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The rate of progression to AIDS is independent of virus dose in simian immunodeficiency virus-infected macaques

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Of the viral factors that are proposed to influence the rate of progression to AIDS, the role of infectious dose remains unresolved. Intravenous infection of outbred *Macaca mulatta* with various doses of simian immunodeficiency virus isolate 8980 (SIV_{8980}) revealed an endpoint from which an infectious dose 50 (ID_{50}) was defined. In the six infected animals, the time to develop AIDS was variable with a spectrum of rapid, intermediate and slow progressors. High and sustained plasma viraemia with marked loss of $CD4^+$ T-cells was a distinguishing feature between rapid versus intermediate and slow progressors. Animals that received the highest doses did not develop the highest sustained viral loads, nor did they progress more rapidly to disease. Similarly, animals infected with lower doses did not uniformly develop lower viral loads or progress more slowly to AIDS. Furthermore, compiled data from more than 21 animals infected with different doses of the same virus administered by the same route failed to reveal any correlation of infectious dose with survival. Indeed, host factors of these outbred animals, rather than dose of the initial inoculum, were probably an important factor influencing the rate of disease progression in each individual animal. Comparison of animals infected with SIV_{B670}, from which SIV_{8980} was derived, revealed marked differences in disease progression. Clearly, although dose did not influence viral loads nor disease progression, the virulence of the initial inoculum was a major determinant of the rate of progression to AIDS.

Introduction

The observation that simian immunodeficiency virus (SIV_{sm}), when transmitted to Asian macaques from sooty mangebeys, caused AIDS similar to that seen in human immunodeficiency virus type 1 (HIV-1)-infected humans has resulted in the development of an important primate model for the understanding of AIDS pathogenesis (Baskin *et al.*, 1988; Heeney, 1996; Letvin *et al.*, 1985; Whetter *et al.*, 1999). The evolution of HIV-1 infection and the development of AIDS varies considerably, with some individuals progressing to AIDS within 3 years of infection while others remain clinically asymptomatic without evidence of $CD4^+$ T-cell decline for more than 18 years (Buchbinder *et al.*, 1994; Keet *et al.*, 1994; Levy, 1993; Munoz *et al.*, 1995; Phair *et al.*, 1992). Studies of rapid progressors and long-term non-progressors have revealed that host immunogenetic (Cameron *et al.*, 1990; Kaslow *et al.*, 1996) and immunologic responses (Buchbinder *et al.*, 1994; Cao *et al.*, 1995; Chaisson *et al.*, 1995; Laurence, 1990; Lifson *et al.*, 1991; Montefiori *et al.*, 1996; Fauci, 1996; Kaur *et al.*, 1998; McCarthy, 1992; O'Brien *et al.*, 1996), as well as certain virological factors (Dykhuizen *et al.*, 1998; Deacon *et al.*, 1995; Marthas *et al.*, 1995) may delay the onset of SIV- or HIV-induced disease (Fultz, 1993; Geretti *et al.*, 1998; Rausch *et al.*, 1999; Zink *et al.*, 1998).

Viral factors such as infectious dose, route of infection, repeated exposure and viral virulence have been proposed to influence the rate of progression to AIDS (Asjo *et al.*, 1986; Coffin, 1986; Kestler *et al.*, 1991; Marthas *et al.*, 1993, 1995; Nielsen *et al.*, 1993; Phair *et al.*, 1992; Tersmette *et al.*, 1989) To date, only the issue of viral virulence has been addressed in depth, but only with regard to attenuation of viral virulence. The Nef protein of SIV and HIV is one of the best defined viral factors and is widely considered to be a critical factor for the pathogenesis of AIDS in both humans (HIV-

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1/HIV-2) and rhesus macaques ($SIV_{sm/mac}$) (Kestler *et al.*, 1991; Kirchhoff *et al.*, 1999; Whetter *et al.*, 1999). The molecular determinants of virulence appear to be encoded by several viral genes in different regions of the genome and in general appear to evolve in an interrelated fashion (Edmonson *et al.*, 1998; Marthas *et al.*, 1993). In a series of SIV studies using single Nef and multiple deletion mutants, Baba *et al.* (1995) showed that AIDS could develop in neonatal animals when given high doses of the virus. Subsequently, the viral threshold hypothesis was proposed to explain in part the pathogenic potential of attenuated viruses if high enough levels of viraemia were achieved (Ruprecht *et al.*, 1998). More recently, a pathogenic threshold of plasma viral load has been defined which not only distinguishes between pathogenic and non-pathogenic infections, but also predicts the rate of disease progression (Ten Haaft *et al.*, 1998). Based on these as well as observations from other infectious disease models, we set out to determine the influence of the dose of the inoculum on the initial viral load, the threshold achieved, and thus the influence on disease progression. To address this question, different dilutions of the SIV_{8980} isolate were administered intravenously to ten mature rhesus monkeys. Animals were monitored for evidence of infection, plasma viral load, $CD4^+$ T-cell decline and the rate of progression to AIDS. These results were compared to data compiled from other animals infected previously with different doses of the same virus stock.

Methods

■ **Animals, clinical and pathological observations.** Two separate studies were performed with mature, outbred Indian rhesus monkeys (*Macaca mulatta*), free of SIV, STLV and type D retroviruses, from captive purpose-bred colonies. The MHC type of these animals was determined as previously described (Baskin *et al.*, 1997). In the first study ten animals, two animals per group, received an intravenous administration of serial tenfold dilutions of 2 ml of SIV_{8980} ranging from 1×10^{-7} to 1×10^{-3} of the original virus stock (Holterman *et al.*, 1999). Following inoculation, animals were monitored daily for clinical evidence of disease. At 2 week intervals for the first 2 months and at monthly intervals thereafter, animals were lightly anaesthetized for physical examination and blood sampling. Haematological analysis included measurements of $CD4^+$ and $CD4^+/CD29^+$ T-cell subsets, in addition to complete blood cell counts including differential leukocyte analysis. Serum and plasma samples as well as peripheral blood mononuclear cells (PBMC) were stored for retrospective virological analysis. Upon clinical and haematological evidence of AIDS animals were euthanized to avoid unnecessary suffering. A full autopsy was performed at the time of euthanasia. Necropsy and histopathological findings were compiled to confirm the diagnosis of AIDS in all cases.

The second study consisted of retrospective data for rhesus monkeys which had been infected with the exact same stock virus diluted with the exact same procedure as before and administered by the same route but given different doses. The clinical and pathological follow-up of each animal was performed using the same standardized criteria. AIDS-defining criteria in macaques included one or more of the following; 10% weight loss, intractable diarrhoea, neurological abnormalities, oral lesions (i.e. thrush or herpes-like lesions); together with evidence of persistently low CD4 T-cell counts, and/or thrombocytopenia, anaemia, neutrophilia, low albumin with evidence of increased plasma RNA loads ($> 1 \times 10^5$ copies/ml).

■ **Virus stock and virological analysis.** All animals were infected with the same SIV_{8980} stock ($TCID_{50}$ $1 \times 10^{-4.8}$/ml) for comparative purposes. The $TCID_{50}$ was determined by the Kärber formula as previously described (Bogers *et al.*, 1997). The stock was derived from animal 8980 (P4) following four *in vivo* passages of SIV_{B670} in juvenile rhesus monkeys born and purpose-bred from the BPRC's breeding colony of Indian rhesus monkeys (Holterman *et al.*, 1999). The SIV_{8980} stock was propagated on autologous PBMCs. Within 10 days of autologous 8980 PBMC cultivation the supernatant was harvested, clarified to remove cellular debris, aliquotted and preserved at −135 °C. The *in vivo* macaque infectious dose 50 (MID_{50}) was determined by intravenous administration of serial fold dilutions of the SIV_{8980} virus stock. The endpoint was based on the following virological criteria. The MID_{50} value was estimated based on the dose of the viral stock which infected approximately half of the animals to which it was administered (Bogers *et al.*, 1997). Virological analysis of inoculated animals included plasma antigen (p27 pg/ml), ELISA for anti-SIV antibodies, virus isolation, DNA-PCR on PBMC and quantitative RT–PCR on plasma. Plasma antigen and anti-SIV ELISA (SIV antigen kindly provided by MRC/programme EVA reagent repository, Potters Bar, UK) were measured as described previously (Bogers *et al.*, 1998). The DNA-PCR assay was performed on genomic DNA isolated from PBMC from inoculated animals. Genomic DNA was isolated from separated and washed PBMC by proteinase K/Triton X-100-based lysis followed by ethanol precipitation. Nested PCR was performed on each sample using SIV-gag primers as follows:

SIV-gag 5′ (outer) TTGAAGCATGTAGTATGGGCAGC (1139–1161 nt)

SIV-gag 3′ (outer) TGCCACCTACTTGCTGCACTGGG (1453–1475 nt)

SIV-gag 5′ (inner) TGGATTAGCAGAAAGCCTGTTGG (1180–1202 nt)

SIV-gag 3′ (inner) CCTCCTCTCGACACTAGGTGGTGC (1424–1446 nt).

Outer reaction PCR mixes contained 1 µg genomic DNA, 20 mM Tris–HCl (pH 8·3), 50 mM KCl, 0·01% gelatine, 2·5 mM $MgCl_2$, 200 µM each dNTP, 250 nM each primer and 2 U *Taq* DNA polymerase (AmpliTaq; PE Biosystems) in a total volume of 50 µl. Cycling conditions for outer primers consisted of an initial denaturation step (95 °C, 3 min), followed by five cycles (95 °C, 30 s; 50 °C, 30 s; 70 °C, 30 s), thirty cycles (95 °C, 30 s; 55 °C, 30 s, 72 °C, 30 s), and finally by one cycle (72 °C, 7 min; 4 °C, 7 min; 20 °C, 1 s). From the outer reaction mix, 5 µl of product was transferred to an inner reaction mix containing 20 mM Tris–HCl (pH 8·3), 50 mM KCl, 0·01% gelatine, 2·5 mM $MgCl_2$, 200 µM each dNTP, 250 nM each primer and 2 U *Taq* DNA polymerase in a total volume of 50 µl. Cycling conditions for inner primers were identical to the first PCR except that twenty cycles (95 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s) were carried out. PCR products were analysed by agarose gel electrophoresis.

■ **Quantification of plasma viral load.** A quantitative competitive RNA-PCR was used to estimate the virus load in plasma (Ten Haaft *et al.*, 1998). In brief, RNA was extracted from 200 µl of serum or EDTA plasma using guanidine isothiocyanate-mediated lysis, followed by propan-2-ol precipitation of the RNA. A known amount of synthetic internal standard RNA was added prior to RNA purification and was co-purified to monitor the efficiency of the purification. The RNA was reverse transcribed and amplified in a single reaction protocol using r*Tth* DNA

Table 1. Virological readouts from *M. mulatta* inoculated with tenfold dilutions of SIV_{8980}

Animal	SIV_{8980} dilution*	Plasma Ag (pg/ml)†			Virus isolation‡		Anti-SIV Ab§		Proviral PCR‖
		0	2	4	2	4	2	4	
2999	10^{-3}	−	+	ND	+	ND	ND	ND	+
3175	10^{-3}	−	+	−	+	ND	+	+	+
4103	10^{-4}	−	+	−	+	+	+	+	+
CK	10^{-4}	−	+	−	+	+	+	+	+
8877	10^{-5}	−	+	+	+	+	+	+	+
8941	10^{-5}	−	−	−	−	−	−	−	−
1ZI	10^{-6}	−	+	+	+	+	−	−	+
1YX	10^{-6}	−	−	−	−	−	−	−	−
8737	10^{-7}	−	−	−	−	−	−	−	−
2704	10^{-7}	−	−	−	−	−	−	−	−

* Diluted virus stock.
† Plasma antigen at 0/2/4 weeks.
‡ Virus isolation from PBMC at 2/4 weeks.
§ Anti-SIV env at 2/4 weeks.
‖ *pol* PCR from PBMC.
ND, Not determined.

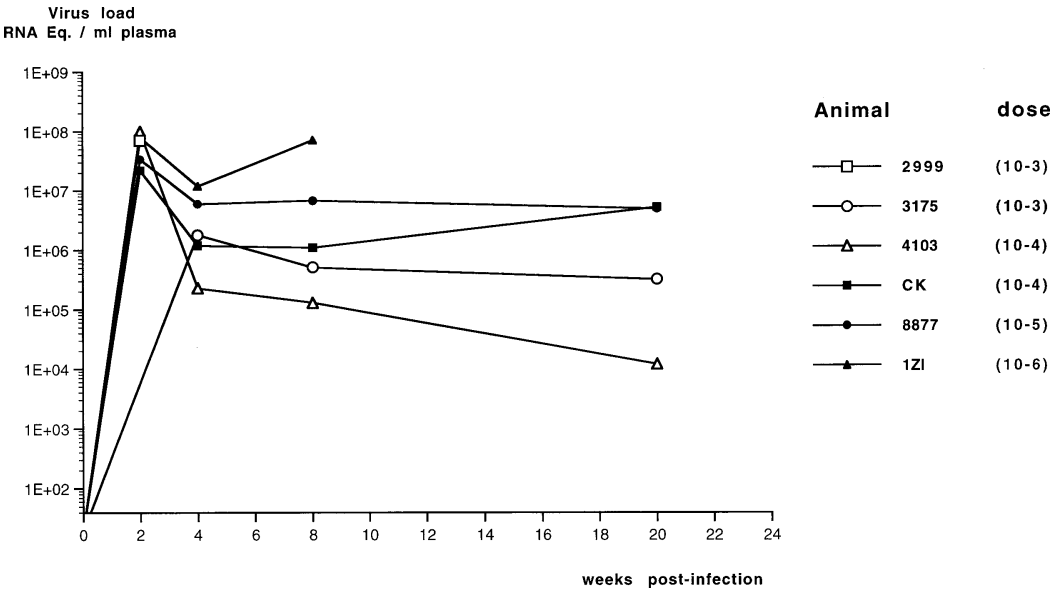

Fig. 1. Plasma viral RNA levels in rhesus monkey after intravenous inoculation with different doses of SIV_{8980} (Table 1). Plasma virus RNA concentrations are presented as RNA equivalents per ml of plasma (RNA Eq./ml plasma).

polymerase (PE Biosystems) and biotinylated primers. The internal standard RNA was co-amplified to monitor the amplification efficiency. The amplified fragments were detected by a capture probe that was covalently bound to Nucleolink microwells (NUNC). The amplification products were detected by a streptavidin–horseradish peroxidase-mediated colorimetric reaction. The amplified internal standard was hybridized to a different capture probe in separate microwells. The number of RNA copies in the sample was calculated from the absorbance of the sample wells compared to that of the corresponding internal standard well.

Results

Each pair of animals receiving each virus dilution became infected (Table 1) until the doses of 1×10^{-5} and 1×10^{-6} were reached at which only one of each pair of animals became infected. This indicated that the ID_{50} was between these two dilutions at approximately $3{\cdot}2 \times 10^{-6}$. Thus, a clear dose-effect on the establishment of infection was achieved. To determine if animals that received higher doses also developed higher

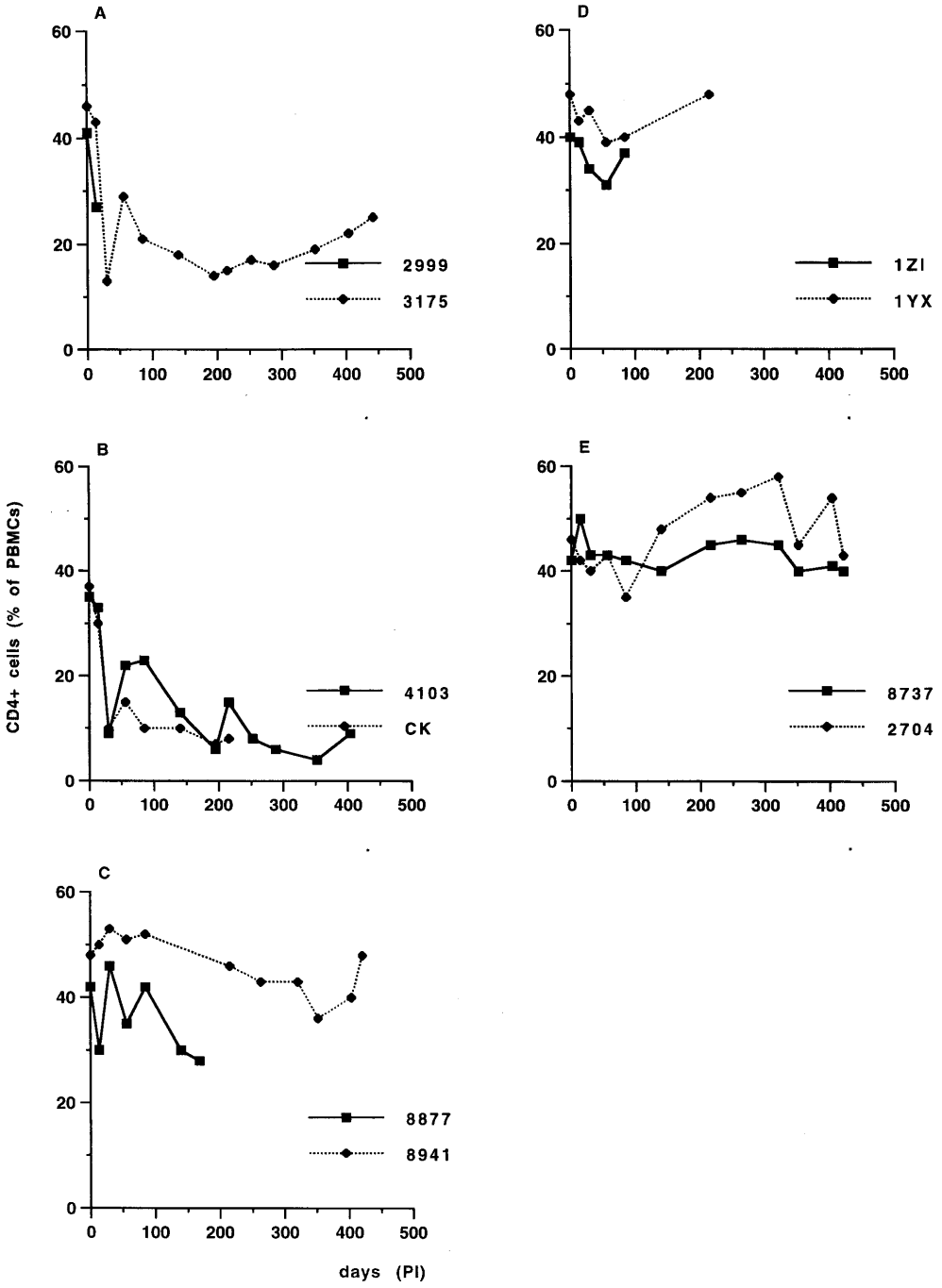

Fig. 2. Percentage of $CD4^+$ T-cells in circulation in mature rhesus macaques following intravenous inoculation of SIV_{8980} at different dilutions: (A) 1×10^{-3}, (B) 1×10^{-4}, (C) 1×10^{-5}, (D) 1×10^{-6}, (E) 1×10^{-7}. Animals 8941 (C), 1YX (D) and 8737 and 2704 (E) remained free of infection and maintained $CD4^+$ T-cell numbers within the normal range.

virus loads in plasma, either at the peak or set-point of infection, we quantified the plasma virus RNA concentration on sequential samples from each of these infected monkeys. The kinetics of the plasma virus RNA load of each of the infected animals listed in Table 1 are plotted in Fig. 1.

Two weeks post-infection there was no difference in the peak of primary plasma (viraemia) viral RNA in animals receiving different doses of virus (Fig. 1). The so-called steady state or set-point established after the initial peak of viraemia has been correlated with the rate of progression to disease in HIV-1-infected patients (Mellors *et al.*, 1996) and SIV- or SHIV-infected rhesus monkeys (Ten Haaft *et al.*, 1998; Smith *et al.*, 1999). Comparison of the set-point viral load levels established after the initial peak did not reveal any correlation

Table 2. Compiled results of survival of rhesus macaques inoculated with different doses of SIV_{8980}

VI, virus isolation performed with a minimum of 4×10^6 PBMC; Ab, antibody titre; Ag, plasma antigen; +, positive response above established cut-off; 2× background at a minimum of 0·1. Based on samples taken between 2 to 12 weeks post-inoculation.

SIV dose (MID_{50})	Survival (months)	DNA-PCR	Results Ab	Ag	VI	Clinical outcome	Macaque
100	18·10	+	−	+	+	AIDS	9001b
100	16·30	+	−	+	+	AIDS	8984b
100	15·00	+	−	+	+	AIDS	8928b
100	10·50	+	+	+	+	AIDS	8771
100	7·00	+	+	+	+	AIDS	8606
100	5·30	+	+	+	+	AIDS	1XI
100	9·00	+	+	+	+	AIDS	1VS
50	0·50	+	−	+	+	AIDS	2999
50	14·50 (euth.)	+	+	+	+	Asymptomatic	3175
5	14·50	+	+	+	+	AIDS	4103
5		−	−	−	−	Not infected	HQ
5	7·20	+	+	+	+	AIDS	CK
5	4·80	+	+	+	+	AIDS	3991
1·6		−	−	−	−	Not infected	4091
1·6		−	−	−	−	Not infected	9001a
1·6		−	−	−	−	Not infected	8986a
1·6		−	−	−	−	Not infected	8984a
1·6		−	−	−	−	Not infected	8977a
1·6	7·40	+	+	+	+	AIDS	8967
1·6		−	−	−	−	Not infected	8928a
1·6	11·00 (euth.)	+	+	+	+	Asymptomatic	8754
1·6	30·75	+	+	+	+	AIDS	8604
1·6	3·50	+	−	+	+	AIDS	1RO
1·6	9·00	+	+	+	+	AIDS	L65
1·6	6·90	+	−	+	+	AIDS	CS
0·5		−	−	−	−	Not infected	8941
0·5	5·50	+	+	+	+	AIDS	8877
0·5		−	−	−	−	Not infected	3215
0·5	9·60	+	+	+	+	AIDS	3049
0·5		−	−	−	−	Not infected	CY
0·5		−	−	−	−	Not infected	CF
0·16		−	−	−	−	Not infected	3532
0·16		−	−	−	−	Not infected	3598
0·05	3·50	+	−	+	+	AIDS	1ZI
0·05		−	−	−	−	Not infected	1YX
0·05		−	−	−	−	Not infected	HT18
0·05		−	−	−	−	Not infected	3974
0·005		−	−	−	−	Not infected	8737
0·005		−	−	−	−	Not infected	2704

with dose. For instance animal 1ZI, which received the lowest dose of virus, had the highest viral load, far above the pathogenic threshold as defined by Ten Haaft *et al.* (1998). Similarly animal 3175, which received the highest dose of virus, had one of the lowest set-point values (Fig. 1). There was also no correlation between dose, the 2 week viral peak or the set-point of the plasma viral load. Furthermore, neither the $CD4^+$ T-cell (Fig. 2), nor the $CD4^+/CD29^+$ T-cell subset (data not shown) declined inversely with the dose of virus.

To ensure that a possible correlation was not overlooked because of the relatively small number of animals studied in this titration study, we retrospectively compiled data from 39 mature rhesus macaques exposed to intravenous doses of SIV_{8980} ranging from 5×10^{-3} to 100 ID_{50}. Of these 39 animals, 21 became infected and the survival time until onset of AIDS, as confirmed by necropsy, was documented (Table 2). The dose and the survival period post-infection clearly did not correlate (correlation coefficient $R = 0{\cdot}025$) in rhesus monkeys which became infected with this pathogenic strain of SIV. Furthermore, with this virus strain there was no apparent role of host MHC on the rate of disease progression as evidenced by a lack of correlation of the MHC serotype (data not shown) in this group of animals.

Discussion

A number of host as well as virus factors interact to influence the rate of progression to AIDS. The effect of the virus dose on the rate of progression to AIDS has been suggested but not proven. The effect of virus dose on disease development in animal models has been reported for cytomegalovirus (Jordan, 1978), adenovirus (Ginsberg *et al.*, 1991), rabies virus (Niezgoda *et al.*, 1997), hepatitis B virus (Jilbert *et al.*, 1998) and herpes simplex virus type 2 (Fowler *et al.*, 1992). To study the effect of HIV-1 dose on disease in humans has proven difficult, even in documented cases of contaminated blood transfusions in cohorts of haemophiliacs. To address the hypothesis that higher doses of HIV-1 may accelerate progression to AIDS we turned to the SIV_{sm} model of AIDS in rhesus macaques. This model mimics the pathogenesis of HIV-1-induced AIDS in almost all respects (Whetter *et al.*, 1999). Two studies were undertaken using this model. Firstly, in a prospective study ten mature rhesus monkeys were inoculated with serial tenfold dilutions of the pathogenic SIV_{8980} isolate. There was a clear correlation with the dose that the animal received and infection (Table 1). However, there was no correlation between the infectious dose and the 2 week peak of primary viraemia or the subsequent set-point (steady-state) plasma viral RNA loads established following seroconversion (Fig. 1). Furthermore, there was no inverse correlation between the decline of $CD4^+$ T-cells (Fig. 2) and the SIV dose the animal received. Lastly, a retrospective analysis of survival data (Table 2) of 39 animals exposed to different doses of SIV_{8980} administered intravenously failed to reveal any statistical correlation between the infectious dose administered and the time to the development of AIDS. Interestingly, there may be exceptions to this observation in situations of pre-existing immune-compromise. Neonates given high doses of virus orally may develop AIDS even if given infectious SIV_{mac} with attenuating mutations (Wyand *et al.*, 1997). Also, animals given low doses by other mucosal routes, such as vaginal

infection, may not develop a systemic primary viraemia and subsequently develop disease, but may acquire an inapparent infection and remain healthy (Miller *et al.*, 1998).

A number of host factors which may influence the rate of progression to AIDS have been documented, including age, concurrent disease and host immunogenetics. We have previously found a correlation between survival and the MHC type of the SIV-infected animals (Baskin *et al.*, 1997; Bontrop *et al.*, 1996). Similarly, several viral virulence factors have been reported to influence the rate of disease progression in man as well as in monkeys. Since the virus used in these studies was a highly virulent, late stage variant (Holterman *et al.*, 1999), it may be less likely to be controlled by host immune responses, in contrast to what we have observed with other strains (Baskin *et al.*, 1997; Bontrop *et al.*, 1996). Indeed, in this study no association between serologically defined Mamu-A, -B and -DR specificities, and susceptibility/resistance to SIV_{8980} was found.

Certain SIV_{mac} *nef* mutations may either cause an attenuated disease course and prolonged survival or cause acute haemorrhagic enteritis, but a viral virulence factor which is actually capable of accelerating the progression to AIDS has not yet been identified. In a previous study (Holterman *et al.*, 1999) the *in vivo* passage of SIV_{B670} led to an acute progression to AIDS following four *in vivo* passages in monkeys. Comparison of the Kaplan–Meier plots of the survival of animals infected with pre-passage stock versus the post-passage stock clearly revealed that the virus had acquired a significant increase in virulence capable of causing an accelerated disease course (Holterman *et al.*, 1999). Our data indicate that the dose of the inoculum is important for establishing SIV infection. However, once systemic primary plasma viraemia is established there is no influence of dose on the rate of disease progression. Once systemic infection has occurred, it is probably the replication rate together with other virulence properties of the dominant viral variant in the viral inoculum which influence the post-primary viraemia 'set-point' of viral load which is established (Kimata *et al.*, 1999; Ten Haaft *et al.*, 1998). It is not the dose of the inoculum. Together, the interaction of viral virulence factors with host responses appear to dictate the rate of progression to AIDS after systemic infection has been established.

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