Table 2).

Clonal analysis of a 7-0 kb fragment [nt 2904–9894 (Regier & Desrosiers, 1990)] comprising sequences spanning SIV pol through nef revealed that the unprotected animals had pol sequences consistent with SIVmac239Δnef, but the original nef deletion was replaced with a full-length nef gene. Taken together, these findings suggest that a recombination event may have occurred between the vaccine and challenge viruses in the unprotected macaques, yielding pathogenic SIVmac239 with wild-type nef that was capable of replicating to high levels.

**DISCUSSION**

Numerous studies over the years have documented the efficacy of live, attenuated SIV vaccines as a means of affording protection against pathogenic SIV or SHIV infection (reviewed by Whitney & Ruprecht, 2004). Whilst this vaccine strategy has not been pursued in humans due to safety concerns, immunization of adult macaques with live, attenuated SIV continues to serve as a valuable model for investigating the possible immune correlates of protection. To this end, potential protective mechanisms, including CD8+ cytotoxic T-lymphocyte (CTL) and CD4+ T-helper cell responses, neutralizing antibodies, chemokines and viral interference have been examined, although the results have yet to yield clear answers.

Several reports point to CD8+ T cells as a critical component in controlling SIV viraemia, with perhaps the most definitive evidence coming from in vivo CD8+ T-cell depletion studies in macaques (Jin et al., 1999; Madden et al., 2004; Metzner et al., 2000; Rasmussen et al., 2002; Schmitz et al., 1999). In these studies, administration of anti-CD8 mAbs to macaques during either acute (Madden et al., 2004) or chronic (Jin et al., 1999; Rasmussen et al., 2002) infection led to a failure to suppress virus replication. In an earlier report, we found that macaques chronically infected with live, attenuated SIV experience a transient rise in viraemia coincident with in vivo depletion of CD8+ T cells (Metzner et al., 2000). Control of virus replication was temporally associated with return of the peripheral CD8+ T-cell population.

Here, we show that this transient period of enhanced antigenic pressure can augment antiviral immune responses by increasing functional CD8+ T-cell responses and SIV-specific antibody titres. However, we observed marked variation in SIV-specific immune-response kinetics and magnitude among different CD8-depleted animals, suggesting underlying determinants that affect the degree of immune reactivity to endogenous viral antigens. Two of six macaques responded with a rapid induction of high titres of antibodies to both SIV gp130 and p27 antigens, and increased numbers of CD8+ T cells secreting IFN-γ. In contrast, three other macaques had low CD8+ T-cell IFN-γ responses (e.g. 1496 and 1520) or inherently high baseline titres to SIV gp130 (e.g. 1522) and had little or no immediate change in immune parameters following antigenic stimulation.

The inability of some macaques to respond rapidly to antigenic stimulation in vivo is indicative of immune dysfunction, most probably resulting from prolonged infection and ongoing replication of the attenuated SIV vaccine virus. Ample evidence now exists documenting the pathogenic potential of live, attenuated SIV in both neonatal and adult macaques (Baba et al., 1995, 1999). A prevailing hypothesis of infection suggests that threshold levels of attenuated SIV replication may be associated with the induction of vaccine protection; however, persistence of the virus over time may lead to the emergence of more aggressive escape variants and presumably gradual erosion of immune function (Whitney & Ruprecht, 2004). Thus, the duration of infection with live, attenuated SIV may in fact compromise the ability of some macaques to respond immunologically, despite having preserved normal CD4+ T-cell counts and low viral loads.

A recent report of HIV-infected individuals undergoing STI indicates that anti-p24 antibody responses may be a reliable marker to validate overall immune responsiveness and may serve as a surrogate measurement for virus-specific CD4+ T-helper cell activity (Trkola et al., 2004). We performed detailed longitudinal analyses of anti-p27 antibody responses in the CD8+ T cell-depleted macaques and found significant differences in the ability of the animals to mount a response to p27 antigen following CD8+ T-cell depletion. Two animals responded rapidly with approximately 2 log10 increases in the titre of anti-p27 antibodies, whilst the remaining macaques had limited or no increase in reactivity to p27. Our findings suggest a waning or absence of immune responsiveness in some macaques that may be indicative of subtle changes in CD4+ T-helper cell activity, with a subsequent reduction in the capacity to mount an antiviral immune response.

Our results measuring anti-gp130 antibodies support the notion of a rapid induction of antiviral immunity in the
high-responding macaques, with each animal demonstrating a 1–2 log_{10} increase in titres to SIV gp130 within 14 days of CD8+ T-cell depletion. Conversely, the low-responding animals had modest or negligible changes in reactivity to gp130 during the same period. Several of the low-responding animals had relatively high baseline titres of anti-gp130 antibodies (e.g. 1496 and 1522), a situation that has been noted in HIV-1-infected humans as a strong negative indicator of immune function and the ability to control viraemia (Trkola et al., 2004). The highest levels of pre-existing anti-gp130 antibodies were found in macaque 1522; this animal also had the highest sustained levels of SIVmac239Δnef plasma viraemia and failed to mount a significant response to either gp130 or p27 antigens following depletion of CD8+ T cells. This observation is consistent with the view that high titres of antibodies to HIV gp120 may arise as a consequence, not a cause, of increased viraemia (Trkola et al., 2004), which coincides with more pronounced immune deficiency.

We also found that neutralizing antibody activity against SIVmac239 increased in four of the five CD8-depleted animals. The significance of neutralizing antibody responses during chronic infection in this model is unclear; however, there is little direct evidence to suggest that they play a dominant role in mediating protection from challenge with pathogenic SIV. Early passive-transfer experiments using sera from protected macaques immunized with live, attenuated SIV failed to prevent infection of naive recipients upon challenge (Almond et al., 1997). Moreover, we have shown that protection against SIVmac251 develops as early as 10 weeks after immunization with SIVmac239Δnef in the absence of detectable neutralizing antibody responses against the challenge virus (Connor et al., 1998). Thus, it seems unlikely that the relatively modest increases in neutralizing antibody responses that we observed here are associated with significant control of viraemia in the CD8-depleted macaques.

Data on the functional capacity of CD8+ CTLs in macaques infected with nef-deleted SIV have recently been reported in MamuA*01 macaques following prolonged infection with attenuated SIV (Sharpe et al., 2004). None of the macaques used in the present study expresses the MamuA*01 major histocompatibility complex class I allele, thus we relied on IFN-γ ELISPOT assays as a measure of CD8+ T-cell function. We found that CD8+ T-cell responses specific for SIV Gag increased above baseline within 14 days of CD8+ T-cell depletion in the two high-responding macaques, whilst the other animals showed no evidence of enhanced CD8+ T-cell responsiveness during the same period. The failure to mount a significant SIV-specific CD8+ T-cell response in these animals may again be indicative of a loss of CD4+ T-cell help resulting from prolonged infection with live, attenuated SIV.

Whilst all macaques given anti-CD8 antibody had consistent repopulation of peripheral CD8+ T cells (Metzner et al., 2000), the most distinguishing feature among the different animals was the kinetics of expansion of SIV-specific immune responses. This may be influenced by several factors, including the initial number of SIV-specific memory cells, the amount of antigen required for cell stimulation, the rate of memory-cell proliferation and, possibly, the balance between cell proliferation and apoptosis of effector cells. The source of returning CD8+ T cells may also provide clues as to the observed differences in immune-response kinetics. Increased numbers of SIV-specific CD8+ T cells would be expected to come from proliferation of residual memory cells, but may also arise as a result of increased thymic output (Sodora et al., 2000). In the majority of macaques, we observed fluctuations in the levels of sjTRECs in peripheral blood cells over several weeks, including points prior to CD8+ T-cell depletion (days −7 to 0), indicating ongoing perturbation within the naive T-cell population.

As CD8+ T cells rebounded in the periphery (days 10–14), expected increases in sjTRECs resulting from enhanced thymic output were not observed consistently, suggesting that other factors must contribute to the peripheral expansion. Most notable is the proliferation of memory-cell populations, which would be expected to dilute the levels of sjTRECs as a consequence of cell division (Chakrabarti et al., 2000). High levels of cell proliferation occurred within 2 weeks, coincident with the return of CD8+ T cells in the circulation, suggesting that peripheral expansion and/or redistribution of proliferating cells from lymphoid tissues into the circulation provides a major source for repopulation. Consistent with the former idea, peripheral expansion has been shown to be a significant contributor to T-cell repopulation in adult animals after T-cell depletion (Mackall et al., 1997).

The immune responses associated with control of endogenous viraemia during chronic infection with SIVmac239-Δnef may differ from those needed to mediate protection against an exogenous challenge with pathogenic SIV. When the six macaques were challenged by intravenous inoculation of pathogenic SIVmac055 approximately 5 months after CD8+ T-cell depletion, we again observed differences in the kinetics and magnitude of SIV-specific immune responses. On the day of challenge, the two high-responding macaques had increased antibody titres to both SIV gp130 and p27 antigens compared with baseline, and these animals experienced further anamnestic increases to both SIV antigens shortly after challenge. Conversely, the low-responding animals maintained significantly suppressed anti-p27 titres relative to gp130 and had little or no enhancement of these responses after challenge. The number of SIV Gag-specific CD8+ T cells also increased in several macaques following challenge; however, these responses were not correlated with the ability of the animals to control replication of the challenge virus. Interestingly, even the unprotected macaques continued to suppress replication of the endogenous vaccine strain, suggesting inherent differences in immune control of chronic infection compared with vaccine-induced protection.
In the unprotected animals, we found high post-challenge viral loads (10^4–10^6 RNA copies ml^-1) and evidence of recombination between the vaccine and challenge viruses, resulting in restoration of a full-length nef gene to SIVmac239Δ nef. One unprotected macaque (1520) gained partial control over SIVmac055 replication. This animal had a moderate increase in antibodies to SIV gp130 and p27 antigens following CD8^+ T-cell depletion, but failed to generate SIV-specific IFN-γ CD8^+ T-cell responses. These results suggest that virus-specific antibodies alone are insufficient to mediate complete protection against an exogenous SIV challenge.

In summary, our results demonstrate marked differences in the magnitude and kinetics of SIV-specific immune responses among rhesus macaques responding to in vivo CD8^+ T-cell depletion and transient enhanced viraemia. Reports using DNA immunization strategies in rhesus macaques have noted a correlation between the magnitude and duration of secondary immune-response kinetics and control of virus replication (Barouch et al., 2000; Egan et al., 2000). Our findings provide additional evidence that a transient increase in endogenous SIV antigenaemia can enhance both SIV-specific antibodies and cellular responses in macaques that are immunocompetent to respond, and underscore the pathogenic potential of live, attenuated SIV to mediate even subtle disruption of immune function in the low-responding animals. These findings may be of interest not only for the evaluation of HIV-1 candidate vaccine strategies, but also for further understanding of the immunological consequences of controlled STI in patients with chronic HIV-1 infection who experience intermittent periods of viraemia.

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