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# Comparative studies on mucosal and intravenous transmission of simian immunodeficiency virus (SIVsm): the kinetics of evolution to neutralization resistance are related to progression rate of disease

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The kinetics of appearance of autologous neutralizing antibodies were studied in cynomolgus macaques infected with simian immunodeficiency virus (SIVsm) by the intravenous (IV) route (six monkeys) or the intrarectal (IR) route (ten monkeys). The SIVsm inoculum virus and reisolates obtained at 2 weeks, 3 or 4 months and later than 1 year were tested in a GHOST(3) cell line-based plaque-reduction assay with autologous sera collected at the same sampling times. All monkeys developed a neutralizing-antibody response to the inoculum virus, those infected by the IV route earlier than monkeys infected by the IR route. Animals were divided into progressor (P), slow-progressor (SP) and long-term non-progressor (LTNP) monkeys, based on progression rate. In P monkeys, neutralization escape could be demonstrated by 3 months post-infection. Neutralization-resistant variants also emerged in SP and LTNP monkeys, but were much delayed compared with P monkeys. Evolution of neutralization resistance was also demonstrated by a positive-control serum in the heterologous reaction. Pooled sera from four LTNP monkeys showed a broad neutralizing capacity, including neutralization of escape variants. These results from a large group of infected monkeys showed that SIV evolves to neutralization resistance in the infected host and that the kinetics of this evolution are related to the route of transmission and the progression rate of SIV disease. The results suggest an important role for neutralizing antibodies in controlling viraemia. Although this control is transient in the infected host, neutralization resistance is relative and variant viruses may be neutralized by a broadly cross-neutralizing serum pool.

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## INTRODUCTION

Neutralizing antibodies serve as markers of protective immunity in many virus infections and are the principal component of the humoral immune response against viral pathogens. However, in human immunodeficiency virus types 1 and 2 (HIV-1 and -2) and simian immunodeficiency virus (SIV) infections, the role of neutralizing antibodies has been unclear. We and others have found an association between neutralizing-antibody responses and delayed disease progression in HIV-1 infections (Cao *et al.*, 1995; Montefiori *et al.*, 1996; Pantaleo *et al.*, 1995; Pilgrim *et al.*, 1997; Zhang *et al.*, 1997). In these studies, serum from asymptomatic long-term non-progressors contained antibodies capable of neutralizing both heterologous and autologous virus isolates, whilst serum from fast progressors had no or only weak neutralizing activity. Although autologous HIV-1-neutralizing antibodies could be detected as early as 1 month after infection, not surprisingly, this restricted

immune response combined with the high variability of HIV-1 was, in many cases, followed by selection of neutralization-resistant variants (Albert *et al.*, 1990; Arendrup *et al.*, 1992; Richman *et al.*, 2003; Wei *et al.*, 2003). In contrast, no escape from neutralization could be demonstrated in HIV-2-infected individuals (Björling *et al.*, 1993). Similar to HIV-1 infection, in the pathogenic SIV infection of macaques, variant viruses resistant to autologous neutralizing antibodies appear repeatedly (Burns *et al.*, 1993; Zhang *et al.*, 1993), whereas HIV-2 reisolates obtained from HIV-2-infected macaques have been shown to remain neutralization-sensitive during the entire course of the non-pathogenic infection (Zhang *et al.*, 1994). Thus, the patterns of neutralization suggest a close relationship to pathogenesis.

Differences in the immune response also may be related to the route of infection. Comparisons of disease progression in HIV-1 infection between injecting drug users and homosexual men have shown that homosexual men have

a significantly accelerated progression rate (Eskild *et al.*, 1997; Pehrson *et al.*, 1997). Although this was not confirmed by others (Hengge *et al.*, 2003; Prins & Veugelers, 1997), Jiang & Bekesi (2001) described higher antibody responses to HIV-1 antigens in HIV-1-positive injecting drug users than in HIV-1-positive homosexual men. It is therefore an open question whether the route of HIV-1 infection influences neutralizing-antibody production. To dissect the effect of transmission, we turned to the monkey model and examined the kinetics of appearance of autologous neutralizing antibodies in cynomolgus macaques infected with SIVsm (of sooty mangabey origin) by the intravenous (IV) route (six monkeys) or by the intrarectal (IR) route (ten monkeys). In addition, we compared the evolution of neutralization resistance using sera collected at 2 weeks and at 3–4 months post-infection (p.i.) and late in infection. Our results from a large group of infected monkeys showed that monkeys infected by the IV route developed a neutralizing-antibody response to the inoculum virus earlier than monkeys infected by the IR route. We also showed that SIVsm evolved to neutralization resistance in all infected hosts, but that the kinetics of appearance of neutralization-resistant variants were related to the severity of infection. This suggests that neutralizing antibodies may have an important role in pathogenesis and that their protective role should be considered in vaccine studies.

## METHODS

**Animals.** Sixteen cynomolgus macaques were inoculated by the IV or IR route with SIVsm (strain SMM-3 from P. Fultz and H. McClure, Yerkes National Primate Research Center, Atlanta, GA, USA). Strain SMM-3 was originally isolated from a naturally infected sooty mangabey monkey (Fultz *et al.*, 1986). The virus stock used for infection was propagated *in vitro* in cultures of peripheral blood mononuclear cells (PBMCs) from cynomolgus macaques (Quesada-Rolander *et al.*, 1996). Cell-free virus stocks (10 MID<sub>50</sub>) were used for infection, which meant that animals infected by the IV route received an approximately 10<sup>3</sup>-fold higher virus dose than the IR-inoculated animals. The monkeys were monitored for general clinical status. Blood samples for virus isolation, sera, viral load determination and CD4<sup>+</sup> cell counts were collected at regular intervals after infection. SIV RNA levels in plasma were measured by using a highly sensitive quantitative competitive RT-PCR assay with a lower detection limit of 100 RNA equivalents (ml plasma)<sup>-1</sup>, as described in detail elsewhere (Ten Haaf *et al.*, 1998). The animals were monitored for changes in their CD4<sup>+</sup> cell counts by using two-colour flow-cytometric analysis as reported previously (Mäkitalo *et al.*, 2000). CD4<sup>+</sup> T-cell values were evaluated as a percentage of the total T-cell count. When observing the rate of change of the CD4<sup>+</sup> lymphocytes, values obtained before infection were set as 100% for each animal and following values were calculated in relation to these set-point values. The rate of change as a percentage of the CD4<sup>+</sup> lymphocyte population was fitted by linear-regression analysis. Animals were kept until development of AIDS or, if asymptomatic, until the end of the study period, when they were euthanized (Table 1).

**Virus reisolates.** Virus isolation was performed by co-cultivation of PBMCs from infected cynomolgus macaques with uninfected macaque or human donor PBMCs (mPBMCs or hPBMCs, respectively). PBMCs were stimulated by phytohaemagglutinin for 3 days

prior to cultivation (Nilsson *et al.*, 1995). Reisolates were passaged no more than twice in mPBMCs or hPBMCs. Cell-free supernatants were screened for reverse transcriptase (RT) activity with a Cavid HS kit Lenti RT (Cavid Tech) and stored frozen at -80 °C until use. Before neutralization, all isolates were tested for replication on GHOST(3)-CCR5 cells [see accompanying paper by Laurén *et al.* (2006) in this issue]. Three reisolates from each monkey were tested for autologous neutralization. The first re isolate was obtained early at 2 weeks p.i., the second re isolate was obtained at 3 or 4 months and the late reisolates shortly before the end of the study (Table 1).

**Sera.** We were interested especially in the kinetics of appearance of the neutralizing-antibody response in the monkeys and therefore analysed sera prior to infection and at 2 weeks and 1, 2 and 3 months after infection. A late serum obtained shortly before the end of the study was also used from each monkey (Table 1). A positive-control serum (H55:16) was obtained from an infected monkey that remained asymptomatic and had high neutralizing titres towards SIVsm. Monkey H55 was one of four monkeys that remained healthy in an earlier study on disease progression of 33 cynomolgus macaques infected with SIVsm (Putkonen *et al.*, 1992). Monkey H55 was inoculated with 1–10 MID<sub>50</sub> SIVsm and a serum sample used in the present study was obtained at approximately 500 days p.i. To remove complement activity, all sera and plasma were heat-inactivated (30 min at 56 °C) before use in the neutralization assay.

### Virus titrations and neutralization assay on GHOST(3) cells.

A similar method based on plaque reduction has been described for U87.CD4 cell lines (Shi *et al.*, 2002). In the present study, we used GHOST(3)-CCR5 cells with minor modifications of the assay (Nordqvist & Fenýö, 2005). The GHOST(3) cell lines were derived from a human osteosarcoma cell line by introducing the genes for human CD4 and the human CCR5 chemokine receptor (Laurén *et al.*, 2006; Mörner *et al.*, 1999). GHOST(3) cells are stably transfected with the green fluorescent protein (GFP) gene driven by the HIV-2<sub>ROD</sub> long terminal repeat. Upon infection, the viral Tat protein activates GFP expression. The GHOST(3) cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 7.5% FBS and antibiotics (penicillin and streptomycin).

One day before infection, GHOST(3) cells were seeded into 96-well plates at a concentration of  $5 \times 10^3$  cells per well in 200 µl medium and incubated overnight at 37 °C. Before infection, the medium was replaced with 50 µl fresh medium containing polybrene (2 µg ml<sup>-1</sup>). Viruses were first titrated on GHOST(3) cells to determine an appropriate virus concentration for the neutralization assays. Titrations were performed as follows: on the day of infection, virus was first diluted fivefold in culture medium, followed by at least four fivefold dilution steps, giving dilutions from 1/5 to 1/3125. Each dilution was added to triplicate wells at a volume of 150 µl per well and cultures were incubated overnight at 37 °C. The day after infection, cultures were washed once with 200 µl PBS and 200 µl fresh medium was added. Three days after infection, cultures were evaluated for results by using a fluorescence microscope.

For the neutralization assay, heat-inactivated sera and virus were diluted and mixed in culture medium to give a final 1:20 serum dilution and an appropriate dilution of virus, which was determined (by previous titration) as the virus dilution required to yield a countable number of fluorescent cells (plaques) in the first or second dilution step. The virus and serum mixtures were incubated at 37 °C for 1 h. After incubation, virus and sera were diluted further in two or three fivefold dilution steps. The virus was titrated in parallel with the neutralization to allow determination of the percentage neutralization. The starting dilution of virus in this titration was the same as that used for the starting dilution of virus in the neutralization assay. A 1:20 dilution of the serum was used as a (serum) control with no virus, a known strongly neutralizing serum was used as positive control and

**Table 1.** Summary of disease progression in macaques

Monkey	Group	Route of inoculation	End of study		CD4 decline (% CD4 per month)†	Viral load [ $\log_{10}$ RNA copies (ml plasma) $^{-1}$ ]		
			Disease*	Time (months)		2 weeks p.i.	3 months p.i.	1 year p.i.
D24	P	IR	sAIDS	12	-8.1	6.9	5.9	6.2
D23	P	IR	sAIDS	12	-7.0	5.9	4.9	5.6
B174	P	IV	sAIDS	15	-5.3	ND	ND	ND
C73	P	IR	sAIDS	18	-3.3	ND	ND	ND
D26	P	IR	sAIDS	18	-2.9	ND	ND	ND
C27	P	IV	Weight loss, diarrhoea	18	-2.8	3.3	2.7	3.4
C26	P	IV	sAIDS	21	-2.7	5.5	5.0	4.7
C39	P	IR	Lymphoma, sAIDS	18	-2.6	6.7	1.9	4.1
C20	P	IV	sAIDS	18	-1.8	3.8	0	3.6
C24	SP	IV	None	38	-1.0	ND	ND	ND
C68	SP	IR	None	53	-1.0	ND	ND	ND
B173	LTNP	IV	None	39	-0.8	ND	ND	ND
C44	P	IR	sAIDS	27	-0.7	7.7	3.5	6.0
C82	LTNP	IR	None	35	-0.5	ND	ND	ND
C93	LTNP	IR	None	50	-0.4	6.1	3.5	3.3
D28	LTNP	IR	None	39	0.5	4.3	3.2	3.3

\*Symptoms of disease at end of study; sAIDS, simian AIDS.

†CD4<sup>+</sup> cell decline is presented as the regression coefficient of linear-regression analysis, taking into account 10–27 determinations per monkey. ND, Not determined.

serum from an uninfected individual was used as a negative control together with virus. The different dilutions of virus/serum mixtures, virus and controls were distributed into triplicate wells in a volume of 150  $\mu$ l per well. Cultures were then treated as described above for virus titration.

**Evaluation of virus titres and neutralization.** Three days after infection, cultures were checked for expression of GFP by using fluorescence microscopy. Individual fluorescent cells or groups of fluorescent cells were regarded as plaques or single infectious units. Fluorescent units were counted at a dilution that gave 10–40 plaques per well. Virus titres were calculated as p.f.u. ml<sup>-1</sup>: (mean number of plaques in triplicate wells  $\times$  virus dilution)/volume in the well (Nordqvist & Fenyő, 2005; Shi *et al.*, 2002). The neutralizing property (plaque reduction) of the serum was calculated by using the formula  $1 - (\text{p.f.u. with serum}/\text{p.f.u. without serum}) \times 100$ , i.e. the percentage of p.f.u. with serum compared with infection without serum. As neutralization is based on plaque reduction in the presence of serum, the intra-assay variation was important. To establish the intra-assay variation of virus titre determinations, three assays were performed on the same day. Four serum/virus combinations were tested on GHOST(3)-CCR5 cells. We calculated the percentage difference for each individual determination relative to the mean of the three repeat determinations. The range of differences was -12.3 to 11% for the negative sera and 48.8–73.4% for the positive sera on GHOST(3)-CCR5 cells, giving an SD of 9.66 or 9.89%, respectively. On the basis of these data, we chose a cut-off point for neutralization (i.e. plaque reduction) of 30%, which represented 3.1 SD in assays performed on the same day. Thus, intra-assay variation in the GHOST(3) assay was similar to that of the U87.CD4 plaque-reduction assay (Shi *et al.*, 2002). By using this cut-off point, the risk of falsely identifying a neutralizing serum should be <1%.

**Statistics.** To compare neutralization in the different groups of monkeys, we used the Mann-Whitney non-parametric test. Statistics were calculated by using SPSS statistical software.

## RESULTS

### Disease progression

Animals were divided into progressor (P), slow-progressor (SP) and long-term non-progressor (LTNP) monkeys, based on disease progression rate. All monkeys showed a sharp decline in CD4 count during the first 3–4 months of infection. There was no difference in CD4 decline or viral load between monkeys infected by the IV or IR routes. The ten P monkeys studied here had a more rapid decline in CD4<sup>+</sup> T-cell counts than SP and LTNP monkeys and developed simian AIDS (sAIDS) or sAIDS-related symptoms (for details of CD4 decline and virus isolation frequencies, see Table 1; Laurén *et al.*, 2006). Due to early disease symptoms, two monkeys (D23 and D24) were euthanized 1 year after infection. Others developed symptoms of disease later, but all monkeys in this group were euthanized by 27 months p.i. and the median survival time was 18 months. In most of the P monkeys, plasma viral load was high initially [ $>10^6$  RNA copies (ml plasma) $^{-1}$ ] and stayed high, except in monkey C39, where viral load declined below  $10^3$  copies (ml plasma) $^{-1}$  within 3 months and then increased slowly again to values  $>10^4$  copies (ml plasma) $^{-1}$ . Monkey C27 had an exceptionally low viral load for this group, but had to be

euthanized at 18 months after infection because of extensive diarrhoea and weight loss and was therefore considered a P monkey. Monkey C44 with the slowest CD4 decline in the P group also showed a viral load pattern similar to that of C39, but with a decrease that was less dramatic and did not last as long as that in monkey C39. Two monkeys (C24 and C68) had a slow disease progression with a CD4 cell decline of  $-1.0\%$  CD4<sup>+</sup> cells per month and did not show disease symptoms during the study periods of 38 and 53 months, respectively. Although the rate of CD4 cell decline was lower, virus isolation frequencies were as high as others in the P group (91 and 100%). Four monkeys were classified as LTNPs based on normal CD4 counts, with a slight decline, if any, over time. These monkeys remained asymptomatic to the end of the observation period (35–60 months). Virus isolation from LTNPs was unsuccessful at many time points and varied between 31 and 72% for individual monkeys. The viral load of the LTNP group decreased after the early peak viraemia to values of between  $10^3$  and  $10^4$  RNA copies (ml plasma)<sup>-1</sup> and remained stable over the entire study period (Table 1).

**Neutralization of inoculum virus**

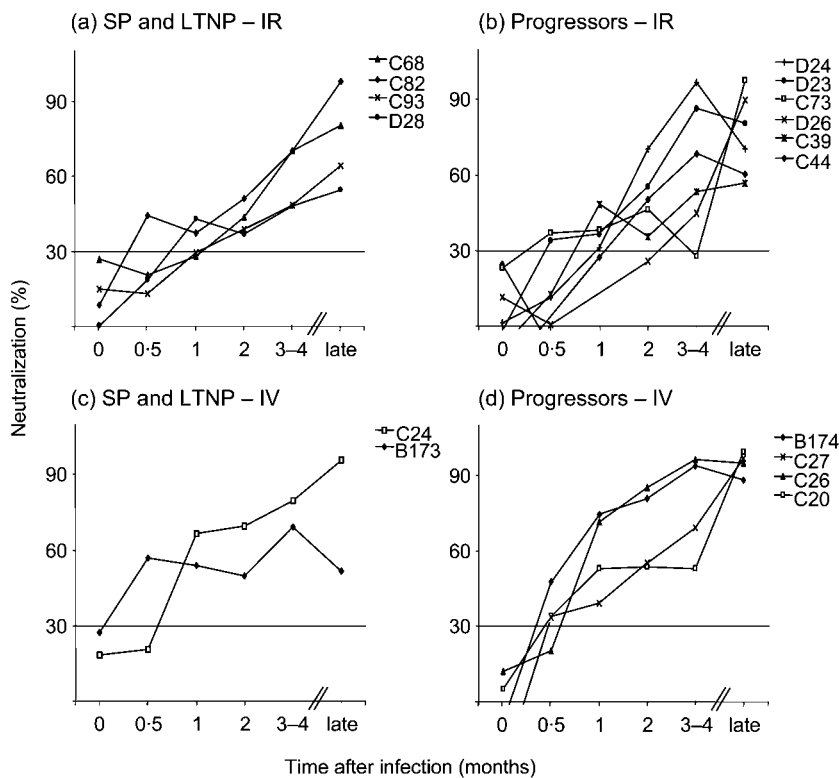
All monkeys developed a neutralizing-antibody response to the inoculum virus. A few monkeys (B173, C27, C20, B174, C82, D23 and C73) from all groups already had detectable neutralizing-serum titres (above the 30% cut-off point) by 2 weeks after infection (Fig. 1). An early neutralizing-antibody response was observed particularly in monkeys infected by the IV route, as four out of six monkeys were

positive in this group, whereas only three out of ten IR-infected monkeys had titres above the cut-off point at 2 weeks. At 1 and 2 months after infection, the IV-infected monkeys had developed a significantly higher capacity to neutralize the inoculum virus than IR-infected monkeys ( $P=0.002$  and  $P=0.022$ , respectively; Mann–Whitney test). Apart from this, there was no pathogenesis-related difference in the kinetics of neutralizing-antibody response when measured against the inoculum virus.

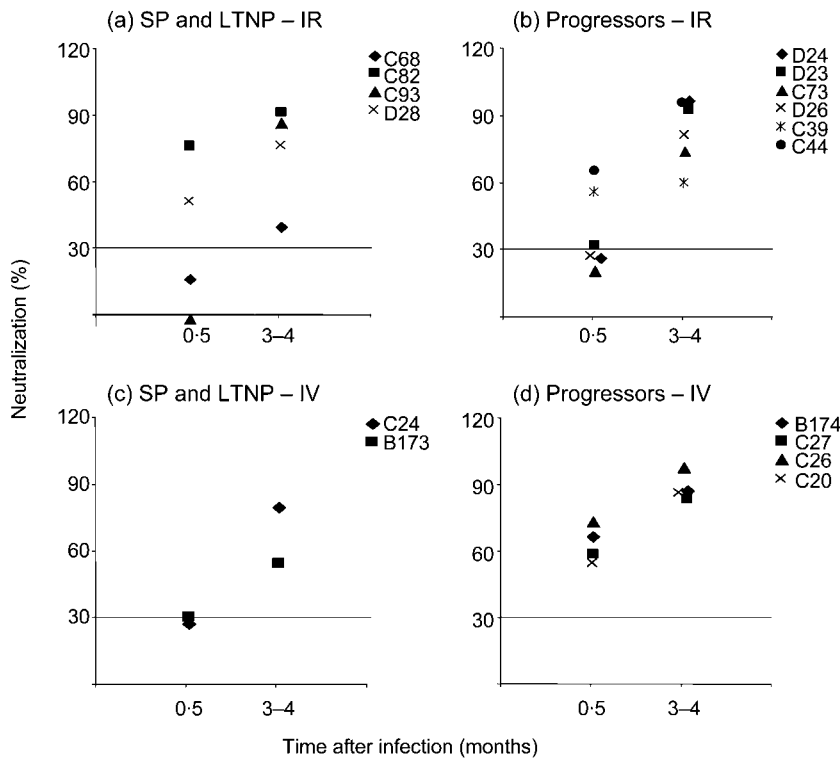
**Neutralization of autologous virus**

First, sequentially collected sera were tested against the 2 week isolate from each monkey. In the autologous neutralization, we also detected a small difference in the kinetics of the neutralizing-antibody response between the IV- and IR-inoculated groups. At 2 weeks after infection, sera from half of the IR-inoculated monkeys did not neutralize autologous 2 week isolates (Fig. 2), whilst in the IV-infected group, five of the six animals showed neutralizing activity against the autologous 2 week isolates. This initial difference between the groups had disappeared by 3–4 months after infection, as, at that time, all monkeys had developed neutralizing antibodies to their own 2 week isolates, regardless of whether virus isolation was carried out on hPBMCs or mPBMCs (monkeys D24, C68 and B173 were tested; data not shown).

Neutralization-resistant isolates could be recovered from both IV- and IR-inoculated monkeys by 3 or 4 months after infection (Fig. 3b). Notably, six out of ten P monkeys escaped neutralization by autologous sera, whilst



**Fig. 1.** Neutralization of the inoculum virus SIVsm strain SMM-3. IR infection (a, b) and IV infection (c, d) of SP and LTNP monkeys (a, c) and P monkeys (b, d). Late indicates serum obtained at the end of the study (see Table 1). Values are means of two independent assays.



**Fig. 2.** Neutralization of 2 week reisolates (hPBMCs) with sera obtained at 2 weeks and 3 or 4 months after infection. IR infection (a, b) and IV infection (c, d) of SP and LTNP monkeys (a, c) and P monkeys (b, d). Values are means of two independent assays.

neutralization-resistant variants could not be observed at this early time point for the SP and LTNP groups of animals. We compared differences in mean values for neutralization sensitivity among isolates from the three time points. The difference between neutralization of 2 week and 3 or 4 month isolates was larger for the P group than for the SP and LTNP groups with serum from 3 months after infection ( $P=0.043$ , Mann-Whitney test). Isolates obtained late in infection (regardless of whether they were obtained on mPBMCs or hPBMCs) were all more or less resistant to neutralization with early sera (Fig. 3). Interestingly, B174, an IV-inoculated P animal, showed the highest neutralization resistance and this monkey developed sAIDS earlier (at 15 months) than the other IV-infected animals. When the capacity of serum to neutralize autologous virus isolated at 3 months p.i. was analysed in relation to viral load, two monkeys (C39 and C44) that did not evolve neutralization-resistant variants at this time point had a large dip in viral load (Table 1). The third P animal (C27) with a latency to develop neutralization-resistant virus had a low viral load throughout the study, similar to the two LTNP animals (C93 and D28). These results suggest a role for neutralizing antibodies in controlling viraemia. This control is transient and in most cases is overridden by the emergence of neutralization-resistant variants.

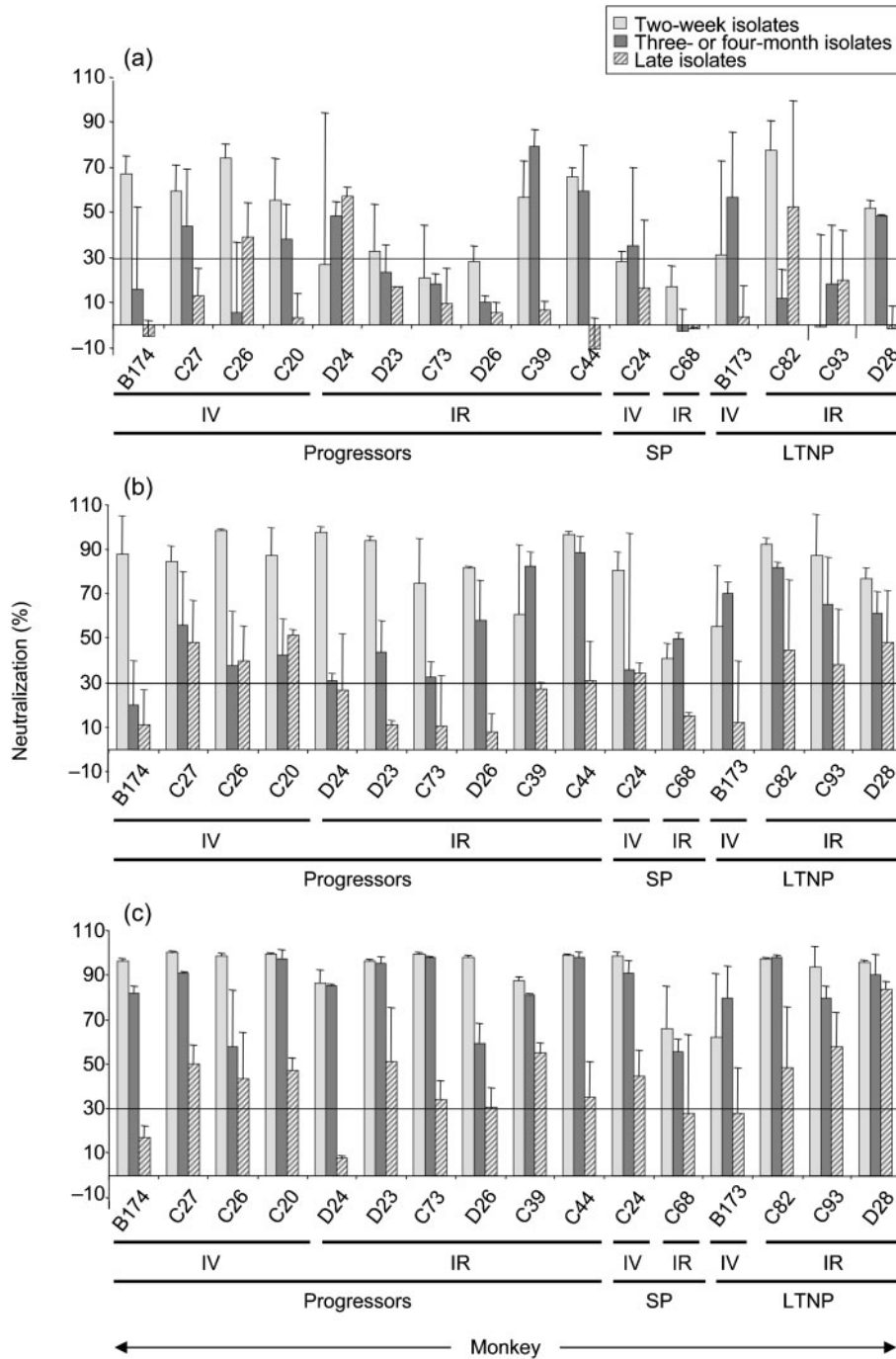
#### Virus isolates that escape neutralization in the autologous reaction also escape neutralization by heterologous serum

Virus isolates from four P monkeys (B174, D23, C39 and C44) were chosen for these experiments because of their

different patterns of evolution to neutralization escape. The 3 month virus isolates from monkeys B174 and D23 were neutralization-resistant, whereas the corresponding isolates from monkeys C39 and C44 were sensitive to neutralization by autologous sera (Fig. 3; Table 2). When tested in the heterologous reaction with sera from the three other animals in a chequerboard reaction, the same pattern was maintained (Table 2). The results indicated that virus variants resistant not only to autologous, but also to heterologous neutralization emerged over time in these animals. This appeared to be a converse relationship between emergence of neutralization-resistant variants and detectable neutralization potency of sera. Sera from monkeys with neutralization-resistant viruses showed broad neutralizing activity in the heterologous reaction, whereas sera from monkeys with neutralization-sensitive viruses had low activity in the heterologous reaction.

#### Evolution of neutralization resistance revealed by a positive-control serum in the heterologous reaction

All viruses were tested with a known high-titre neutralizing serum from an LTNP monkey (H55:16, Fig. 4). The results further indicated that viruses resistant to autologous neutralizing activity were also resistant to neutralization in the heterologous reaction. This serum neutralized the inoculum virus by  $>80\%$  (data not shown). Early virus isolates (obtained 2 weeks after infection) were also neutralized in 11/16 cases by  $>80\%$  (Fig. 4). However, the 3 month isolates from the IV-infected P monkeys were completely or partially resistant to neutralization with this



**Fig. 3.** Autologous neutralization of viruses obtained on hPBMCs with 2 week serum (a), 3 or 4 month serum (b) and late serum (c). Late time-point isolates indicate samples obtained at the end of the study (see Table 1). Values are means ( $\pm$  SD) of two independent assays performed in triplicate.

high-titre neutralizing serum, whereas the IR-infected P monkeys did not show the same pattern. Similar to the autologous neutralization, we compared differences in mean values for neutralization sensitivity between isolates from the three time points. The difference between neutralization of 2 week and 3 month isolates was significantly larger for IV-infected than for IR-infected P monkeys ( $P=0.001$ ).

Virus isolates tested from one SP and one LTNP monkey did not change during the first 3 months. In the SP group, the 3 month isolate from the IV-infected monkey (C24) showed neutralization escape, whereas the IR-infected SP monkey did not show escape until late in infection. All late isolates, except one obtained from an LTNP monkey, were highly resistant to neutralization. Comparison of viruses isolated

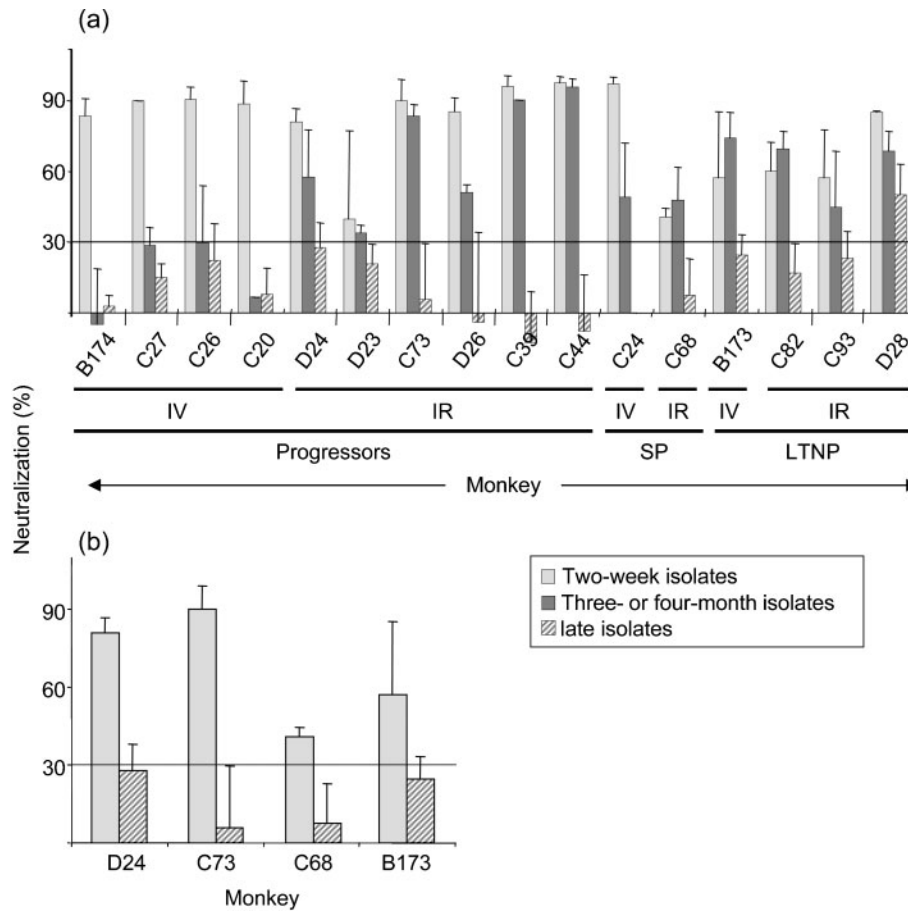
**Table 2.** Heterologous neutralization compared with autologous neutralization in four P monkeys

Autologous neutralization is indicated in bold. <, Values below the cut-off point of 30 %; ND, not determined.

Monkey	Serum Months p.i.	Neutralization (%) of virus isolates from:											
		Monkey B174 (IV)			Monkey D23 (IR)			Monkey C39 (IR)			Monkey C44 (IR)		
		0.5 months	3 months	15 months	0.5 months	3 months	12 months	0.5 months	4 months	18 months	0.5 months	3 months	27 months
B174	0.5	<b>67</b>	<	<	ND	<	<	ND	74	<	ND	<	<
	3	<b>88</b>	<	<	99	<	<	96	96	<	90	84	54
	15	<b>96</b>	<b>82</b>	<	94	93	46	99	95	<	91	88	83
D23	0.5	<	<	<	<b>33</b>	<	<	77	63	<	82	87	<
	3	98	<	<	<b>94</b>	<b>44</b>	<	100	85	<	96	96	78
	12	ND	83	<	<b>96</b>	<b>95</b>	<b>51</b>	ND	91	<	ND	99	75
C39	0.5	38	<	<	40	<	<	<b>56</b>	<b>79</b>	<	80	87	<
	4	ND	<	<	ND	<	<	<b>61</b>	<b>82</b>	<	ND	90	<
	18	79	<	<	58	<	<	<b>87</b>	<b>81</b>	<b>55</b>	85	83	51
C44	0.5	<	<	<	<	<	<	71	66	<	<b>66</b>	<b>59</b>	<
	3	79	38	<	49	<	<	90	87	<	<b>97</b>	<b>88</b>	<b>31</b>
	27	89	31	<	50	<	<	96	91	<	<b>99</b>	<b>98</b>	<b>35</b>
Serum H55:16		<b>84</b>	<	<	<b>40</b>	<b>34</b>	<	<b>96</b>	<b>90</b>	<	<b>98</b>	<b>96</b>	<
Serum pool*		95	89	50	95	93	37	99	ND	43	92	91	80

\*Pooled late serum from LTNP monkeys D28, C93, B173 and C82.





**Fig. 4.** Neutralization of isolates from hPBMCs (a) and mPBMCs (b) with a known high-titre neutralizing serum from an LTNP monkey (H55 : 16). Late time-point isolates indicate samples obtained at the end of the study (see Table 1). Values are means ( $\pm$ SD) of two independent assays performed in triplicate.

on mPBMCs with viruses isolated on hPBMCs showed the same pattern of evolution. Early isolates from monkeys D24, C68 and B173 were all sensitive to neutralization, but this sensitivity decreased over time.

To analyse the evolution of neutralization resistance further, we used a pool of late sera from the four LTNP monkeys (B173, C82, C93 and D28; Table 2) and tested virus isolates from four P monkeys (B174, D23, C39 and C44). The virus isolates were chosen to represent two monkeys with neutralization escape at 3 months and two monkeys with no escape at this time point. Interestingly, the serum pool could neutralize the 3 or 4 month isolates from all four monkeys with neutralization close to or greater than 90%. Whilst the single high-titre neutralizing serum H55 : 16 could not neutralize the late virus isolates from these four monkeys, the serum pool neutralized well above the cut-off point (50, 37, 43 and 80 %, respectively). These results indicated that a serum pool, presumably containing a spectrum of neutralizing antibodies, is more potently neutralizing than one serum from a single animal. Neutralization resistance can thus be overcome by a reagent with broad neutralizing capacity.

## DISCUSSION

In the present work, we examined the kinetics of the neutralizing-antibody response in relation to the route of infection, IV versus IR infection of cynomolgus macaques with SIVsm. In this model system, neutralizing activity of serum could be detected early after infection, in some animals by 2 weeks p.i. Unexpectedly, neutralizing activity against the inoculum virus and, to a smaller extent, to the autologous 2 week isolate, appeared significantly earlier in monkeys infected by the IV route than in monkeys infected by the IR route. This was in spite of a  $10^3$ -fold lower inoculum dose for the IV than for the IR route (equal to 10 MID<sub>50</sub> for both routes). Our results were in line with the observations of Rybarczyk *et al.* (2004), who found that neutralizing antibodies against the challenge virus SIVsmE660 could be detected at 7 weeks after infection in IV-challenged macaques, whereas at this time the IR-challenged macaques did not have detectable neutralizing antibodies (Rybarczyk *et al.*, 2004). It has also been found that, in HIV-1 infection, antibody responses to HIV-1 antigens are higher in HIV-1-positive injecting drug users

than in HIV-1-positive homosexual men (Jiang & Bekesi, 2001). It is tempting to speculate that these results reflect differences in antigen presentation. IV-transmitted virus may infect target cells directly and thereby produce large amounts of viral antigen within a short time, whilst the IR-transmitted virus may encounter antigen-presenting cells before infection of virus-permissive cells. Alternatively, the capacity or response of B cells or T-helper cells may differ according to the compartment of virus entry. With regard to CD4<sup>+</sup> T cells, both activated and a surprisingly large number of resting cells are infected productively shortly after IV or mucosal transmission of SIV (Hirsch *et al.*, 1998; Li *et al.*, 2005; Mattapallil *et al.*, 2005; Stahl-Hennig *et al.*, 1999; Veazey *et al.*, 1998; Zhang *et al.*, 1999). According to these studies, the gastrointestinal tract appears to be the major site of CD4<sup>+</sup> T-cell depletion and virus replication after both intravaginal and IV infection with SIV. After mucosal transmission, SIV DNA and RNA are also detected in macrophages and dendritic cells (Hu *et al.*, 2000; Spira *et al.*, 1996). In our study, massive early infection of target cells by SIVsm in all animals tested was reflected by plasma viral load detectable by 2 weeks p.i.

Our results showed that neutralization-resistant SIVsm variants emerged in all monkeys. However, there was a pathogenesis-related difference in the timing of appearance of variants resistant to neutralization by autologous serum. In P monkeys, neutralization-resistant variants appeared earlier than in SP or LTNP monkeys. The difference between the groups was evident at 3 months after infection and was statistically significant. This is in line with our previous observation on a small group of SIVsm-infected monkeys where lack of neutralization was associated with fast disease progression (Zhang *et al.*, 1993). In that study, the emergence of neutralization-resistant variants was also demonstrated. Similar results were obtained by Burns *et al.* (1993) when testing chimeric viruses expressing envelope proteins from sequentially collected SIVmac variants on an SIVmac239 backbone. Rybarczyk *et al.* (2004) showed that the timing of detectable neutralizing antibodies in macaques correlated with the timing of V1/V2 diversification, used by the authors as a marker for measuring the complexity of viral populations. It is known that the initial complexity of the viral population detected in plasma is higher after IV transmission than after transmission through a mucosal barrier (Greenier *et al.*, 2001; Sodora *et al.*, 1998). In the system explored by Rybarczyk *et al.* (2004), macaques challenged by the IV route displayed subsequent V1/V2 diversification from homogeneous V1/V2 variants significantly earlier than macaques challenged by the IR route. This led to the suggestion that the route of virus entry affects the diversification and heterogeneity of the SIV envelope and that this is correlated with a type-specific antibody response, with the potential to select neutralization-resistant variants. Interestingly, it also appears that, in HIV-1 subtype A and C infections, viruses with fewer N-linked glycans and condensed V1/V2 loop sequences are selected during

heterosexual transmission (Chohan *et al.*, 2005; Derdeyn *et al.*, 2004).

Evolution of neutralization resistance in our isolates was also demonstrated by a positive-control serum in the heterologous reaction. In this heterologous system, we also found differences between monkeys in the two transmission groups, in that escape from neutralization of 2 week and 3 month isolates was significantly greater for IV- than for IR-infected P monkeys. However, the virus isolates did not escape from neutralization with pooled sera from four LTNP monkeys, indicating that a set of different antibodies can be protective for infection. Further analysis of the heterologous reaction revealed that monkeys B174 and D23 had broadly cross-neutralizing antibodies, even though the monkeys harboured neutralization-resistant viruses. Conversely, monkeys C39 and C44 did not seem to elicit any broadly neutralizing antibodies and virus isolates from these monkeys were sensitive to neutralization. This inverse relationship between detectable neutralizing activity in the sera and neutralization sensitivity of isolated viruses may be explained by binding of antibodies by the neutralization-sensitive viruses, thereby depleting sera of antibodies. Viruses that show evolution to neutralization resistance would no longer consume the neutralizing antibodies and the antibodies would remain detectable in the heterologous reaction.

Our results suggest an important role for neutralizing antibodies in controlling viraemia. A decrease of over 4 log<sub>10</sub> at 3 months from the initial peak of viraemia could be detected in two monkeys whose virus remained sensitive to neutralization by autologous sera at this time. However, control was transient and, in P monkeys, was soon overridden by the emergence of neutralization-resistant variants, followed by an increase in viral load. LTNP monkeys could control viraemia better and the low levels detected at 3 months were maintained up to 1 year. In line with this, neutralization-resistant variant viruses were only detected in LTNP monkeys late in infection (35 months or later). In other studies, the importance of neutralizing antibodies was shown by passive immunization with neutralizing sera that prevented HIV-2 and SIVsm infection of cynomolgus macaques (Putkonen *et al.*, 1991). Similarly, in subsequent studies, administration of neutralizing antibodies before virus challenge with chimeric SIV and HIV viruses has shown protection from disease and even protection from virus infection at high titres (Baba *et al.*, 2000; Mascola *et al.*, 1999; Nishimura *et al.*, 2002; Veazey *et al.*, 2003). The importance of the humoral immune response has also been investigated by depleting B cells from macaques before exposure to SIV (Johnson *et al.*, 2003; Schmitz *et al.*, 2003). Johnson *et al.* (2003) found that depletion of B cells around the time of infection resulted in less immunological control of infection and much higher viral loads at set points after infection than in controls, suggesting a role for neutralizing antibodies early in infection. On the other hand, Schmitz *et al.* (2003) concluded that neutralization plays a limited

role during acute infection, although the humoral immune response may contribute towards control of SIV replication in the post-acute phases of infection.

Many groups have emphasized the importance of the other arm of the adaptive immune response, the cellular response, governed by CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs). The appearance of CTLs in HIV and SIV infections correlates with the decline and control of viral load from peak levels (Jin *et al.*, 1999; Koup *et al.*, 1994; Schmitz *et al.*, 1999). As in the humoral immune response, where viruses resistant to neutralization by autologous sera emerge over the entire course of infection, HIV- and SIV-specific CTL responses select for viral escape variants during chronic infection (Allen *et al.*, 2002; Goulder *et al.*, 1997; O'Connor *et al.*, 2002; Price *et al.*, 1997; Van Baalen *et al.*, 1998). It therefore seems that both arms of the adaptive immune response play important roles in HIV and SIV pathogenesis, although the true correlates of protective immunity need to be defined further. A vaccine against HIV infection will probably have to induce both humoral and cellular immunity (Letvin & Walker, 2003; Moore & Burton, 2004).

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