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Determinants of disease in the simian immunodeficiency virus-infected rhesus macaque: characterizing animals with low antibody responses and rapid progression

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Clinical and laboratory markers of simian immunodeficiency virus (SIV) infection were studied during the first 3 months after intravenous inoculation of rhesus macaques. Virus-binding serum antibody titres were correlated strongly with disease progression ($P < 0{\cdot}005$) and were predictive of disease outcome by 7 weeks after inoculation. Low virus-binding serum antibody responses to SIV occurred in animals that also showed acute depletion of circulating CD20 B cells. Acute damage to the CD4 T cell and CD20 B cell populations rendered some animals incapable of mounting virus-specific antibody responses and these macaques became the rapidly progressing cases comprising approximately 20–30% of infected animal cohorts.

Introduction

Simian immunodeficiency virus (SIV) infection of rhesus macaques initiates a disease course similar to HIV-1 infection in human beings and is an important model for transmission, pathogenesis, vaccine and treatment studies (Chalifoux *et al.*, 1986; Chargelegue *et al.*, 1995). Rhesus macaques are readily infected with SIVmac by intravenous, intrarectal, vaginal and oral routes for inoculation (Kindt *et al.*, 1992; Letvin & King, 1990). Initial virus exposure leads to a recognizable, acute viraemia that resolves into a persistent infection phase typified by progressively declining $CD4^+$ T cells, increasing immunodeficiency, wasting disease and death. A consistent finding in several SIV infection studies was the existence of 'non-responding, rapid progressing' animals after intravenous inoculation (Letvin & King, 1990; Otsyula *et al.*, 1996; Zhang *et al.*, 1988). These animals are persistently infected with SIV, make a low or undetectable serum antibody response to virus (nonresponding), and succumb rapidly to AIDS sequelae (Daniel *et al.*, 1985; Fultz *et al.*, 1990; Kannagi *et al.*, 1986).

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Although nonresponding animals have been observed in most SIV infection studies, there have been few systematic efforts to define this group or to understand the reasons for low immune responses and rapid disease progression. We performed a detailed, statistical analysis of rapid progressing animals within a single cohort of SIV-infected macaques. This cohort was sufficient to acquire significant data on disease progression and all animals progressed to end-stage disease without unusual deaths. Our objectives were to define better the group of nonresponding, rapidly progressing animals and to correlate other markers of infection with the short survival period. These analyses provide a comprehensive and quantitative view of rapid disease progression after SIV infection, and show that an early effect on circulating B cells is an important factor in the lack of serum antibody responses and concomitant rapid disease progression.

Methods

■ **Animal infections and sampling intervals.** Eleven juvenile rhesus macaques were inoculated intravenously with 40 TCID of SIVmac. The virus stock (originally provided by R. C. Desrosiers, Harvard University and New England Regional Primate Research Center) was amplified by growth on rhesus PBMC with a final passage on CEMx174

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to increase the titre, as was reported previously (Pauza *et al.*, 1993; Trivedi *et al.*, 1994). Animals were confirmed positive for infection by two independent virus isolation assays and two tests for p27 antigenaemia.

Venous blood samples were collected at 1, 3, 5, 7 and 13 weeks, and then every 4 weeks until euthanasia. All samples were tested to measure p27 plasma antigenaemia, virus isolation efficiency (cells and plasma), antibody levels, and $CD4^+$, $CD8^+$ and $CD20^+$ lymphocyte subpopulations. For statistical evaluation, we concentrated on samples from 7 and 13 weeks after inoculation. Data from samples earlier than 7 weeks were not significantly correlated with disease progression rates. We did not pursue a detailed analysis of samples later than 13 weeks because this was past the point where the most rapid progressors were euthanized due to clinical deterioration.

■ **Virus isolation.** We employed virus isolation assays to measure: (i) the frequency of infectious cells using 10-fold dilutions (10^6–10^1 cells per well) of purified, phytohaemagglutinin-stimulated monkey PBMC and (ii) the concentration of infectious plasma virus using serial dilutions of plasma (200, 100, 50, 10, 2, or 0 μl per well). In both cases, 2×10^5 CEMx174 cells were added to each well and cultures were maintained for 28 days with weekly monitoring for cytopathic effects. p27 antigen levels in plasma or culture fluids were detected with a commercially available kit (Coulter/Immunotech).

■ **Markers for disease progression.** Animals were monitored for clinical status and laboratory measures of disease progression that could be correlated with length of survival (see Table 2). We used a commercially available ELISA kit to titre virus-binding antibodies (Genetic Systems HIV2 EIA from Sanofi/Pasteur). Neutralizing antibody titres were measured with a laboratory-adapted stock of SIVmac251 in a CEMx174 cell-killing assay (Montefiori *et al.*, 1996, 1988). Western blot assays were performed as described previously (Pauza *et al.*, 1993) except that: CEMx174 cells were co-cultured with PBMC from a SIV-positive animal (R95027) and these infected co-cultures provided the source for viral antigens. Samples of uninfected plasma or plasma from 13 weeks after infection were diluted 1:100 then overlaid on the filter membrane. Bound, rhesus IgG were detected with alkaline-phosphatase-conjugated, anti-human IgG (Sigma, St. Louis, MO) diluted 1:10000.

$CD4^+$, $CD8^+$ and $CD20^+$ lymphocyte subsets in PBMC were identified by flow cytometry. We stained 5×10^5 paraformaldehyde-fixed white blood cells with 5 μl of the appropriate monoclonal antibody (anti-CD4 or anti-CD8, both from Antigenix America, anti-CD20 from Becton-Dickinson, or the relevant isotype controls). Samples were analysed on a FACScan flow cytometer. Absolute counts were determined by multiplying the percentage for each subset (%CD4, %CD8 or %CD20) and the absolute (total) lymphocyte count obtained from clinical haematology data (Pauza *et al.*, 1997). Relative changes in lymphocyte subsets were calculated after dividing the absolute count at each time-point by the average of two pre-infection cell counts. CD4/CD8 ratios were also calculated for each sample.

Results and Discussion

By 1 year of infection, 8 of the 11 rhesus macaques had been sacrificed. Euthanasia was performed when an animal showed a rapidly deteriorating clinical condition (determined by a veterinarian), usually including rapid weight loss, poor fluid intake, loss of appetite and unresponsiveness to stimuli. Simian AIDS was confirmed by both the major pathological diagnoses and the lymphoid tissue status at necropsy (Table 1). Necropsy findings included diseases commonly associated with simian AIDS, such as giant-cell interstitial pneumonia (Letvin & King, 1990). Macaques euthanized because of presumed terminal disease progression showed advanced stages of lymphoid depletion (Letvin & King, 1990). Tissue pathology and clinical laboratory data confirmed that euthanasia was appropriate for these animals owing to advanced disease. Necropsy studies are essential for SIV/nonhuman primate studies where natural death is unacceptable and the time to death is influenced by decisions of the attending veterinarians.

Correlating laboratory test data with duration of survival

Laboratory test data were correlated with survival after infection to assess the predictive value for each parameter. We concentrated on samples from 7 and 13 weeks after infection; both time-points are subsequent to the acute infection interval and they coincide with early signs of clinical disease. For statistical analysis, animals alive at 1 year after infection were assigned an arbitrary life span of 52 weeks. Even though some animals survived longer than 1 year, this is a conservative approach to data analysis. Several tests correlated ($P < 0{\cdot}05$) with overall survival times (Table 2). For 7 week samples, only SIV-binding antibody titre and neutralizing antibody titre correlated with the time to death (survival) after infection. At 13 weeks, p27 antigenaemia, plasma virus titre, change in CD4 counts and change in CD20 counts all correlated with survival. Among all tests that were performed, disease outcome and antibody titre at 13 weeks were best correlated ($P < 0{\cdot}005$). The rank order of antibody titre at 13 weeks was identical to the rank order of survival times (Table 3).

The kinetics of antibody response were also related to eventual antibody titre and to survival (Fig. 1). In general, animals with an early positive serology maintained a high level of response and had slow disease progression. As the interval increased between infection and first positive serology, it was increasingly likely that the animals would mount a poor antibody response and tend to more rapid disease progression. Animals unable to mount a sustained, positive antibody response became the most rapid progressors.

Western blot data showed a similar hierarchy of responses (Table 4). Animals with shorter lifespans showed a less diverse antibody response. In particular, animals with the highest antibody titre and slowest disease progression produced antibodies to both Gag and Env proteins. Animals with faster disease progression, but still having a positive antibody response, responded to either Gag or Env proteins but not both. Animals with the fastest disease progression showed, at best, weak responses to only one SIV protein.

Table 1. Pathological findings at necropsy for SIV-infected rhesus macaques

Animal ID	Lymphoid organs*	Major diagnoses at necropsy
AS73	Depleted	Wasting, interstitial pneumonia
AS53	Depleted	Meningitis (cytomegalovirus), giant cell interstitial pneumonia
AS69	Involution	Peritonitis with multiple abscesses
AS75	Depleted	Wasting, giant cell interstitial pneumonia, colitis, gastritis
AR36	Involution	Wasting, anaemia, colitis, giant cell interstitial pneumonia
AS34	Depleted	Wasting, glomerulonephropathy
AS40	Involution	Severe oedema
AS44	Involution	Lymphosarcoma, giant cell interstitial pneumonia, peritonitis, cholangiohepatitis

* Depleted: depletion of follicles, paracortex, and periarteriolar lymphoid sheaths. Involution: follicular involution with normal or expanded paracortex and periarteriolar lymphoid sheaths.

Table 2. Correlating laboratory markers of infection and survival after SIV infection of rhesus macaques

Monitoring procedure	Time procedure performed (weeks)	Average value (range)	Correlation to lifespan*	Significance probability†
Antibody titre	7	1:100 (1:5–1:6400)	0·6908	$P < 0·05$
	13	1:400 (1:5–1:25 600)	0·7778	$P < 0·005$
Neutralizing antibody titre	7	1:500 (< 1:20–1:4193)	0·6036	$P < 0·05$
	13	1:2000 (1:30–1:18 113)	0·5571	
Antigen level (ng/ml)	7	6·5 (0·01–38·0)	−0·5661	
	13	8·1 (0·00–35·8)	−0·7057	$P < 0·05$
Virus isolation – plasma‡	7	4 µl (> 200–2 µl)	−0·4948	
	13	11 µl (> 200–2 µl)	−0·7348	$P < 0·05$
Virus isolation – PBMC‡	7	10^3 (10^1–10^5)	0·2173	
	13	10^3 (10^1–10^4)	0·3157	
Change in CD4 counts	7	132% (34–365%)	−0·5193	
	13	88% (117–204%)	−0·7548	$P < 0·01$
Change in CD8 counts	7	206% (43–405%)	−0·5177	
	13	138% (29–298%)	−0·5079	
Change in CD20 counts	7	74% (34–149%)	0·5167	
	13	73% (21–198%)	0·6394	$P < 0·05$
CD4/CD8 ratio	7	1·09 (0·45–2·55)	−0·5140	
	13	1·09 (0·53–2·71)	−0·5920	

* Pearson Product-Moment correlation coefficient (lifespan for alive animals set at 52 weeks).
† Only those which had significant probabilities are listed.
‡ Plasma and PBMC titres only available for 9 of 11 animals at 13 weeks.

Grouping animals based on the 13 week antibody titres

We divided animals into three groups based on virus-binding serum antibody titres: a low antibody titre group (at or below 1:100 titre), a moderate antibody titre group (1:400–1:1600) and a high antibody titre group (1:6400 and above) (Table 3). Differences in survival were tested for each group using Kaplan–Meier analysis (Fig. 2). Groupings based on 13 week antibody titre were highly predictive of disease pro-

Table 3. Comparing the rank order of virus-binding serum antibody responses and survival after SIV infection in rhesus macaques

Animal ID	Antibody titre: 7 weeks after infection	Antibody titre: 13 weeks after infection	Lifespan after infection (weeks)
AS73	1:25	1:5*	12
AS53	1:5	1:5*	12
AS69	1:100	1:5	18
AS75	1:400	1:100	22
AR36	1:100	1:400	25
AS34	1:100	1:400	28
AS40	1:400	1:1600	30
AS44	1:5	1:1600	33
AR37	1:6400	1:6400	Alive
AR34	1:400	1:25 600	Alive
AS30	1:6400	1:25 600	Alive

* Both animals died at 12 weeks after infection; antibody titre reported is for last available serum sample, taken at 12 weeks for AS73 and 10 weeks for AS53.

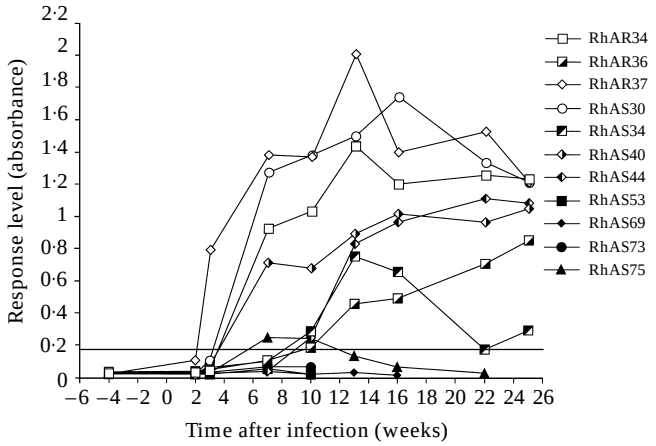

Fig. 1. SIV-binding antibody levels determined by ELISA using 1:400 serum dilutions. Serum samples were collected before infection and up to 25 weeks after SIVmac inoculation. Data for individual animals are plotted in terms of absorbance versus weeks after infection. Open symbols designate slow progressors (high antibody response), half-filled symbols indicate moderate progressors (moderate antibody response), and filled symbols represent fast progressors (antibody nonresponders).

Table 4. Summary of Western blot patterns showing for 11 rhesus macaques using serum samples from 13 weeks after SIV infection

Animal	p27	gp41	p55	gp120	gp160
AS73			+		
AS53					
AS69					
AS75					
AR36		+		+	
AS34	+				
AS40			+		
AS44		+		+	+
AR37	+	+	+	+	+
AS34	+	+	+	+	
AS30	+	+	+	+	+

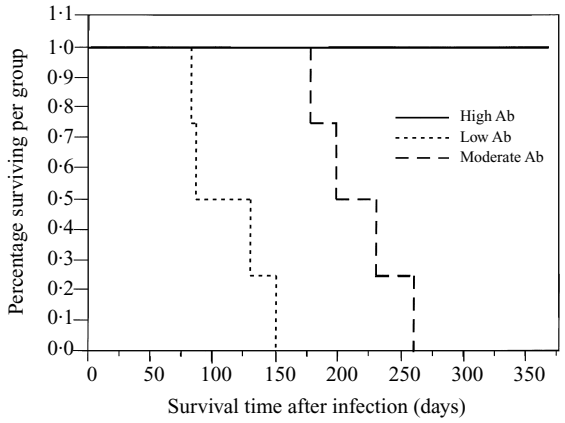

Fig. 2. Kaplan–Meier survival analysis for animals grouped according to antibody titres at 13 weeks after infection. Animals in the 'high Ab' group had titres < 1:6400 at 13 weeks after inoculation (solid line). Animals in the 'moderate Ab' group had titres in the range of 1:400 to 1:1600 at 13 weeks (dashed line). Animals in the 'low Ab' group all had 13 week antibody titres < 1:100 (dotted line).

gression, with a chi-square probability of $P = 0{\cdot}0014$. The low antibody titre group included the first animals to show disease, with euthanasia being required 3–5 months after infection. The moderate antibody titre group included animals that succumbed to disease between 6 and 9 months after infection. One year after infection, animals in the high antibody titre group remained alive.

Groupings based on antibody responses help to understand correlations for survival time and other laboratory measures of disease progression. We observed (Table 2) a negative correlation for peripheral blood virus antigen levels and survival, and a negative correlation for concentration of infectious virus in plasma and survival. Higher cell-free virus burdens occurred in low antibody responders than in moderate antibody responders, and cell-free virus burdens were higher in moderate antibody responders than in high antibody responders. Elevated levels of cell-free virus were associated with rapid disease progression. These findings are consistent with results from others (Kannagi *et al.*, 1986), and provide a quantitative view of disease progression after SIVmac infection.

Changes in the CD20 B cell population may account for the low antibody responses in rapid and moderate progressor animals. Average B cell counts for the entire cohort declined

Table 5. $CD20^+$ B cell and $CD4^+$ T cell counts at 7 and 13 weeks after infection in rapid, moderate and slow progressing animals

Values are the average absolute cell counts (cells/μl blood) for animals in each category with the range of values shown in parentheses. Rapid, moderate and slow progressors are defined in the text.

Sample	Entire cohort ($n = 11$)	Rapid progressors ($n = 3$)	Moderate progressors ($n = 5$)	Slow progressors ($n = 3$)
$CD20^+$ at preinfection	1842 (365–5435)	1835 (365–5435)	1956 (1562–2388)	1712 (516–2455)
$CD20^+$ at 7 weeks	1113 (145–2793)	834 (145–2739)	908 (734–1173)	1493 (767–2793)
$CD20^+$ at 13 weeks	1060 (196–3525)	921 (196–1645)	717 (504–915)	1844 (1023–3525)
$CD4^+$ at preinfection	1439 (548–2301)	1288 (548–2200)	1172 (789–2083)	1841 (1432–2301)
$CD4^+$ at 7 weeks	1660 (475–4442)	2112 (475–4422)	1412 (715–2918)	1114 (702–2399)
$CD4^+$ at 13 weeks	760 (239–2070)	1155 (239–2070)	1039 (475–1842)	517 (383–868)

slightly at 7 weeks after infection and remained low throughout 13 weeks of infection (Table 5). However, these changes were not representative of all animals in the cohort. Macaques shown to be slow progressors with high virus-binding antibody titres experienced a slight increase in CD20 cell counts by 13 weeks after infection despite a transient decline at 7 weeks. Substantial and sustained declines in CD20 cell counts were noted in both the moderate and fast progressor groups (Table 5). CD20 cell counts for these latter groups at 13 weeks after infection were approximately one-half of the preinfection values. These data demonstrate a direct link between bystander cell depletion during acute SIV infection, low virus-binding serum antibody responses and rapid disease progression. Declining B cell pools could be due to an overall loss of $CD4^+$ T cell help, or due to indirect (bystander) cell killing such as that described for HIV-1 infection (Finkel *et al*., 1995). In either case, B cell depletion would be expected to occur in proportion to the magnitude of virus replication and this is consistent with the observed high virus burdens in rapid progressing, antibody nonresponding animals.

CD4 counts declined with advancing disease as expected. However, CD4 levels were negatively correlated with survival at 13 weeks. This unusual finding was due to increased CD4 counts for rapid progressors during the terminal phase. Surprisingly, we were unable to show a clear relationship between blood $CD4^+$ T cell counts and disease progression; by 13 weeks, CD4 counts were actually lowest for animals with slow disease progression.

Neutralizing antibody titre was the only parameter that correlated significantly with disease progression at 7 weeks but was not significant at 13 weeks. It should be noted that at both 7 and 13 weeks, animals with most rapid disease progression had lowest neutralizing antibody titres (AS53, AS69 and AS73) while animals that were still alive had highest neutralizing antibody titres (AR34, AR37 and AS30). Animals surviving 5–9 months, or the moderate antibody titre group plus AS75, were the animals for which neutralizing antibody titres at 13 weeks were not predictive of survival times. A previous study noted low neutralizing antibody responses in rapid progressors at 88–104 days and high neutralizing antibody titres in animals with slower disease progression (Daniel *et al*., 1987). In the previous and present studies, it was noted that moderate progressors showed a poor correlation between survival times and neutralizing antibody responses. It remains confusing as to why binding antibody levels are tightly correlated to survival while discrepancies exist in the neutralizing antibody data. Levels of antibody by 13 weeks after inoculation, the point when the fastest progressing animals were beginning to succumb to simian AIDS, provided the strongest individual predictor of disease progression.

Laboratory tests predict survival after SIV infection

SIV-specific antibody titres at 13 weeks provide a quantitative and reliable method for grouping animals in any infection or treatment study. Individual groups within a study cohort can be normalized to include equal numbers of high, moderate and low virus-binding antibody responder animals. By incorporating these criteria when animal groups are assigned, the reliability of data is increased for disease progression or survival studies. Alternatively, 13 week antibody titres can be used to predict survival for individual animals and treatment effects can be assessed on the basis of

whether observed survival times deviate from expected survival times. Both approaches will improve experimental power for macaque infection studies and provide a statistically valid method that normalizes data among groups with high variation in disease progression rates.

A recent study (Watson *et al*., 1997) assessed plasma viral RNA, plasma viraemia, p27 antigenaemia and frequency of infected cells in PBMC as possible predictors of survival after infection. Plasma viral RNA levels were correlated with survival for samples at 6 weeks after infection but not earlier. Our data compare favourably in that survival times were predicted by serum antibody titres 7 weeks after infection. Results were similar for biological assays of plasma viraemia or frequency of infectious cells. Watson *et al*. did not observe a significant correlation for p27 antigenaemia and survival until 28 weeks after infection. In our animal group, plasma p27 levels were significantly correlated with survival by 13 weeks after infection. By determining the titre of SIV-specific serum antibodies at 7 weeks after infection, one obtains a valid prediction of outcome that has a greater statistical significance than was reported for 6 week plasma viral RNA measurements. However, the virus-binding antibody titres would not be valid as predictors for vaccination challenge experiments and viral RNA measurements (Watson *et al*., 1997) or 13 week p27 plasma antigenaemia tests would be more appropriate in these cases.

Implications for viral pathogenesis mechanism

The time of 6–7 weeks after intravenous inoculation may be considered the 'set point' (Mellors *et al*., 1996) for SIVmac infection in rhesus macaques. Two separate studies using SIVmac (this report) or SIVsmE660 (Watson *et al*., 1997) in *Macaca mulatta* reported laboratory markers of infection by around 6–7 weeks of infection that were highly correlated with survival. Disease progression rates and survival times were similar for these separate rhesus cohorts infected with distinct virus stocks. Survival times are comparable to a previous study of SIVmac infection (Daniel *et al*., 1987) and the frequency of antibody nonresponding animals is similar to other reports in the literature (Letvin & King, 1990). All of these studies are similar for using intravenous infection routes and SIV isolates. When we used intrarectal inoculation of SIVmac (Trivedi *et al*., 1996) or switched to a pathogenic SHIV isolate (Steger *et al*., 1998), disease progression rates were slowed or accelerated respectively. However, in all cases the relative distribution remained the same for rapid, moderate and slow progressing groups and we identified approximately the same frequency of antibody non-responding animals in each infection study. In general, infections with similar virus stocks using the same route of infection gave comparable outcomes. These data argue that virus type and route of inoculation have greater impact on disease progression than details of the preparation for each stock (Pauza *et al*., 1998). In our hands, *in vitro* passage on immortalized cell lines decreases the ratio of animal infectious doses to tissue culture doses and introduces larger numbers of defective or avirulent viral species but does not affect outcome as long as the inoculum contains at least the minimal animal infectious dose (Trivedi *et al*., 1996).

Evaluation of data from separate studies relating early markers of infection to survival strongly supports the view that the set point is controlled by virus replication dynamics and lymphocyte depletion during the interval of acute infection. Significant damage to the B-cell compartment is an important and unexpected consequence of events during acute infection. Virus-binding antibody levels reflect the residual immune response capacity that survives the interval of acute infection. This capacity is likely affected by the kinetics and magnitude of $CD4^+$ T cell and $CD20^+$ B cell depletion. We found no evidence for direct SIV infection of circulating B cells (not shown) and we conclude that loss of these cells occurs through an indirect mechanism, probably involving apoptotic death. The observation of acute B cell loss is similar to our findings from the model of SHIV infection of macaques (Steger *et al*., 1998). In the SHIV infection studies, B cell losses approached 90% of the pre-infection values and were greatest in animals that progressed most rapidly in disease. Similarities between the SIV and SHIV infection studies show that indirect mechanisms of cell depletion are prominent during acute infection and are likely to be important in AIDS pathogenesis. Some of the earliest studies on HIV infection documented the ability of virus antigens to stimulate polyclonal activation of peripheral blood B cells (Schnittman *et al*., 1986). More recently, bystander killing of B cells in HIV-positive lymph nodes was reported to proceed by an apoptotic mechanism (Carbonari *et al*., 1997). Our studies of acute SHIV infection in macaques showed apoptotic death of B cells in parallel with $CD4^+$ T cell depletion (M. Wallace & C. D. Pauza, unpublished). Accordingly, we believe that apoptotic death of B cells and possibly other lymphocyte subsets is an important mechanism for cell depletion. This pathway for cell death is most apparent during acute infection and may be an important part of the mechanism for establishing the viral set point.

Progression of HIV disease also may be characterized by three groupings: fast progression, progression and long-term nonprogression (Haynes *et al*., 1996). Differences in anti-HIV antibody levels relate to disease progression rates (Chargelegue *et al*., 1995; Strathdee *et al*., 1995; Zwart *et al*., 1994), and a recent report documented the rapid clinical course for HIV infection where there was only a low-level antibody response (Michael *et al*., 1997). The rapid disease course for SIV infection in macaques with poor immune responses to virus emphasizes the importance of virus replication and cell destruction during the acute infection interval. It is likely that similar mechanisms may control the set point and subsequent disease progression for HIV-1 infection in man.

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