**In vivo resistance to simian immunodeficiency virus superinfection depends on attenuated virus dose**

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Infection of macaques with attenuated simian immunodeficiency virus (SIV) induces potent super-infection resistance that may be applicable to the development of an AIDS vaccine but little information exists concerning the conditions necessary for the induction of this vaccine effect. We report that only a high dose of attenuated SIVmac protected macaques against intravenous challenge with more virulent virus 15 weeks after primary infection. Three of four animals given 2000–20 000 TCID50 of SIVmacC8, a molecular clone of SIVmac251(32H) with a 12 bp deletion in the nef gene, essentially resisted superinfection with uncloned SIVmac. In two animals challenge virus was never detected by PCR and in one animal challenge virus was detected on one occasion only. Although animals given 2–200 TCID50 of attenuated virus were super-infected they were spared from the loss of CD4 cells seen in infected naive controls. Protection from superinfection did not correlate with immune responses, including the levels of virus-specific antibodies or virus-neutralizing activity measured on the day of challenge; although, after superinfection challenge, Nef-specific CTL responses were detected only in animals infected with high doses of attenuated SIV. Unexpectedly, cell-associated virus loads 2 weeks after inoculation were significantly lower in animals infected with a high dose of attenuated SIV compared to those in animals infected with a low dose. Our results suggest that the early dynamics of infection with attenuated virus influence superinfection resistance.

**Introduction**

The development of an effective AIDS vaccine requires investigation of multiple strategies. This task is complicated by the pathobiology of immunodeficiency virus infection and the high degree of genetic variability exhibited by these viruses. Simian immunodeficiency virus (SIV) shares many of the biological properties of HIV and infection of macaques with SIV has proved invaluable for modelling AIDS vaccine strategies. One of the most promising observations to emerge from the use of this model is the finding that macaques infected with live attenuated SIV are resistant to superinfection (Daniel et al., 1992). Importantly, such protection works not only against intravenous challenge with cell-free virus, but also against challenge with virus-infected cells (Almond et al., 1995) and against challenge via mucosal surfaces (Putkonen et al., 1996; Cranage et al., 1997; Miller et al., 1997).

The use of a live attenuated immunodeficiency virus as a vaccine in man is controversial, principally because of safety concerns. The observation that infant macaques given SIV that is attenuated for growth in adult macaques can develop disease (Baba et al., 1995) underscores the importance of host factors as well as viral determinants in the definition of virulence. Despite these important concerns, the potency of protection elicited by infection with attenuated virus cannot be ignored. The parameters and mechanisms of protection with live attenuated virus are still to be fully defined: an essential step if such a strategy is to be used safely in man.

Our previously reported studies and those of our collaborators, as part of a European Community Concerted Action Programme, have utilized the SIVmacC8 molecular clone, a naturally occurring attenuated variant of SIVmac251-32H derived and characterized by Rud et al. (1994). The principle
difference between this virus and the more virulent SIVmac5 molecular clone is a 12 bp deletion in the nef/3' LTR. Infection of macaques with SIVmacC8 results in an early, high, cell-associated viraemia followed by a rapid clearance of virus from the peripheral circulation, so that by 12 weeks after infection virus can only be recovered intermittently from peripheral blood mononuclear cells (PBMC) (Whatmore et al., 1995). Infection of macaques with this virus conferred resistance to superinfection with closely related and heterologous virus following intravenous challenge (Whatmore et al., 1995) and rectal challenge (Cranage et al., 1997). Furthermore, a high degree of protection against intravenous challenge with cell-free virus has been seen as early as 10 weeks after infection (Norley et al., 1996).

To investigate the relationship between dose of attenuated virus, the dynamics of infection and the outcome of superinfection challenge early after vaccination we inoculated ten macaques in pairs with graded doses of SIVmacC8. Fifteen weeks after primary inoculation, animals were challenged intravenously with SIVmac220, a cell-free virus stock derived from a macaque infected with the SIVmac5 molecular clone (Polyanskaya et al., 1997). We show here that only animals primed with high doses of the attenuated virus resisted superinfection. Furthermore, cell-associated virus loads in these animals, 2 weeks after inoculation, were lower than in those animals given low doses of attenuated virus. Protection was correlated neither with humoral nor cellular immune responses, although Nef-specific CTL were detectable after challenge only in animals given high doses of attenuated SIV.

Methods

**Virus stocks and animal inoculations.** The derivation of SIVmacC8 from the uncloned stock of SIVmac251(32H) and its characterization have been described previously (Rud et al., 1994). This molecular clone has an attenuated phenotype in vivo (Rud et al., 1994; Whatmore et al., 1995; Almond et al., 1995), differing from the more virulent SIVmac5 molecular clone by a 12 bp deletion in the nef/3' LTR (nucleotides 9501–9512) and six nucleotide substitutions. Two of the three substitutions in the overlapping nef/3' LTR result in conservative amino acid changes and one is a silent change. The inoculum stock, designated 9/90, was produced from the cell-free supernatant of infected C8166 cells as described previously (Rud et al., 1994) and has a titre of 10^6 TCID_{50}/ml on C8166 cells.

The derivation of the cell-free SIVmac220 challenge stock, designated 6/94, from the spleen of a rhesus macaque infected with SIVmac5 was described by Polyanskaya et al. (1997). The stock has a titre of 10^4 TCID_{50}/ml on C8166 cells and the MID_{50} in rhesus macaques is equal to or less than 0.3 TCID_{50}.

Juvenile rhesus macaques used in this study were bred within the United Kingdom and housed according to the Home Office Code of Practice. Ketamine hydrochloride sedation was used for all procedures requiring the removal of animals from their cages. Animals were challenged with virus by inoculation into the femoral vein.

**Virus isolation and cell-associated virus loads.** PBMC were isolated from heparinized blood by centrifugation onto Ficoll–Paque (Pharmacia). Virus isolation was assayed by co-cultivation of PBMC with C8166 cells either at a single concentration of 10^6 PBMC or additionally in a 5 fold dilution range from 4 x 10^5 cells to 130 cells. PBMC/C8166 cell cultures were put up in duplicate in 25 cm^2 flasks and replenished with fresh medium and C8166 cells every 3–4 days maintaining the total volume at approximately 15 ml. Cultures were examined regularly for cytopathic effect (CPE) and where none was evident cultures were maintained for 28 days. Cultures not resulting in CPE were tested for the presence of virus antigen by immunofluorescent staining of acetone–methanol-fixed cells using an antisera from an SIV-infected macaque. Fifty percent end-points were calculated using the Karber formula and cell-associated virus loads expressed as the number of virus-infected cells per 10^6 PBMC.

**PCR.** DNA was extracted from PBMC essentially as described by Kitchin et al. (1990). SIV nef sequences were amplified by nested PCR using primer sets SE9044N/SN9866C and SN9272/SN9763C. PCR products having the full-length nef sequence (SIVmac220) were distinguished from those with the truncated sequence encoded by SIVmacC8 by restriction enzyme digestion with RsaI and the specificity of PCR products was confirmed by Southern blotting as described by Rose et al. (1995).

**Assay of anti-SIV antibodies and virus infectivity neutralization.** Binding antibody titres against SIV were determined by ELISA using standard methods. Plates were coated with recombinant SIV-mac251 gp140 (Replicon; MRC AIDS Reagent Project AR625.1) or recombinant SIVmac251 p27–GST fusion protein (MRC AIDS Reagent Project AR643) at predetermined optimal concentrations. Immunoblots were prepared by standard methods using lysates of crude virus pellets prepared from the culture supernatants of SIVmac251(32H)-infected C8166 cells that were concentrated approximately 1000-fold by high-speed centrifugation. Binding of SIV-specific antibodies to SIV-coated membranes was detected with rabbit anti-mouse IgG HRP and visualized by enhanced chemiluminescence (ECL, Amersham).

Virus infectivity serum neutralizing activity was determined using a yield reduction assay based on the comparison of virus titre in the presence and absence of constant serum dilution. Briefly, 25 µl of a 1/50 dilution of each serum was aliquoted into each well of a microtitre plate; 25 µl aliquots of SIVmac32H, titrated in a 2-5-fold dilution series, were then added to each plate with eight replicates per dilution. After mixing, plates were incubated at 37 °C for 1 h after which 150 µl of a suspension of C8166 cells (2000 cells per well) was added. Plates were incubated for 7 days at 37 °C before detection of virus by antigen capture ELISA. Cells were lysed by addition of 20 µl of 2% (v/v) Tween 20 per well before transferring 50 µl of the lysates to the corresponding wells of ELISA plates coated with an anti-SIV mac Gag monoclonal antibody. The presence of trapped viral antigen was determined by addition of diluted plasma from an SIVmac-infected rhesus macaque followed by anti-human IgG-peroxidase conjugate and finally substrate/chromogen buffer. The number of positive wells was used to calculate the TCID_{50}. The yield reduction was calculated as the virus titre in the absence of serum divided by the titre in the presence of serum.

**PBMC phenotype enumeration.** A whole blood lysis method was used to analyse PBMC for CD4^+ (OKT4, Ortho Diagnostics) and CD8^+ (DK25, Dako). Fifty µl volumes of heparinized whole blood were combined with 15 µl of monoclonal or isotype-matched control antibody, mixed and incubated at room temperature for 1 h. The cells were washed once with 3 ml PBS, resuspended in 1 ml of 4% (v/v) ImmuneLyse in PBS (Coulter) and mixed vigorously. After 30 s, 3 ml PBS containing 1% (w/v) formaldehyde was added and the cells pelleted (8 min at 400 g). Cells were resuspended in 1 ml of this medium and analysed using an EPICS Profile I flow cytometer (Coulter). The lymphocyte population
was delineated by forward scatter versus side scatter and a bitmap placed around this population was used to gate the fluorescence signal. Total white cell counts and blood smear differential counts were determined manually for each animal, allowing the calculation of the absolute numbers of each lymphocyte subset.

**Assay of cytotoxic T cell activity.** Ficoll–Hypaque isolated PBMC were stimulated in vitro with autologous SIV-infected PHA blasts (Gallimore et al., 1995). One-fifth of the isolated PBMC were prepared for use as stimulator cells while the remaining cells were maintained overnight at 37°C. Stimulator cells were treated overnight with 1% (v/v) PHA (Gibco) and infected with SIVmacC8. The resulting blasts were washed twice with serum-free RPMI, resuspended and inactivated with mitomycin C (Sigma) at 25 µg/ml for 1 h at 37°C. The inactivated, SIV-infected PHA blasts were washed three times prior to addition to the culture of unstimulated, autologous PBMC. On day 3, 10 Units of Lymphocult T (Biostat) was added to the culture. Cultures were fed subsequently every 3 days and assayed for CTL activity after 10–14 days. Later in the study the in vitro stimulation method was modified to increase the efficiency of pCTL expansion. Autologous, SIV-infected, PHA-induced blasts were prepared from PBMC isolated 7 days before the cells to be stimulated. Blasts were prepared and infected as previously and then cultured at 1 × 10⁶ cell/ml at 37°C for 3–4 days. Cells were resuspended at 10⁷ cells/ml in medium containing one-tenth (v/v) 0.5 M Tris pH 8.0; β-propiolactone (Sigma) was added to a final concentration of 0.2% and cells were incubated in the dark at 37°C for 2 h. The reaction was stopped by the addition of sodium azide to a final concentration of 0.056 M and cells were washed prior to addition to effector cell cultures.

The inactivated, SIV-infected PHA-induced blasts were combined with freshly isolated PBMC at ratios between one to two and one to five. On day two, Lymphocult T was added at 10 Units/ml. Cultures were fed on day four and tested for CTL activity on day seven.

Target cells were prepared from herpesvirus papio-transformed autologous B-lymphoblastoid cell lines (B-LCL) by overnight infection with 5 p.f.u. per cell of recombinant vaccinia viruses expressing either SIV Nef (VV 9011 MRC AIDS Reagent Programme, ARP274.1) or influenza virus [H1N1] A/WSN/33 (Holland et al., 1984). Infected cells were harvested from each well and the radioactivity determined by liquid scintillation counting. Tests were performed in triplicate and the geometric mean c.p.m. were determined for each test.

Cell-associated virus loads were measured by limiting dilution of PBMC and cultivation with the C8166 cell line, a sensitive indicator of SIV infection. As shown in Fig. 1, a peak of cell-associated viraemia was detected 2 weeks after infection and virus loads had decreased by week 6 in all animals. The mean virus load at 2 weeks in animals inoculated with 20 000 (4S, 14S) and 2000 (33S, 21S) TCID₅₀ was significantly lower than in animals inoculated with 200 (23S, 33R) or 20 (22S, 7S) TCID₅₀ (131 virus infected cells per 10⁶ PBMC compared to 4627 virus infected cells per 10⁶ PBMC; P = 0.04, Student’s t-test). At week 6, no significant difference in virus load was seen between these two groups (6–7 infected cells per 10⁶ PBMC).

Numbers of CD4 and CD8 cells in the peripheral circulation increased following inoculation of animals with SIVmacC8 and then declined so that mean levels at the time of superinfection challenge were similar to pre-infection levels (Fig. 2). This pattern was seen in all animals inoculated with the attenuated virus, including animal 15S from which virus could not be recovered but which was positive by PCR, and animal 13S, which showed no evidence of infection. When data from individual animals were analysed there was no obvious relationship between CD4 or CD8 cell numbers and the dose of attenuated virus with which each animal was infected.

**Results**

**Cell-associated virus loads measured early after infection with attenuated virus are dependent upon inoculum dose**

Five pairs of macaques were inoculated intravenously with 10-fold dilutions of a cell-free virus stock of the attenuated molecular clone SIVmacC8 (Table 1). Virus was recovered 2 and 4 weeks later from the PBMC of all animals receiving 20 000 — 20 TCID₅₀. At weeks 13 and 15 six of these animals had become virus isolation negative and in the remaining two virus was isolated on a single occasion only. Infection of all of these animals was confirmed by PCR detection of SIVmacC8 proviral DNA. PCR also revealed that one of the animals (15S) that had received 2 TCID₅₀ was infected, despite being virus isolation negative.

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**Outcome of superinfection challenge**

Fifteen weeks after inoculation with the attenuated SIV-macC8, all ten macaques and four naive control animals were inoculated intravenously with 30 MID₅₀ of a cell-free stock of virus designated SIVmac220. This virus stock was derived
Table 1. Effect of dose on infection of macaques with attenuated SIVmacC8 and resistance to superinfection challenge with SIVmac220

VI, virus isolation on C8166 cells; PCR, polymerase chain reaction for PBMC-associated proviral DNA; S, seroconversion.

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from the spleen of a rhesus macaque 167 days after infection with the J5 molecular clone of SIVmac251-32H. Virus was isolated consistently from week 2 through to week 24 from the four control animals and from the animal that had received 2 TCID<sub>50</sub> of SIVmacC8 but was not infected (13S). Differential PCR confirmed that all these animals were infected with the challenge virus (SIVmac220). In contrast, virus was recovered with relatively high frequency in only six of nine animals that had been primed with attenuated virus and in three of these animals virus isolation was delayed relative to the controls. Differential PCR revealed that three of four animals primed with the two highest doses of attenuated virus essentially resisted superinfection. Challenge proviral DNA was never detected in two of these animals (4S and 33S) and was detected on a single occasion only, 4 weeks after challenge, in the third (21S). Furthermore, the profile of virus isolation from these three animals was consistent with the presence of attenuated virus only. Both challenge and attenuated provirus signals were detected in the remaining animals, although in only one animal (15S) did the challenge virus become consistently the predominant proviral species. With the exception of animal 7S, the frequency of virus recovery from PBMC was high (75–100%) in dually infected animals, consistent with the presence of the more virulent challenge virus.

Following superinfection challenge, mean CD4 and CD8 cell numbers in the peripheral circulation increased to values
similar to those determined in the naive controls. Although individual values in each animal fluctuated over time (data not shown) there was no sustained loss or increase of either CD4+ or CD8+ PBMC in animals infected with SIVmacC8 and challenged with SIVmac220. In all animals except one (15S), CD4 cell numbers had increased by 2 weeks after superinfection challenge, regardless of protection status. In animal 15S, the CD4 cell count remained unaltered after challenge but had risen by week 8. CD4 cell counts in animal 13S, which remained uninfected following primary inoculation with SIVmacC8, were below pre-challenge levels on two occasions following infection with SIVmac220.

The majority of SIVmacC8-infected animals also showed an increase in CD8+ cell numbers 2 weeks after superinfection challenge, with the exception of animal 23S where the cell count fell but nevertheless rebounded by 8 weeks after challenge. CD8 cell numbers in animal 13S changed in a similar manner to those of 23S.

In contrast to animals already infected with SIVmacC8, numbers of circulating CD4 and CD8 cells in controls fell following infection with SIVmac220. CD4 cell numbers fell in three animals to values below 0.5 x 10^6 cells/ml. In a fourth animal (26S) the CD4 cell count was low on the day of challenge (0.39 x 10^6 cells/ml) and fell to 0.26 x 10^6 cells/ml at 16 weeks after challenge. The mean CD4 cell count in the control group was significantly different from the SIVmacC8-infected group at 23 weeks and 31 weeks after challenge (P ≤ 0.005, P ≤ 0.006 respectively; Student’s t-test). CD8 cell numbers in controls did not show a sustained reduction and were not significantly different from those of SIVmacC8-infected animals.

**Immune responses following infection with live attenuated SIVmac and superinfection challenge**

Immunoblotting revealed all but one animal (13S) to have seroconverted to the SIV major structural proteins following inoculation with attenuated virus (data not shown), including animal 15S which failed to yield virus upon culture of PBMC. Only in animals given high doses (2000 and 20000 TCID_50) of attenuated virus (three of four) were binding antibodies to SIV envelope detectable 2 weeks after inoculation (Fig. 3). Initially, the anti-envelope response in 15S was lower than in other animals of the group. Following superinfection challenge, the four control animals and animal 13S seroconverted to SIV antigens. In general, it was not possible to distinguish animals that were superinfection resistant from those that became dually infected on the basis of anamnestic antibody responses to SIV envelope.

Neither titre of binding antibody nor virus neutralizing activity, measured on the day of superinfection challenge, correlated with protection (Table 2).

In order to determine how quickly CTL responses were generated after infection with live attenuated virus, PBMC from SIVmacC8-infected animals were restimulated in vitro using SIV-infected autologous PHA blasts. CTL activity was measured against autologous B-LCL targets expressing SIV Nef from recombinant vaccinia virus. This protein was chosen since it is known to contain CTL epitopes and a strong anti-Nef CTL response has been associated with a vaccine effect (Gallimore et al., 1995).

A transient Nef-specific CTL response was detected in three of nine animals tested following infection with SIVmacC8. Later, at the time of superinfection challenge, Nef-specific CTL responses could no longer be detected in any of
**Table 3. SIV Nef specific CTL activity in PBMC following infection of macaques with SIVmacC8 and superinfection challenge at week 15**

CTL activity was determined after restimulation of PBMC using autologous short-term infected PHA blasts. Activity of less than 10% after subtraction of activity against control targets expressing influenza A virus PB2 was considered as not significant. *nd*, Not done.

<table>
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<tr>
<th>Animal*</th>
<th>SIVmacC8 dose (TCID&lt;sub&gt;50&lt;/sub&gt;)</th>
<th>Percent specific release (Nef-specific CTL) (E:T = 50:1)</th>
<th>Weeks after SIVmacC8 inoculation:</th>
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* Animals shown in bold were protected from superinfection challenge with SIVmac220.

**Fig. 4.** CTL activity against SIV Nef. The percent specific release of <sup>51</sup>Cr from autologous B cell lines infected with recombinant vaccinia virus expressing SIV Nef (○) or recombinant vaccinia virus expressing influenza A virus PB2 (●) is shown for a range of effector to target ratios (E:T) in five attenuated SIVmac-infected macaques tested between 17 and 25 weeks after challenge with virulent virus.

the animals tested (Table 3). Furthermore, Nef-specific CTLs could not be detected following superinfection challenge when tested up to 14 weeks after challenge. This result suggested that the in vitro restimulation method may have been inefficient in expanding CTL precursors. Between 17 and 25 weeks after superinfection challenge, a modified restimulation method was adopted, using a higher stimulator to effector ratio and PHA blasts that had been inactivated after a longer exposure to
virus. This method revealed the presence of Nef-specific CTL in three of three animals tested that had been primed with the higher doses of attenuated virus. Nef-specific CTL were absent in two of two (33R and 22S) animals tested given lower doses of attenuated virus, despite their earlier detection after primary infection (Fig. 4). The presence of Nef-specific CTL did not, however, correlate directly with protection since animal 14S became infected upon superinfection challenge.

When sufficient PBMC were available, lymphoproliferative responses against whole, heat-inactivated SIV and recombinant envelope were determined. Although the magnitude of responses in individual animals varied on each occasion tested PBMC from each of the protected animals (4S, 21S and 33S) had whole SIV antigen-driven responses on two of three occasions tested (SI range 2.7–22). A response to gp140 was seen on one occasion with PBMC from 33S (SI 57) and on two occasions with PBMC from 21S (SI 2.9 and 9), corresponding to times when responses to whole virus were detected. PBMC from four animals that were infected with both attenuated virus SIVmacC8 and SIVmac220 (14S, 22S, 7S and 15S) responded to whole SIV on at least one occasion (SI range 2.7–46). PBMC from 14S, 7S and 15S also responded to gp140 (SI range 2.1–7.5).

Discussion

In general, live attenuated vaccines against virus infections are extremely successful. The efficacy of this type of vaccination is believed to be due to the stimulation of immune responses that mimic those seen during natural infection and thus the immune system is primed for encounter with the natural pathogen. It is therefore important to determine the parameters of infection that influence superinfection resistance. One study of macaques infected with attenuated SIV has suggested that superinfection resistance developed fully only after a considerable period of time (Wyand et al., 1996). Other studies, however, indicated a high degree of protection at 10 and 20 weeks (Norley et al., 1996) or 22 weeks (Stahl-Hennig et al., 1996) after primary infection. The results of the present study show that this apparent discrepancy may be related to the dose of attenuated SIV administered. Furthermore, different doses of attenuated SIV influenced the virus recovery profile prior to superinfection challenge, suggesting that the early dynamics of infection relate to the ‘vaccine’ efficacy.

Dose-related vaccine efficacy has been reported with other live attenuated viruses including rabies virus (Flamand et al., 1984), rotavirus (McNeal et al., 1992; Feng et al., 1994) and herpes simplex virus (Mercadal et al., 1993). The results of the present study also show a dose-related effect but are unusual in that animals receiving high doses of attenuated SIV had lower cell-associated virus loads 2 weeks after infection than did animals receiving lower doses, suggesting that less virus replication had occurred in these former animals. The possibility that the peak of cell-associated viraemia had shifted in these animals cannot be excluded. The results from Norley et al. (1996), using a high dose of SIVmacC8, who found a similar level of early viraemia that had declined by 4 weeks after inoculation, indicate that any shift in replication dynamics is, therefore, likely to be towards an earlier peak in viraemia. The earlier rise in anti-SIV envelope antibodies in animals given a high dose of attenuated virus may indeed support this notion. Taken together, it is clear that further experiments with a higher frequency of sampling and including plasma RNA determination are now needed to define the relationship between virus dose and early replication dynamics.

The level of protection from superinfection with virulent virus was similar to that reported in two previous studies (Norley et al., 1996; Stahl-Hennig et al., 1996), both of which used high doses of SIVmacC8. An effect on pathogenesis was also found in the present study in the majority of animals infected with lower doses of attenuated virus. Although these animals became superinfected, as evidenced by PCR detection of challenge virus-derived proviral DNA, there was a sparing from CD4 cell loss, delayed detection of challenge virus in all but one animal and strong SIV-driven lymphoproliferative responses. Normally, SIV-driven lymphoproliferative responses are weak in animals infected with virulent SIV (McGraw et al., 1990; Ahmed-Ansari et al., 1990; Dittmer et al., 1994; Sharpe, 1997). Protection from the consequences of superinfection with virulent virus in the absence of ‘sterilizing’ protection has been reported in macaques infected with SIVmac 1A11 or HIV-2 (Marthas et al., 1990; Putkonen et al., 1990), both of which replicate relatively poorly in macaques.

Interestingly, in the animal infected following inoculation with the lowest dose of attenuated virus the outcome was different with no recovery of virus but evidence of infection by PCR and seroconversion. Wyand et al. (1996) reported transient infection in animals given an apparently low dose of SIVmac239 ∆nef intravenously and others have reported transient infections following high-dose mucosal exposure (Pauza et al., 1993; Clerici et al., 1994; Miller et al., 1994; Trivedi et al., 1996). Exposure to low doses of virus has been associated with protection from subsequent virus challenge (Clerici et al., 1994; Trivedi et al., 1996); however, in the present study no such association was found. Neither the animal with modified SIVmacC8 infection (15S) nor the animal that remained uninfected (13S) resisted superinfection. Although detection of challenge virus was delayed in 15S, the challenge virus proviral DNA subsequently became the predominant species and CD4 cells declined relative to pre-challenge levels. Similarly, Wyand et al. (1996) found no protection in animals transiently infected with attenuated virus after intravenous exposure, suggesting that the low-dose protection effect may be unique to mucosal exposure.

The mechanism of superinfection resistance remains unknown. No direct correlate of immune protection was evident in the study described here. The importance of serum neutralizing antibodies in superinfection resistance has been
proposed (Clements et al., 1995; Wyand et al., 1996) but no association between such activity and protection was found in this study in agreement with a previous study using SIVmacC8 (Norley et al., 1996).

The detection of CTL activity, early after infection with attenuated virus is consistent with a role for CTL in the early clearance of virus infected cells and the inability to detect activity at later time points may have been due to inefficient in vitro restimulation of precursor CTL (Sharpe, 1997; Cranage et al., 1997). A longer period of stimulation in vitro may have revealed low frequency CTL precursors. The detection of CTL activity only in animals primed with high doses of attenuated virus, regardless of protection status, together with the tendency for an earlier antibody response to envelope in these animals, suggests that these animals may have been primed for qualitatively or quantitatively different responses to those in animals that received lower doses of attenuated virus. Infection of macaques with a high dose of SIVmacC8 has been associated with an overexpression of IL-2, IL-4 and IFN-γ mRNAs in PBMC during the first few weeks of infection (Benveniste et al., 1996) and it remains to be determined if this effect is virus-dose-related.

The lack of definitive correlates of protective immunity in the study described here may simply reflect the limitations of the assays employed with respect to breadth of antigen recognition, specific function and sampling sites. Alternatively, superinfection resistance induced with attenuated SIV may be a non-immunological phenomenon. It is possible that a high dose of attenuated virus saturates a critical population of target cells creating a bottleneck to the establishment of subsequent infection. This may involve virus interference as has been described for murine retroviruses (Mitchell & Risser, 1992) or may be associated with the induction of intrinsic antiviral factors such as the CD8 suppressor factors (Mackowicz & Levy, 1992; Cocchi et al., 1995; Paxton et al., 1996). PBMC from macaques infected with SIVmacC8 are relatively less sensitive to infection with virulent virus in vitro than are cells from uninfected macaques and this difference is influenced by the presence of CD8 cells (P. Silvera, personal communication).

Finally, defective particles in the primary inoculum may influence superinfection resistance. Such material may modulate the early dynamics of infection by the induction of cell factors as discussed above and may account for the threshold effect of protection seen in the current experiment. Such a model would not exclude classical immunity in the superinfection resistance effect, since the primary inoculum may determine the optimal environment for the stimulation of subsequent protective immunity. Experiments using defined particle to infectivity ratios would test this hypothesis.

The dose of attenuated virus needed to obtain protection from challenge with virulent virus is a particularly important parameter in the light of the findings that, at least in neonates infected orally, high doses of virus may induce disease (Baba et al., 1995; Wyand et al., 1997). It is clear that the relationship between virus dose and time to superinfection resistance requires further investigation to better understand the parameters and mechanisms of protection elicited with live attenuated vaccines against immunodeficiency viruses.

We are grateful to Dr E. J. Stott, Dr N. Almond and Dr E. W. Rud for useful discussions and to Dr Harvey Holmes and the MRC AIDS Reagent Programme for the provision of many of the reagents used. The work was supported by a grant from the UK Medical Research Council and by the UK Department of Health.

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Received 12 February 1998; Accepted 23 April 1998