Macaques infected with attenuated simian immunodeficiency virus resist superinfection with virulence-revertant virus

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Macaques infected with attenuated simian immunodeficiency virus (SIVmac) can resist superinfection challenge with virulent virus, showing the potential of live attenuated virus as an AIDS vaccine. Superinfection resistance does not, however, prevent the generation of virulent virus *in vivo*, suggesting that such virus may circumvent the resistance effect. Here, we show that three macaques already infected with the attenuated molecular clone SIVmacC8 were resistant to superinfection with virulent virus that arose *in vivo* following repair of a 12 bp attenuating lesion in the *nef*/3′ LTR. In contrast, four naive animals became infected following inoculation with blood taken from the macaque in which virulent virus arose. Loss of *nef*-specific cytotoxic T lymphocyte (CTL) responses followed repair of the attenuating lesion within *nef* in the donor animal, suggesting the possibility of escape from CTL-driven selection pressure.

The work of Daniel *et al*. (1992) showing that preinfection of macaques with attenuated SIVmac can induce a state of superinfection resistance to intravenous challenge with high doses of pathogenic virus highlighted the possibility of using attenuated virus as a vaccine against HIV infection. Infection with attenuated virus also protects macaques against superinfection with SIV-infected cells (Almond *et al*., 1995) and cell-free infection via mucosal routes (Putkonen *et al*., 1996; Van Rompay *et al*., 1996; Cranage *et al*., 1997). Despite the power of this protective effect, it is unlikely that a live attenuated vaccine will be used in humans in the foreseeable future, principally because of safety concerns. Retroviruses have a high mutation rate, in part due to the error-prone nature of reverse transcription and high frequency of recombination. Point mutations attenuating SIV and HIV may quickly revert *in vivo* (Kestler *et al*., 1991; Quillant *et al*., 1993), as may small deletions (Whatmore *et al*., 1995). Even attenuated mutants carrying multiple deletions, which are therefore unlikely to revert (Wyand *et al*., 1996), may induce disease in neonatal macaques (Baba *et al*., 1995). Nevertheless, investigation of the mechanisms of superinfection resistance may reveal insights into lentivirus infection and immunity that may be exploitable for the design of an effective vaccine against AIDS.

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At present it is not clear whether superinfection resistance is or is not an immunological effect. Several studies support the view that serum-neutralizing antibodies are important for superinfection immunity (Clements *et al*., 1995; Wyand *et al*., 1996; Van Rompay *et al*., 1996). However, in a study of macaques given short-term exposure to live attenuated SIVmac, Norley *et al*. (1996) could find no correlation between the levels of binding or neutralizing antibodies and protection from superinfection challenge. Infection of macaques with live attenuated SIVmac also induces virus-specific cytotoxic T lymphocytes (CTL) (Dittmer *et al*., 1995; Cranage *et al*., 1997). However, profound depletion of circulating $CD8^+$ lymphocytes failed to ablate superinfection immunity in such animals (R. Stebbings, personal communication).

In the study described here, we have utilized the molecular clones derived from SIVmac32H (Rud *et al*., 1994) to investigate the protective mechanisms involved in superinfection resistance. Molecular clone SIVmacC8 has an attenuated phenotype in rhesus macaques. After an initial cell-associated viraemia, virus loads become much reduced and virus recovery from peripheral blood mononuclear cells (PBMC) is infrequent (Whatmore *et al*., 1995). This clone is isogenic with its more virulent partner SIVmacJ5 except for a 12 bp deletion in the *nef*/3′ LTR overlapping region (nucleotides 9501–9512) and six nucleotide substitutions, two of which result in coding changes within *nef* (Rud *et al*., 1994). Because SIVmacC8 has only a small attenuating lesion, the virus is susceptible to mutation *in vivo* resulting in the restoration of full-length *nef*. Paradoxically, reversion to virulence, concomitant with *nef* repair, occurred in a SIVmacC8-infected macaque despite the animal being resistant to exogenous superinfection with virulent SIVmacJ5 (Whatmore *et al*., 1995). This result suggested that *in vivo*-selected virulence-revertant virus may be able to bypass the super-

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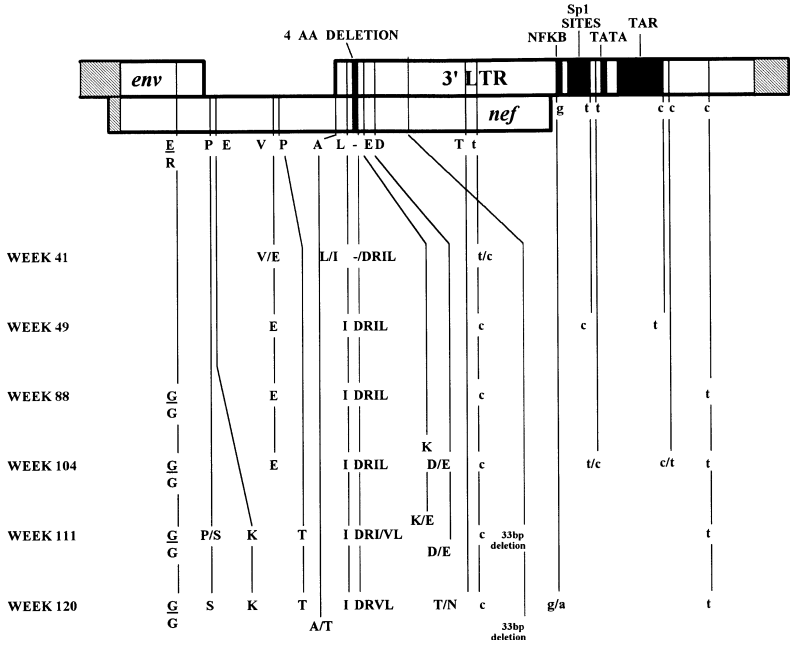

Fig. 1. Schematic representation of sequence changes accumulating over time in the *nef*/3′ LTR region of proviral DNA amplified from the PBMC of SIVmacC8-infected macaque 29R. Predicted amino acid changes in *nef* are shown in capital letters. Predicted amino acid changes in the envelope are shown underlined. Nucleotide substitutions resulting in synonymous changes or within non-coding regions are shown in lower case letters. Mixed sequence populations are shown as alternatives. Regulatory regions within the 3′ LTR are indicated. Regions shown in diagonal shading were not sequenced.

infection resistance effect, perhaps by escape from a SIVmacC8-driven immune response. This possibility has been investigated further in the experiments described here.

Four Indian rhesus macaques had been infected with SIVmacC8 as part of a mucosal superinfection resistance study (Cranage *et al*., 1997). All animals became negative or intermittent for virus isolation by week 17, seroconverted to SIV and made virus-neutralizing responses. In one animal, 29R, direct sequencing of PBMC-derived proviral DNA revealed that by week 49 after infection the 12 bp deletion associated with the attenuated phenotype had been replaced by an almost exact duplication of the upstream flanking region. This resulted in a predicted amino acid sequence of DRIL restoring *nef* to full-length (Whatmore *et al*., 1995). Sequence analysis of plasmid clones generated at week 41 revealed that the revertant population had already arisen by this stage, with 35 % of clones having the repaired sequence and the remainder having the deleted sequence of the parental virus. Thus, the repaired sequence became predominant between weeks 41 and 49. By week 59, virus isolation from PBMC had become positive and remained so during the subsequent course of infection.

Fig. 1 summarizes changes seen in the consensus sequence of the *nef* and 3′ LTR regions occurring during the course of infection. A total of 18 base substitutions was recorded but none of these was associated with known regulatory sequence elements. All but one of the 12 base substitutions occurring in the *nef* ORF resulted in predicted amino acid changes. At week 111, further evolution of the repaired sequence was detected, and by this stage a 33 bp deletion in *nef* (nucleotides 9652–9684) had occurred. These latter nucleotide sequence changes were concurrent with the onset of clinical decline. The animal showed progressive weight loss and a decline in both $CD4^+$ and $CD8^+$ lymphocytes. By week 145, axillary lymph nodes were enlarged and by week 150, inguinal lymph nodes were also enlarged. Autopsy at 158 weeks after infection with SIVmacC8 revealed follicular and paracortical hyperplasia in all lymph nodes examined. The follicles of the spleen were markedly hyperplastic and hyalinization was common. *Pneumocystis carinii* and associated pathology was seen in the lungs.

To determine if revertant virus had circumvented superinfection resistance, three rhesus macaques that had been infected with SIVmacC8 for 93 weeks (11R, 19R and 28R), together with four naive control animals (47T, 47S, 41S and 27S), were each inoculated intravenously with 2 ml of blood from 29R. The three animals previously infected with SIVmacC8, in which no repair of the *nef* deletion had occurred, had been shown to resist superinfection with SIVmacJ5 and SHIV4 (a chimeric virus of SIVmac239 expressing the HIV-1 HXB2c *env*, *tat* and *rev*; Li *et al*., 1992) following intrarectal exposure at weeks 60 and 80 respectively (Cranage *et al*., 1997).

On the day of challenge, the cell-associated virus load in the blood of 29R was found to be 10 infected cells per 10^6 PBMC, but cell-free virus was undetectable in plasma, even when added undiluted to indicator cells. Following challenge, the four naive macaques all became persistently viraemic (Table 1), and the presence of 29R proviral DNA was confirmed by direct sequencing of *nef*, PCR-amplified from PBMC DNA. In contrast, in the SIVmacC8-infected animals virus was isolated only from animal 28R after challenge with 29R blood. Virus isolation from 28R PBMC was always slower (18–27 days) compared to isolation from PBMC of the control animals

Table 1. Outcome of virus challenge

Virus recovery and PCR following challenge of macaques previously infected with attenuated virus (C8) and naive control animals (CON). PBMC were cocultured with C8166 cells and virus isolation confirmed by immunofluorescent antibody-staining (Whatmore *et al.*, 1995). DNA extracted from PBMC was used to distinguish vaccine and challenge virus by a nested PCR procedure using primer sets SE9044N/SN9866C and SN9272/SN9763C, as described by Rose *et al.* (1995). Where PCR products were obtained, their identity was confirmed by direct sequencing of the product (Sequanase PCR product sequencing kit, Amersham) using primer SN9272N to sequence across the region of the SIVmacC8 deletion/29R repair. Numbers of weeks after challenge with blood from macaque 29R are indicated.

Animal no.	Status	Week …	Virus isolation (SIV *nef* PCR)									
			0	2	5	6	8	10	12	16	18	22
28R	C8		+	−	+ (−)	+	+	+	− (−)	− (−)	− (−)	−
11R	C8		−	−	− (−)	−	−	−	− (−)	− (−)	− (−)	−
19R	C8		−	−	− (−)	−	−	−	− (−)	− (−)	− (−)	−
47T	CON		−	+	+ (+)	+	+	+	+ (+)	+ (+)	+ (+)	+
47S	CON		−	+	+ (+)	+	+	+	+ (+)	+ (+)	+ (+)	+
41S	CON		−	+	+ (+)	+	+	−	+ (+)	− (+)	+ (+)	+
27S	CON		−	+	+ (+)	+	+	+	+ (+)	+ (+)	+ (+)	+

(7–15 days), probably reflecting a lower virus load. Virus was also recovered intermittently from 28R before challenge, whereas the other two infected macaques had been isolation-negative for several months prior to challenge. PCR amplification of proviral DNA from 28R and the other SIVmacC8-infected macaques failed to reveal the presence of 29R *nef* sequences. The failure to detect provirus in animal 28R by routine PCR screening was somewhat surprising, given that virus was isolated intermittently from this animal by culture. A PCR product could be obtained, however, when using the primer set amplifying the virtual full-length *nef*/3′ LTR. Further investigation revealed that a deletion had occurred in the 28R *nef*/3′ LTR, removing the binding site for primer SN9763C used in the routine PCR screening. The provirus in 28R was clearly SIVmacC8-derived as it still had the characteristic 12 bp deletion. Furthermore, SIV-specific antibodies in the SIVmacC8-infected animals were unaffected by challenge, whereas control animals developed rising titres of antibody in response to infection (data not shown).

Post-mortem examination of tissues taken from animals 11R, 19R and 28R, at 26, 29 and 34 weeks respectively after challenge with blood from 29R revealed the presence of virus, and in all cases this was found to be SIVmacC8 (Cranage *et al.*, 1997). Taken together, these results show that there was no detectable superinfection with the 29R challenge.

Humoral immunity is unlikely to be critical for superinfection resistance. A population of viruses that had arisen to escape a SIVmacC8-specific humoral response would be expected to escape recognition in other SIVmacC8-infected animals, thereby resulting in superinfection. Furthermore, as we and others have shown, animals infected with attenuated SIVmac resist superinfection with SIVmac chimeric virus expressing HIV-1 envelope, even though HIV-1 envelope-binding or neutralizing antibodies were not detectable (Stott *et al.*, 1994; Bogers *et al.*, 1995; Cranage *et al.*, 1997). Also, passive transfer of serum from SIVmac wild-type or SIVmacC8-infected macaques failed to protect naive recipients from subsequent challenge with virulent virus (Kent *et al.*, 1994; N. Almond, personal communication). Taken together, these data strongly suggest that antibodies are not responsible for superinfection resistance in this system.

It is possible that virulent virus arose by escape from an immunodominant CTL response. CTL responses to *nef* can efficiently limit early virus replication and may even prevent the establishment of infection when precursor cells are present in sufficient numbers (Gallimore *et al.*, 1995). Consistent *nef*-specific CTL activity was detected in macaque 29R on four occasions when tested between weeks 50 and 61 after infection with SIVmacC8 (Table 2). A response was detected against a pool of peptides covering the whole of the *nef* protein, as well as against pools of peptides covering both the amino- and carboxy-terminal halves. This response was present, therefore, at a time when virus isolation frequency became high, following the emergence of virus sequences having a full-length *nef*. After week 66, *nef*-specific CTL activity could not be demonstrated in *in vitro*-restimulated PBMC against *nef* peptide-pulsed B cell targets or B cell targets infected with recombinant vaccinia virus expressing SIVmacJ5 *nef*. Despite this lack of response to *nef*, when tested at week 90, an *env*-specific CTL response was detected against target cells infected with recombinant vaccinia virus expressing SIVmacJ5 *env* (19% specific lysis at an effector to target ratio of 50:1), suggesting that the loss of CTL activity may have been *nef*-specific.

The challenge experiment described here would not detect CTL-driven escape because CTL epitope recognition is MHC-

Table 2. *Nef*-specific CTL responses in macaque29R

PBMC were restimulated in bulk culture with SIV-infected autologous phytohaemagglutinin blasts as described previously (Cranage *et al.*, 1997). CTL activity was determined over a range of effector to target ratios (E:T) on ^{51}chromium-labelled herpesvirus papio-transformed B cell lines. Target cells were pulsed with peptide pools covering the whole (pool 0), the amino-terminal half (amino acids 1–131; pool 1) or the carboxy-terminal half (amino acids 122–263; pool 2) of the *nef* sequence (20 mer peptides with 10 amino acid overlaps; EVA777, EC programme EVA) for assays before week 80. Subsequently, target cells were infected at 5 p.f.u. per cell with recombinant vaccinia virus expressing SIV *nef* (vv *nef*; VV9011, MRC AIDS Reagent Programme, ARP274.1). ND, Not done.

	% Specific lysis										
	Week ...	50	52	60		61		66	68		80	82
E:T	Pool ...	0	0	1	2	1	2	0	1	2	vv *nef*	vv *nef*
100:1		42	ND	54	ND	ND	ND	−1	−2	−2	ND	−5
50:1		30	25	50	58	44	34	ND	−2	−3	5	0
25:1		28	21	ND	ND	34	31	ND	ND	ND	ND	ND
5:1		11	2	46	50	18	13	0	−2	−4	0	0

restricted. Escape would, therefore, only be detected were the animals to share specific MHC alleles. To further investigate the possibility of *nef* CTL-driven immune escape, PBMC from animal 29R were expanded *in vitro* with stimulator cells pulsed with peptides made to the two regions of *nef* showing major mutation, i.e. the 12 bp deletion repair and the 33 bp deletion. This efficient peptide-driven restimulation method failed to reveal memory for SIVmacC8 *nef*-specific CTL. However, the assays were performed late in the course of infection when $CD8^+$ cell numbers were already low and the animal was in clinical decline. Other regions of *nef*, where point mutations occurred, may represent CTL epitopes. Alternatively, loss of *nef* CTL response may have been due to functional anergy induced by peptide antagonism, for example, rather than escape mutation.

The observation that macaques infected with attenuated virus are resistant to challenge with *in vivo*-reverted virus suggests another hypothetical model for superinfection resistance, where immune escape need not be invoked to explain the virulence-reversion effect. In this model, the resident virus occupies a critical niche, and resistance to superinfection would be dependent upon the replication dynamics of this primary virus.

In summary, the observations of virulence-reversion late in infection with SIVmac and of superinfection resistance against reverted virus pose fundamental questions regarding SIV (HIV) infection dynamics and immunity. Dissociating the relative importance of primary replication of virus from the generation of specific and innate immune responses may prove problematic. If, however, the ability of attenuated virus to exclude incoming virus from a critical niche is the dominant mechanism of superinfection resistance, it may prove to be very difficult to exploit this effect in safe and effective vaccines for use in man.

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