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Virology 330 (2004) 249–260

VIROLOGY

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Vaccination with live attenuated simian immunodeficiency virus for 21 days protects against superinfection

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Received 27 April 2004; returned to author for revision 12 July 2004; accepted 15 September 2004
Available online 12 October 2004

Abstract

The identification of mechanisms that prevent infection with human immunodeficiency virus (HIV) or simian immunodeficiency virus (SIV) would facilitate the development of an effective AIDS vaccine. In time-course experiments, protection against detectable superinfection with homologous wild-type SIV was achieved within 21 days of inoculation with live attenuated SIV, prior to the development of detectable anti-SIV humoral immunity. Partial protection against superinfection was achieved within 10 days of inoculation with live attenuated SIV, prior to the development of detectable anti-SIV humoral and cellular immunity. Furthermore, co-inoculation of live attenuated SIV with wild-type SIV resulted in a significant reduction in peak virus loads compared to controls that received wild-type SIV alone. These findings imply that innate immunity or non-immune mechanisms are a significant component of early protection against superinfection conferred by inoculation with live attenuated SIV.

Keywords: Simian immunodeficiency virus; Live attenuated virus; Superinfection

Introduction

In simian models of human immunodeficiency virus (HIV), inoculation with live attenuated simian immunodeficiency virus (SIV) elicits potent and durable protection against superinfection (Almond et al., 1995; Daniel et al., 1992; Desrosiers, 1990; Lohman et al., 1994; Wyand et al., 1996). This protection is effective against a diverse range of variants and multiple routes of infection (Almond et al., 1995; Cranage et al., 1997; Daniel et al., 1992; Johnson et al., 1999; Nilsson et al., 1998; Norley et al., 1996; Sharpe et al., 1997; Wyand et al., 1999). Furthermore, inoculation with live attenuated SIV can confer protection against infection with chimeric SIV/HIV (SHIV), in which the *env*, *tat*, *vpu* and *rev* genes of SIV have been replaced with those of HIV, even though this vaccine approach does not elicit neutralising antibodies against HIV (Bogers et al., 1995; Dunn et al., 1997; Shibata et al., 1997; Wyand et al., 1999). However, there are limits to this protection and live attenuated SIV vaccines have failed to protect against certain heterologous challenge viruses or failed to protect against a challenge performed several years post inoculation (Hofmann-Lehmann et al., 2003; Wyand et al., 1999). Unfortunately, safety concerns over the stability of attenuating mutations, which can result in reversion to pathogenicity in the long-term, have precluded the clinical evaluation of live attenuated HIV vaccines (Baba et al., 1995; Hofmann-Lehmann et al., 2003; Ruprecht, 1999; Whatmore et al., 1995). Nevertheless, if the mechanism(s) of protection

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0042-6822/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2004.09.026

conferred by this potent vaccine approach could be unravelled, then it may facilitate the development of a safe and effective HIV vaccine. However, there is controversy as to whether this protection is mediated through acquired immune responses, innate immunity or non-immune mechanisms such as viral interference (Abel et al., 2003; Johnson, 2002; Nixon et al., 2000; Rud et al., 1994b; Stebbings et al., 2002; Tenner-Racz et al., 2004).

Inoculation with live attenuated SIV has been reported to elicit a number of acquired anti-SIV immune responses, including neutralising antibodies (Langlois et al., 1998; Wyand et al., 1996), cytotoxic T lymphocyte (CTL) responses (Johnson et al., 1997; Nixon et al., 2000; Vogel et al., 1998) and helper T cell responses (Gauduin et al., 1999; Sarkar et al., 2002). Many current HIV vaccine strategies aim to reproduce these responses. Yet, passive transfer experiments designed to evaluate the role of serological responses to inoculation with live attenuated SIV did not transfer protection (Almond et al., 1997). However, this contrasts with post-challenge immunotherapy with immune globulin, purified from the plasma of SIV infected long-term nonprogressor macaques, which has been reported to reduce virus burdens and alter disease outcome (Haigwood et al., 1996). More recently, it has been reported that infusion of high levels of anti-HIV neutralising monoclonal antibodies can protect macaques against SHIV infection (Baba et al., 2000; Ferrantelli et al., 2004; Mascola et al., 2000, 2003; Parren et al., 2001), although this still does not account for the protection observed following inoculation with live attenuated SIV.

With certain SIV vaccine approaches, an inverse correlation between the precursor frequency of anti-SIV CTL responses elicited by immunisation and virus load following challenge has been demonstrated (Gallimore et al., 1995; Vogel et al., 2002). Furthermore, supporting evidence for the importance of CD8+ CTL in controlling SIV infection has been derived from increased plasma viremia following CD8+ cell depletion of infected macaques (Jin et al., 1999; Lifson et al., 2001; Metzner et al., 2000; Schmitz et al., 1999). However, attempts to obtain more direct evidence by depletion of CD8+ lymphocytes in attenuated SIVmacC8 vaccinees at the time of challenge with wild-type SIVmacJ5 failed to abrogate protection against superinfection, suggesting that factors that control infection may be distinct from those that protect against challenge (Stebbings et al., 1998).

One step towards identifying vaccine responses that confer protection is to perform a time-course study, in order to identify responses whose appearance correlates with the development of protection. In time-course experiments, the onset of protection against heterologous challenge has been evaluated using several attenuated SIV vaccines, with varying results. For SIVmac239Δ3, the earliest reported protection after vaccination is 8 weeks, when two out of four vaccinees resisted superinfection with heterologous wild-type SIV (Wyand et al., 1996). Yet, with SIVmac239Δ*nef* and SIVmacC8, the earliest reported protection after vaccination is 10 weeks, when two out of four and three out of four vaccinees resisted superinfection with wild-type SIV, respectively (Connor et al., 1998; Norley et al., 1996). Generally, it is accepted that there is a trend towards greater protection with increased time between inoculation with live attenuated SIV and subsequent challenge with wild-type SIV (Connor et al., 1998; Wyand et al., 1996). From these observations, it has been inferred that increased protection with time is due to the development or maturation of acquired anti-SIV immune responses (Cole et al., 1997; Wyand et al., 1996). Nevertheless, when superinfection at times early after inoculation occurs, then a significant reduction in primary viremia compared with naive controls has been observed (Connor et al., 1998; Stahl-Hennig et al., 1996). Here we have assessed resistance to superinfection with homologous wild-type SIVmacJ5 in vaccinees inoculated with live attenuated SIVmacC8 for 70, 42, 21, 10 and 0 days prior to challenge.

Results

Time course to protection following inoculation with live attenuated SIV

Over two studies, we have determined the time interval between inoculation of live attenuated SIVmacC8 and challenge with pathogenic, wild-type SIVmacJ5 required to protect against superinfection. Groups of four cynomolgus macaques were inoculated in an initial study at either 70, 42 or 21 days prior to challenge (groups A–C, respectively; individuals S169–S180) and, in a subsequent study, at either 21, 10 or 0 days prior to challenge (groups C–E, respectively; individuals T317–T328). All vaccinees (groups A–D) were successfully inoculated with attenuated SIVmacC8 as determined by virus isolation (Table 1) and DNA polymerase chain reaction (PCR) (Table 2). At 14 days post challenge, the presence of wild-type SIVmacJ5 was detected by DNA PCR in the peripheral blood mononuclear cells (PBMC) of all naïve challenge controls (group F; individuals S181–S184 from the first study and T329–T332 from the second study) and all co-inoculated vaccinees (group E; individuals T325–T328). At this time, apart from a single macaque in group D (T317), there was no evidence of superinfection with wild-type SIVmacJ5 in any vaccinees (Table 2). However, further analysis of lymphoid tissues taken post mortem, between 132 and 168 days post challenge, revealed the presence of wild-type SIVmacJ5 superinfection in a single vaccinee from group A (S170) and a total of three vaccinees from group D (T317, T319 and T320; Table 2). Where superinfection occurred, then two bands were detected by DNA PCR, suggesting dual infection with both vaccine and challenge virus. Formal automated DNA sequencing confirmed mixed sequence populations, confirming the results of

Table 1
Detection[a] and titration[b] of infectious virus by co-culture with C8166 cells

Vaccine group and time of SIVmacC8 inoculation	Animal	Viral load (log_{10} SIV producing cells per 10^6 PBMC)													
		Days post SIVmacJ5 challenge										Post mortem[c]			
		−70	−56	−42	−28	−21	0	14	28	56	84	Bld	Spl	Mln	Pln
(A) Day −70	S169	–	1.0	–	n.d.	–	0.5	–	–	–	–	–	+	–	–
	S170	–	1.5	0.5	n.d.	0.5	–	0.1	–	–	–	–	+	–	+
	S171	–	0.5	0.1	n.d.	–	–	–	–	–	–	–	–	n.d.	–
	S172	–	0.5	–	n.d.	–	–	–	–	–	–	–	–	–	–
(B) Day −42	S173			–	0.5	–	0.5	–	–	–	–	–	–	–	+
	S174			–	0.5	0.5	–	0.1	–	–	–	–	+	+	+
	S175			–	0.5	0.1	0.1	0.1	–	+	–	–	–	–	+
	S176			–	1.0	n.d.	–	–	–	–	–	–	+	–	+
(C) Day −21	S177					–	0.5	0.1	–	–	–	–	–	–	–
	S178					–	1.0	0.5	+	+	n.d.	–	+	+	+
	S179					–	1.0	0.1	+	+	+	–	+	–	–
	S180					–	–	–	–	+	–	–	–	–	–
	T321					–	0.1	–	–	–	–	–	–	–	–
	T322					–	0.1	0.1	+	–	–	–	–	–	–
	T323					–	0.5	0.5	–	+	–	–	–	–	–
	T324					–	0.1	0.1	–	–	–	–	–	–	+
(D) Day −10	T317					–	3.0	1.5	+	+	–	–	–	n.d.	–
	T318					–	1.5	0.1	–	–	–	–	–	–	–
	T319					–	2.0	0.1	+	+	–	–	+	–	+
	T320					–	3.0	1.0	+	+	+	+	+	+	+
(E) Day 0	T325						–	1.0	+	–	–	–	+	–	+
	T326						–	2.0	+	–	–	+	+	+	+
	T327						–	1.0	+	–	–	–	+	–	–
	T328						–	1.5	+	+	–	–	+	–	+
(F) Naïve controls	S181						–	2.5	+	+	+	+	+	+	+
	S182						–	3.0	+	+	–	–	+	+	+
	S183						–	2.5	+	+	+	+	+	+	+
	S184						–	3.0	+	+	–	–	+	+	+
	T329						–	2.5	+	+	–	–	–	–	+
	T330						–	3.0	+	+	+	+	+	+	+
	T331						–	2.0	+	+	–	–	+	–	+
	T332						–	4.0	+	+	+	+	+	+	+

n.d. denotes that the result was not determined.
[a] – and + symbols denote negative and positive isolation of infectious virus, respectively, from either 5×10^6 PBMC or lymphoid tissue cells.
[b] Virus loads shown are log_{10} SIV infectious cells per 10^6 PBMC. The detection limit for this assay is log_{10} 0.5 cells per 10^6 PBMC. A value of 0.1 is given were results were positive by virus isolation but below the detection limit of the titration, where tested.
[c] Post mortem tissues were taken between days 132 and 168 post SIVmacJ5 challenge. Bld denotes blood. Spl denotes spleen. Mln denotes mesenteric lymph nodes. Pln denotes peripheral lymph nodes.

Rsa 1 analysis. Despite superinfection of three out of four vaccinees from group D, cell-associated virus and plasma vRNA loads at 14 days post challenge were significantly lower than amongst challenge controls (group F; Figs. 1A and B, respectively). Furthermore, amongst co-inoculated individuals (group E), a significant suppression of cell-associated virus and plasma vRNA loads was observed 14 days later, when compared with challenge controls that received wild-type virus alone (group F; Figs. 1A and B, respectively). Indeed, viral load amongst co-inoculated individuals (group E) was very similar to controls infected with SIVmacC8 alone (group G, cell associated virus load $P = 0.7663$ and vRNA $P = 0.1722$, unpaired Student's t test, Figs. 1A and B, respectively). Moreover, clearance of virus-infected cells from the periphery of co-inoculated individuals (group E) was accelerated compared to challenge controls (group F) that had received wild-type SIVmacJ5 alone, as determined by virus isolation (Table 1) and plasma vRNA analysis (Table 3). Thus, by 56 days post challenge, it was possible to recover infectious virus by co-culture of PBMC from all of the challenge controls (group F) compared with only one out of four co-inoculated individuals (group E, Table 1).

Detection of vaccine-induced immune responses do not coincide with early protection

Characterisation of the time interval between inoculation with live attenuated SIVmacC8 and challenge with pathogenic, wild-type SIVmacJ5 required to protect against superinfection, provided the opportunity to characterise vaccine-induced immune responses that coincide with the onset of protection. On the day of challenge with wild-type SIVmacJ5, virus-neutralising antibody activity with a titre

Table 2
Detection[a] and discrimination[b] of attenuated SIVmacC8 from wild-type SIVmacJ5

Vaccine group and time of SIVmacC8 inoculation	Animal	Days post SIVmacJ5 challenge										Post mortem[c]			
		−70	−56	−42	−28	−21	0	14	28	56	84	Bld	Spl	Mln	Pln
(A) Day −70	S169	–	+	+	n.d.	+	C8	C8	C8	C8	C8	C8	C8	C8	–
	S170	–	+	+	n.d.	+	C8	C8	C8	C8	C8	C8	C8/J5	C8/J5	C8
	S171	–	+	+	n.d.	+	C8	C8	C8	C8	C8	C8	C8	C8	C8
	S172	–	+	+	n.d.	+	C8	C8	C8	n.d.	C8	C8	C8	C8	C8
(B) Day −42	S173			–	+	+	C8	C8	C8	C8	C8	C8	C8	C8	C8
	S174			–	+	+	C8	C8	C8	C8	C8	C8	C8	C8	C8
	S175			–	–	+	C8	C8	C8	C8	C8	C8	C8	C8	C8
	S176			–	–	+	C8	C8	C8	C8	C8	C8	C8	C8	C8
(C) Day −21	S177					–	C8	C8	C8	C8	C8	C8	C8	C8	C8
	S178					–	C8	C8	C8	C8	–	C8	C8	C8	C8
	S179					–	C8	C8	C8	C8	C8	C8	C8	C8	C8
	S180					–	C8	C8	C8	C8	–	C8	C8	C8	C8
	T321					–	C8	C8	C8	C8	C8	C8	C8	C8	C8
	T322					–	–	C8	–	–	C8	C8	C8	C8	C8
	T323					–	C8	C8	C8	–	C8	C8	C8	C8	C8
	T324					–	–	C8	–	–	C8	C8	C8	–	C8
(D) Day −10	T317					–	C8	C8/J5	C8	–	C8	C8/J5	n.d.	C8	C8/J5
	T318					–	C8	C8	C8	–	C8	C8	C8	C8	C8
	T319					–	C8	C8	C8	C8	–	C8/J5	C8	C8	C8/J5
	T320					–	–	C8	C8	–	C8	C8/J5	C8/J5	C8/J5	C8/J5
(E) Day 0	T325						–	C8/J5	C8	C8	C8	C8/J5	C8/J5	n.d.	C8/J5
	T326						–	C8/J5	C8/J5	–	C8/J5	C8/J5	C8/J5	C8/J5	C8/J5
	T327						–	C8/J5	C8/J5	–	C8	C8/J5	C8/J5	C8/J5	C8/J5
	T328						–	C8/J5	C8	C8	C8	C8/J5	C8/J5	C8/J5	n.d.
(F) Naïve controls	S181						–	J5	J5	J5	J5	J5	J5	J5	J5
	S182						–	J5	J5	J5	J5	J5	J5	J5	J5
	S183						–	J5	J5	J5	J5	J5	J5	J5	J5
	S184						–	J5	J5	J5	J5	J5	J5	J5	J5
	T329						–	J5	J5	J5	J5	J5	J5	J5	–
	T330						–	J5	J5	J5	J5	J5	J5	J5	J5
	T331						–	J5	J5	J5	J5	J5	J5	J5	J5
	T332						–	J5	J5	J5	J5	J5	J5	J5	J5

n.d. denotes that the result was not determined.
[a] – and + symbols denote negative and positive SIV gag DNA PCR, respectively.
[b] C8 or J5 denote that only SIVmacC8 or SIVmacJ5, respectively, were detected by differential SIV nef PCR. C8/J5 denotes that both SIVmacC8 and SIVmacJ5 were detected by SIV nef PCR.
[c] Post mortem tissues were taken between days 132 and 168 post SIVmacJ5 challenge. Bld denotes blood. Spl denotes spleen. Mln denotes mesenteric lymph nodes. Pln denotes peripheral lymph nodes.

greater than 1.5 $\log_{10}$ was detected in all individuals vaccinated for 70 days (group A) and in half of the individuals vaccinated for 42 days (group B), but in none of the individuals vaccinated for 21 days or less (Table 4). This was not due to any inability of vaccinees to generate SIV neutralising antibody activity, as titres greater than 1.5 $\log_{10}$ were detected in all individuals by day 84 post SIVmacJ5 challenge (Table 4). Binding antibody to recombinant SIV envelope gp130 on the day of challenge was detected in two out of four vaccinees from group A, but not in vaccine groups B through F (Table 5). By 140 days post challenge, binding antibodies to recombinant SIV envelope gp130 could be detected in all vaccinees, except S171 from group A (Table 5). Binding antibody to recombinant SIV p27 on the day of challenge was detected in three out of four vaccinees from group A and two out of four vaccinees from group B, but not in groups C through F (Table 6). By 140 days post challenge, binding antibodies to recombinant SIV p27 could be detected in all vaccinees (Table 6).

Analysis of cytotoxic T cell responses following inoculation with live attenuated SIVmacC8 was performed on PBMC from individuals in group C (T321–T323) and additional subjects W58–W61 and X53–X56. These additional subjects were all demonstrated to be infected with SIVmacC8 (data not shown). By chromium release assay, anti-Nef specific memory CTL responses could be detected 20 days after inoculation of attenuated SIVmacC8 in T321–T323 (Fig. 2B), but not 10 days after inoculation of W58–W61 (Fig. 2A). Using intracellular interferon-γ (IFNγ) staining of CD3+ CD8+ T cells, significant numbers of IFNγ-positive cells were detected in response to stimulation with individual 15mer peptides across the Nef protein in three out of four individuals (X53–X55), tested 19 days after inoculation with attenuated SIVmacC8 (Fig. 2D). By comparison, at 10 days post inoculation, an IFNγ response

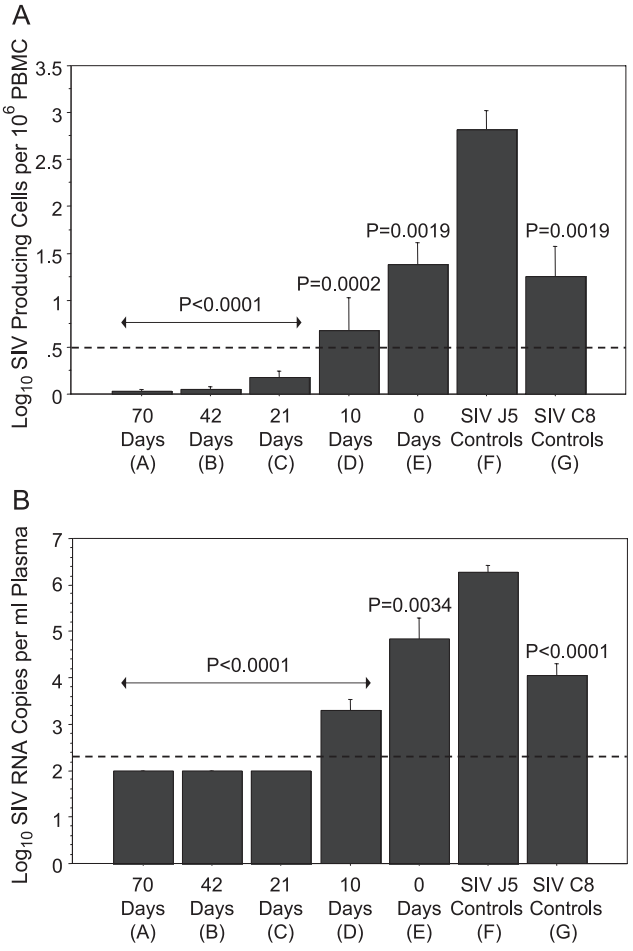

Fig. 1. Virus loads at 14 days post wild-type SIVmacJ5 challenge. (A) Titration of SIV-producing cells by co-culture. (B) Plasma vRNA loads. Groups A to E were inoculated with attenuated SIVmacC8 at 70, 42, 21, 10 or 0 days prior to challenge with wild-type SIVmacJ5, respectively. Group F are naïve controls challenged with wild-type SIVmacJ5. Group G present virus loads at 14 days post inoculation with attenuated SIVmacC8. Assay detection limits, denoted by a dashed line, are $\log_{10}$ 0.5 cells per 10^6 PBMC for cell-associated SIV loads and $\log_{10}$ 2.3 copies per milliliter of plasma for SIV RNA loads. The statistical significance of the results was evaluated individually by unpaired Student's *t* test, comparing the result of each vaccine group with the challenge control group F. Multiple comparison by Dunnett's test using an overall error rate of $P < 0.05$ gives an individual error rate of $P < 0.0097$ as significant. Error bars shown are 1 standard error.

was only detected to a single Nef 15mer peptide in just one of these vaccinees, X55 (Fig. 2C).

Discussion

This study is part of a series that has characterised protection conferred by the *nef* disrupted virus SIVmacC8 (Almond et al., 1995, 1997; Silvera et al., 2001; Stebbings et al., 1998, 2002). It describes that protection against super-infection with pathogenic, wild-type SIVmacJ5 can be obtained within 21 days of inoculation with live attenuated SIV, at a time when neutralising antibodies were not present and limited CD8+ T cell responses were detected. Furthermore, it describes blunting of the primary viremia of wild-type, pathogenic SIVmacJ5 when co-inoculated with live attenuated SIVmacC8. Thus, the key conclusion of these observations is that innate immunity or non-immune mechanisms, such as retroviral interference or target cell depletion, are likely to contribute to the vaccine protection observed here.

Failure to identify any correlation between neutralising antibody responses elicited through inoculation with live attenuated SIV and the appearance of protection concurs with previous observations (Almond et al., 1997; Connor et al., 1998; Langlois et al., 1998; Nilsson et al., 1998; Norley et al., 1996). Furthermore, our observation of poor correlation between anti-SIV CTL responses and super-infection resistance is consistent with the previous findings of some groups (Nilsson et al., 1998; Nixon et al., 2000; Stebbings et al., 1998, 2002), but at variance with those of

Table 3
Plasma vRNA loads[a] post SIVmacJ5 challenge

Vaccine group and time of SIVmacC8 inoculation	Animal	Days post SIVmacJ5 challenge	
		0	14
(A) Day −70	S169	n.d.	<2.3
	S170	n.d.	<2.3
	S171	n.d.	<2.3
	S172	n.d.	<2.3
(B) Day −42	S173	n.d.	<2.3
	S174	n.d.	<2.3
	S175	n.d.	<2.3
	S176	n.d.	<2.3
(C) Day −21	S177	n.d.	<2.3
	S178	n.d.	<2.3
	S179	n.d.	<2.3
	S180	n.d.	<2.3
	T321	2.5	<2.3
	T322	2.7	<2.3
	T323	2.3	<2.3
	T324	n.d.	<2.3
(D) Day −10	T317	4.5	3.9
	T318	4.3	2.8
	T319	4.4	3.0
	T320	5.3	3.5
(E) Day 0	T325	–	4.4
	T326	–	6.1
	T327	–	4.8
	T328	–	4.1
(F) Naïve controls	S181	–	6.9
	S182	–	6.8
	S183	–	6.2
	S184	–	6.3
	T329	–	6.0
	T330	–	6.3
	T331	–	5.5
	T332	–	6.0

n.d. denotes that the result was not determined.
[a] Virus loads shown are $\log_{10}$ SIV RNA copies per 1 ml of plasma. The detection limit for this assay is $\log_{10}$ 2.3 copies per 1 ml of plasma. Values below the detection limit of this assay are given as <2.3 for animals positive by SIV DNA PCR and by the − symbol for animals negative by SIV DNA PCR.

Table 4
Neutralising antibody titres[a] post SIVmacJ5 challenge

Vaccine group and time of SIVmacC8 inoculation	Animal	Days post SIVmacJ5 challenge		
		0	56	84
(A) Day 70	S169	1.5	2.4	n.d.
	S170	1.9	2.8	n.d.
	S171	1.5	2.1	n.d.
	S172	2.5	2.7	n.d.
(B) Day −42	S173	–	2.7	n.d.
	S174	–	2.5	n.d.
	S175	1.8	2.7	n.d.
	S176	1.5	3.1	n.d.
(C) Day −21	S177	–	2.4	n.d.
	S178	–	1.9	n.d.
	S179	–	2.1	n.d.
	S180	–	2.5	n.d.
	T321	–	–	3.6
	T322	–	1.5	2.4
	T323	–	–	2.1
	T324	–	1.5	2.8
(D) Day −10	T317	–	2.7	2.5
	T318	–	–	1.8
	T319	–	1.8	3.4
	T320	–	1.6	3.6
(E) Day 0	T325	–	1.9	3.3
	T326	–	3.6	3.9
	T327	–	2.1	3.0
	T328	–	2.5	3.0
(F) Naïve controls	S181	–	2.5	n.d.
	S182	–	2.7	n.d.
	S183	–	2.1	n.d.
	S184	–	2.2	n.d.
	T329	–	2.1	3.0
	T330	–	2.4	2.7
	T331	–	2.4	3.4
	T332	–	3.0	3.4

n.d. denotes that the result was not determined.
[a] Neutralisation end-point titres are expressed as the reciprocal ($\log_{10}$) of the highest dilution of antibody, in the serum–virus mixture, that demonstrates 75% inhibition of the mean p27 antigen production of the challenge virus controls. Assay cutoff is $\log_{10}$ 1.5. Values below the cutoff are denoted by the − symbol.

others (Johnson et al., 1999; Vogel et al., 1998). Nevertheless, here potent early protection against wild-type SIVmacJ5 challenge was established in a total of eight out of eight vaccinees within 21 days (group C; two studies) and in one out of four vaccinees within 10 days (group D; single study) of inoculation with live attenuated SIVmacC8. In spite of superinfection of three out of four vaccinees challenged 10 days after inoculation (group D), replication of wild-type, pathogenic SIVmacJ5 in those individuals was effectively contained at or below the level of a non-pathogenic attenuated SIVmacC8 infection. This superinfection resistance at 10 days post attenuated SIVmacC8 inoculation was achieved prior to the development of significant anti-SIV Nef CTL responses, which could not be detected readily until 19 and 20 days post inoculation. More intriguing perhaps is the observation of a significant reduction in peak viral load and shorter primary viremia following co-inoculation of homologous attenuated SIVmacC8 with wild-type SIVmacJ5, compared with challenge controls that received wild-type SIVmacJ5 alone. Although during the first week of infection, the primary viremia of both attenuated SIVmacC8 and wild-type SIVmacJ5 are similar, peak vRNA loads between days 10 and 14 are significantly higher for wild-type virus (Clarke et al., 2003). Thus, it would appear that competition between attenuated SIVmacC8 and wild-type SIVmacJ5 during the first week of co-infection is sufficient to blunt the primary viremia of the wild-type virus. It is possible that the generation of revertant virus during co-infection could have contributed to observed reduction in viremia; however, protection against superinfection is different to protection against reversion, live attenuated SIV vaccinees can resist superinfection with virulent virus but are not protected against the generation of revertant virus in vivo (Sharpe et al., 1997; Whatmore et al., 1995). Alternatively, an effective dilution of the wild-type SIV inoculum in the co-infection experiment may have produced the observed reduction in primary viremia, although this would mean that in each macaque there are only a few thousand susceptible target cells capable of establishing a persistent infection.

Immunisation with live attenuated SIV is reported to elicit SIV-specific CD8+ T cells expressing the $\alpha4\beta7$ mucosa-homing receptor, which traffic to the intestinal mucosa (Cromwell et al., 2000). It could be argued, therefore, that our inability to detect anti-SIV Nef CTL responses in the blood of live attenuated SIVmacC8 vaccinees at day 10 post inoculation is due to their sequestration at sites of initial infection, such as the intestinal tract (Veazey et al., 1998). However, it has been demonstrated that anti-SIV CTL responses develop at a similar rate and magnitude in both peripheral and mucosal lymphoid tissue during primary SIV infection (Veazey et al., 2003). Our inability to detect CTL responses to SIV regulatory proteins at 10 days post SIV inoculation is in agreement with the observations of others. It has been reported that anti-SIV gag CTL responses are not detected at 10 days post SIV inoculation, but arise later between days 14 and 21 when control of the primary viremia is first detected (Veazey et al., 2001, 2003). Thus, our observations would support those of others that CTL responses can control SIV replication (Jin et al., 1999; Lifson et al., 2001; Metzner et al., 2000; Schmitz et al., 1999). However, whether these same immune responses can also mediate protection against superinfection is not proven (Stebbings et al., 1998).

Previous studies that have investigated the time interval between inoculation of live attenuated SIV and challenge with pathogenic, wild-type SIV required to protect against superinfection, all used heterologous or uncloned virus for challenge (Connor et al., 1998; Norley et al., 1996; Wyand et al., 1996). Here, we have challenged our live attenuated SIV vaccinees with a wild-type homologous clone. The advantage of using a homologous challenge to investigate the underlying mechanism(s) of protection is that it excludes differences in immunological recognition and tropism for

Table 5
Anti-SIV gp130 antibody titres[a] following SIVmacC8 inoculation and SIVmacJ5 challenge

Vaccine group and time of SIVmacC8 inoculation	Animal	Days post SIVmacJ5 challenge									
		−70	−42	−21	−10	0	14	28	56	84	140
(A) Day −70	S169	–	–	–	n.d.	–	–	–	2.1	2.2	2.2
	S170	–	–	2.2	n.d.	2.4	2.3	2.4	2.6	2.4	2.4
	S171	–	–	–	n.d.	–	–	–	–	–	–
	S172	–	–	–	n.d.	2.2	2.4	2.4	2.2	2.3	2.3
(B) Day −42	S173		–	–	n.d.	–	–	–	–	–	2.4
	S174		–	–	n.d.	–	–	–	–	2.2	2.3
	S175		–	–	n.d.	–	–	–	–	2.0	2.1
	S176		–	n.d.	n.d.	–	–	2.1	2.3	2.5	2.6
(C) Day −21	S177			–	n.d.	–	–	2.1	–	2.4	2.3
	S178			–	n.d.	–	–	2.2	2.3	2.8	2.7
	S179			–	n.d.	–	–	–	–	2.1	2.2
	S180			–	n.d.	–	–	–	2.8	2.6	2.6
	T321			–	n.d.	–	2.0	2.2	2.4	2.7	2.5
	T322			–	n.d.	–	2.2	2.3	2.6	2.4	2.4
	T323			–	n.d.	–	1.9	1.9	2.4	2.3	2.1
	T324			–	n.d.	–	–	1.6	1.8	1.9	2.0
(D) Day −10	T317				–	–	2.0	2.2	2.5	2.5	2.9
	T318				–	–	1.7	2.3	2.6	2.8	2.9
	T319				–	–	–	2.3	3.1	3.2	3.0
	T320				–	–	–	–	2.7	3.0	3.1
(E) Day 0	T325					–	–	1.6	2.4	2.4	2.7
	T326					–	–	2.1	2.6	3.0	3.1
	T327					–	–	2.1	2.5	2.5	2.6
	T328					–	–	2.2	2.6	2.9	3.2
(F) Naïve controls	S181					–	–	–	2.8	2.6	n.d.
	S182					–	–	–	2.4	2.8	n.d.
	S183					–	–	–	2.4	2.6	n.d.
	S184					–	–	–	–	2.2	n.d.
	T329					–	–	–	2.9	2.8	2.9
	T330					–	–	–	2.4	2.5	3.0
	T331					–	–	–	2.3	2.7	2.9
	T332					–	–	2.3	2.4	2.8	3.0

n.d. denotes that the result was not determined.
[a] End-point titres of anti-SIV gp130 antibodies were calculated by linear regression analysis and expressed as $\log_{10}$ values. Assay cutoff is $\log_{10}$ 1.5. Values below the cutoff are denoted by the − symbol.

different target cells. This maximizes the chance for an immune response to work and allows identification of the earliest possible onset of superinfection resistance. Our findings indicate that the onset of early protection against superinfection with a homologous challenge occurs between 10 and 21 days post inoculation with live attenuated SIVmacC8. Nevertheless, the considerable resistance to superinfection observed in vaccinees 10 days post inoculation (group D) and the significant blunting of the wild-type SIVmacJ5 primary viremia observed following co-inoculation (group E) indicates that significant superinfection resistance arises between 0 and 10 days post inoculation with live attenuated SIVmacC8. That partial protection can be established so rapidly implies that early protection in this model is not based upon acquired immune mechanisms. Furthermore, similar early detection of both wild-type and attenuated virus in the lymphoid organs considered the primary sites of SIV replication (Canto-Nogues et al., 2001) would suggest that early resistance to superinfection could be attributed to viral interference or innate immune responses.

The role of CTL responses in mediating the protection conferred by inoculation with live attenuated immunodeficiency virus vaccines is still controversial. The results obtained here correlate vaccine protection poorly with CTL responses, but do not completely rule them out. Further attempts to correlate protection against challenge with the appearance of detectable immunity are unlikely to yield conclusive results. Therefore, alternative approaches should be pursued. The previous attempt by this group to deplete CD8+ CTL following inoculation with live attenuated SIVmacC8 was challenged on the grounds that the degree of depletion obtained in lymphoid tissues may not have been sufficient and that the temporal removal of CD8+ effectors would not have prevented the establishment of effective CD4+ memory T cells that would rapidly drive the reappearance of CD8+ effectors (Dittmer and Hasenkrug, 1999; Stebbings et al., 1998). The availability of less immunogenic humanised anti-CD8 monoclonal antibody that can be administered for longer periods and a model of protection using live attenuated SIV that would encompass the period of effective CD8+ cell depletion could make it

Table 6
Anti-SIV p27 antibody titres[a] following SIVmacC8 inoculation and SIVmacJ5 challenge

Vaccine group and time of SIVmacC8 inoculation	Animal	Days post SIVmacJ5 rechallenge									
		−70	−42	−21	−10	0	14	28	56	84	140
(A) Day −70	S169	–	–	–	n.d.	2.3	2.3	2.3	2.2	2.3	2.3
	S170	–	–	–	n.d.	2.2	–	2.3	2.2	2.3	2.3
	S171	–	–	–	n.d.	–	–	–	–	2.3	–
	S172	–	–	–	n.d.	2.2	2.4	2.3	2.2	2.7	2.3
(B) Day −42	S173		–	–	n.d.	2.2	2.2	–	2.1	–	2.2
	S174		–	–	n.d.	–	2.2	2.2	–	2.2	2.3
	S175		–	–	n.d.	–	–	–	–	2.2	2.1
	S176		–	n.d.	n.d.	2.2	2.3	–	2.7	2.3	2.4
(C) Day −21	S177			–	n.d.	–	–	2.2	2.1	2.3	2.3
	S178			–	n.d.	–	–	2.2	2.3	2.3	2.7
	S179			–	n.d.	–	–	–	–	2.8	2.3
	S180			–	n.d.	–	–	–	2.3	3.4	2.2
	T321			–	n.d.	–	–	2.0	2.3	2.3	2.0
	T322			–	n.d.	–	–	–	1.8	1.9	2.0
	T323			–	n.d.	–	–	–	2.1	1.8	2.2
	T324			–	n.d.	–	–	–	–	2.0	1.7
(D) Day −10	T317				–	–	–	1.9	2.1	2.3	2.6
	T318				–	–	–	–	2.0	2.5	1.8
	T319				–	–	–	1.5	2.4	2.7	2.6
	T320				–	–	–	–	1.9	2.3	2.8
(E) Day 0	T325					–	–	–	–	1.9	2.2
	T326					–	–	–	1.7	2.4	2.5
	T327					–	–	–	1.7	2.2	2.3
	T328					–	–	–	–	2.3	2.6
(F) Naïve controls	S181					–	–	2.2	2.2	–	n.d.
	S182					–	–	2.3	2.4	2.8	n.d.
	S183					–	–	–	2.1	2.3	n.d.
	S184					–	–	–	–	–	n.d.
	T329					–	–	–	2.3	1.6	2.1
	T330					–	–	–	–	2.7	3.0
	T331					–	–	–	2.1	2.8	2.6
	T332					–	–	2.1	1.6	2.0	2.5

n.d. denotes that the result was not determined.
[a] End-point titres of anti-SIV p27 antibodies were calculated by linear regression analysis and expressed as $\log_{10}$ values. Assay cutoff is $\log_{10}$ 1.5. Values below the cutoff are denoted by the − symbol.

possible to address this question again more effectively. Whatever the outcome of this future experiment, the demonstration here of partial protection 10 days after inoculation has implicated innate immunity and non-immune mechanisms as potential components of the protection conferred by live attenuated SIV vaccines. Together, our data strongly advocate research into innate immunity and non-immune mechanisms of protection, in order that novel prophylactic vaccine approaches can be developed to safely reproduce the protection conferred by live attenuated SIV and complements the approaches being investigated in current clinical trials.

Materials and methods

Animals and virus challenges

A total of 52 naïve, D-type retrovirus free, juvenile, purpose-bred cynomolgus macaques (*Macaca fascicularis*) were used in this study. Macaques were housed and maintained in accordance with United Kingdom Home Office guidelines for the care and maintenance of nonhuman primates. The attenuated SIVmac32H (SIVmacC8) virus clone differs from the wild-type SIVmac32H (SIVmacJ5) clone by a 12-bp deletion and two nonsynonymous nucleotide changes, resulting in conservative amino acid changes in the *nef* open reading frame (Rud et al., 1994a). For all live attenuated SIV vaccinations, macaques were inoculated intravenously with 5000 $TCID_{50}$ of the 9/90 pool of SIVmacC8, which has an end-point titre of 10^4 $TCID_{50}$/ml on C8166 cells (Cranage et al., 1998). For all wild-type SIV challenges, macaques were inoculated intravenously with 500 $TCID_{50}$ of the J5C stock of SIVmacJ5, which has an end-point titre of 10^3 $TCID_{50}$/ml on C8166 cells (Canto-Nogues et al., 2001).

Experimental outline

In an initial study, attenuated SIVmacC8 inoculation was carried out in macaques S169–S172, S173–S176 and S177–S180 at 70, 42 and 21 days before challenge, respectively. In

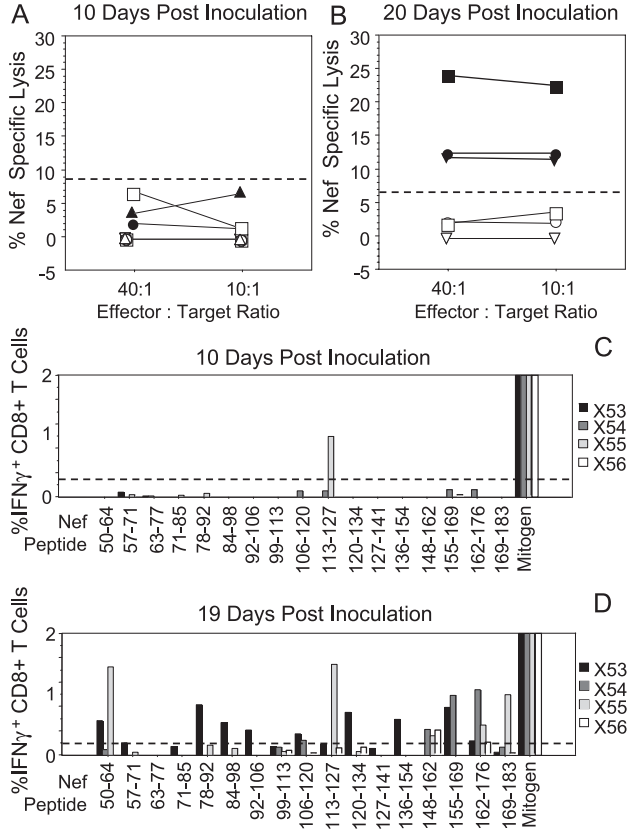

Fig. 2. Detection of anti-SIV Nef cytotoxic T cell and interferon-γ responses following attenuated SIVmacC8 inoculation. Anti-SIV Nef cytotoxic T cell responses were assessed by chromium release assay at 10 (A) and 20 (B) days post SIVmacC8 inoculation, respectively. Solid symbols denote SIVmacC8 inoculated individuals (W58–W61 in A, T321–T323 in B) and empty symbols denote unvaccinated naïve controls (W62–W65 in A, T329, T330 and T332 in B). SIV Nef specific lysis was deemed significant if greater than 3 standard deviations above the mean response of uninfected controls (8.79% in A, 6.52% in B), denoted here by a dashed line. Anti-SIV Nef peptide responses were assessed by intracellular interferon-γ staining at 10 (C) and 19 (D) days post inoculation. Anti-SIV Nef peptide responses measured by intracellular interferon-γ staining of gated CD3+ CD8+ PBMC were considered significant if the percentage of positively stained cells was greater than 3 standard deviations above background staining of unstimulated PBMC, denoted here by a dashed line. The mitogens phorbol 12-myristate 13-acetate (PMA) with ionomycin was used as a positive control.

a follow-up study, attenuated SIVmacC8 inoculation was carried out in macaques T321–T324, T317–T320 and T325–T328 at 21, 10 and 0 days before challenge, respectively. Day 0 vaccinees were inoculated intravenously with a total of 1 ml of pre-mixed vaccine (5000 $TCID_{50}$ of SIVmacC8 in 0.5 ml) and challenge virus (500 $TCID_{50}$ of SIVmacJ5 in 0.5 ml), for simultaneous infection. Macaques S181–S184 and T329–T332 were used as naïve challenge controls in the initial and follow-up study, respectively. Attenuated SIVmacC8 vaccinees W250–W253 were used as vaccine virus-only controls (Clarke et al., 2003). For additional CTL studies, macaques W58–W61 and X53–X56 were inoculated with attenuated SIVmacC8. Macaques W62–W65 and X57–X60 were used as uninfected controls for CTL analysis.

Virus detection and quantification

The presence of SIV in PBMC or tissue samples was determined using SIV *gag* DNA PCR assays, as previously described (Rose et al., 1995). The two molecular clones SIVmacC8 and SIVmacJ5 were differentiated using a *nef*-specific nested PCR protocol, encompassing the 12-bp deletion and subsequent Rsa 1 restriction endonuclease digestion (Rose et al., 1995). Intermediate revertants lacking the Rsa 1 restriction site are not distinguished from SIVmacC8 by this protocol. The overall kinetics of SIV RNA levels in plasma were determined as previously described (Clarke et al., 2003). The sensitivity of the assay is 200 SIV RNA copies per milliliter of plasma. Virus isolation from PBMC was determined by co-culture with C8166 cells, and the presence of replicating virus was confirmed by syncytia identification or by antigen capture at 28 days (Stebbings et al., 1998).

Serology

Neutralising antibody end-point titres were determined as the dilution of serum in the serum and virus mixture inhibiting p27 antigen production by at least 75%, expressed as the $\log_{10}$ of the reciprocal of the end-point dilution (Kent et al., 1994). Titres of binding antibodies to SIV envelope gp130 (EVA670 CFAR/NIBSC, Potters Bar, UK) or recombinant SIV p27 (EVA643 CFAR/NIBSC, Potters Bar, UK) were determined from heat inactivated (56 °C for 1 h) plasma samples by ELISA, as previously described (Almond et al., 1990; Silvera et al., 1994; Stott et al., 1990).

CTL detection by chromium release assay

Autologous targets were prepared by incubating *Herpesvirus papio* immortalized B-lymphoblastoid cell lines with 5 PFU/cell of vaccinia virus expressing SIV *nef* (ARP274, CFAR/NIBSC, Potters Bar, UK). Effectors were generated by re-stimulation of PBMC with psoralen/UV inactivated autologous targets and 10 IU/ml of recombinant human IL-2 for 10 days. Targets pulsed for 2 hours with 50μCi of ^{51}Chromium (Amersham Life Science, Buckinghamshire, UK) per 1×10^6 cells were incubated for 5 h at 37 °C + 5% CO_2 with effectors at E:T ratios of 40:1 and 10:1. Supernatants were collected onto 96-well Lumaplates for reading on a Topcount scintillation counter (Packard Instruments BV, Groningen, Netherlands). The percentage specific lysis was calculated as follows: (experimental release − minimum lysis) / (maximum lysis − minimum lysis) 100. Spontaneous release from target cells was less than 27% in all assays.

Intracellular interferon-γ detection by flow cytometry

For intracellular cytokine staining isolated PBMC in RPMI 1640 medium (Gibco BRL, Paisley, UK), supple-

mented with 10% foetal calf serum + 50 IU/ml penicillin streptomycin (Gibco), were incubated overnight with a range of SIV Nef 15mer peptides at 10 μg/ml (EVA7067, CFAR/NIBSC, Potters Bar, UK). One hour into the incubation, monensin, at 2 μg/ml (Sigma-Aldrich Ltd, Dorset, UK), was added. Subsequently, PBMC were fixed and permeabilised with Permeafix (Ortho Diagnostics Systems Inc.) and stained with anti-monkey CD3 FITC conjugate, anti-human IFNγ PE conjugate and anti-human CD8 PerCP conjugate, as previously described (Stebbings et al., 2002). A FACSCalibur cytometer was used for acquisition and data was analysed using CellQuest Pro software (Becton Dickinson, Oxford, UK).

Acknowledgments

We thank Dr. Harvey Holmes, the AIDS Reagent Programme and Programme EVA for essential materials. This work was funded in part by grants from the U.K. Medical Research Council (G9025730 and G9419998).

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