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Decline of Simian Immunodeficiency Virus (SIV)–Specific Cytotoxic T Lymphocytes in the Peripheral Blood of Long-Term Nonprogressing Macaques Infected with SIVmac32H-J5

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The evolution of simian immunodeficiency virus (SIV)–specific cytotoxic T lymphocyte precursors (CTLps) and their relationship with virus replication were studied in SIV-infected macaques. After primary viremia, 3 of 8 macaques lost culturable virus and polymerase chain reaction–detectable provirus in peripheral blood. Although proviral DNA persisted in the spleen and lymph nodes, virus loads were below or barely above detection levels. Throughout the study, the 3 macaques remained asymptomatic, with stable CD4$^+$ cell counts. These findings were associated with the detection of CTLps directed against both structural and regulatory SIV proteins. The response peaked during the first 7 months of infection but waned subsequently. CTLps increased after rechallenge of 1 macaque, suggesting that limited antigenic stimulation contributed to their disappearance from circulation. Transient viremia with increasing CTLp frequencies and antibody titers also suggested at least partial susceptibility to reinfection. These findings bear implications for vaccination strategies aimed at inducing protective CTLs against lentiviruses.

Although the role of cytotoxic T lymphocyte (CTL) responses in the host defense against lentiviruses has not yet been firmly established, several observations indirectly support the view that CTLs are important correlates of protection against human immunodeficiency virus (HIV) infection and disease, as reviewed elsewhere [1]. CTLs against HIV antigens have been detected in persons exposed to infectious body fluids but lacking in evidence of infection [1], suggesting, although not proving, that virus clearance by CTLs is indeed possible. A temporal association has also been described between the development of HIV-specific CTLs and the containment of primary viremia [1]. Furthermore, during the early stage of infection, plasma RNA loads show an inverse correlation with the levels of Env-specific CTLs [2], whereas the detection of Rev-specific CTL precursors (CTLps) predicts a subsequent slow rate of disease progression [3].

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After the early phase of infection, the relationship between HIV-specific CTLs and the rates of virus replication and disease progression appears to be less than clear. Earlier studies have proposed that high levels of CTL activity are associated with the maintenance of the asymptomatic status, whereas CTL responses decline with disease progression [4]. Consistent with these observations, an inverse correlation has been found between plasma RNA load and the levels of CD8$^+$ T lymphocytes that recognize HIV Gag or Pol peptides in the context of major histocompatibility complex (MHC) class I tetrameric complexes [5]. Nevertheless, strong CTL responses have occasionally been detected in persons with advanced disease [6], and, conversely, low levels of CTL activity have been observed in persons with slow rates of disease progression [7]. In fact, declining CTL responses to HIV antigens have been observed in a long-term nonprogressor carrying a defective HIV provirus and lacking detectable viral RNA expression [8].

Macaque simian immunodeficiency virus (SIVmac) shares with HIV many biological and structural features, a similar genomic organization, and extensive genetic homology. The virus establishes a persistent infection in macaques and induces an immunodeficiency syndrome closely resembling human AIDS [9]. Simian AIDS, however, runs a more rapid course, as most animals develop overt disease within 1–4 years of infection. Nevertheless, animals of the same species and origin show a considerable variation in the rate of disease progression; some develop symptoms and die within a few months, whereas

others remain asymptomatic for a few years, thus resembling long-term nonprogressors with HIV infection.

Because of its similarities with HIV infection of humans, SIVmac offers a unique model to explore the relationship between antiviral immune responses and the long-term containment of lentiviral infections. Here, we investigated the evolution of SIV-specific CTLps in macaques infected intravenously with a molecular clone of SIVmac and followed up prospectively for up to 22 months. We identified 3 animals that, in coincidence with effective virus containment, progressively lost their CTL response against SIV. This finding bears implications for the design and evaluation of vaccines against SIV or HIV infection.

Materials and Methods

Macaques. Eight cynomolgus macaques were infected intravenously with either 50 (primary infection) or 200 (reinfection) monkey median infectious doses of cell-free SIVmac32H-J5 [10]. Monkeys K77, K80, K83, and K88 had been previously immunized with an SIV subunit vaccine but became infected upon challenge [10]. Observation lasted for at least 13 months (K73 and K79) and up to 22 months (K66, K71, K77, K80 and K88). Monkey K83 was euthanized 18 months after infection upon the development of symptomatic disease with diarrhea and weight loss. Macaques were anesthetized with ketamine for blood sampling and lymph node biopsies. Spleens were removed at the end of observation.

Mononuclear cell preparation. Lymph nodes and spleen, in complete culture medium (RPMI 1640 with 100 U/mL penicillin, 100 μg/mL streptomycin, 10^{-5} *M* β-mercapto ethanol, and 2 m*M* L-glutamine) containing 10% fetal calf serum (FCS), were teased with forceps to place cells in suspension and were filtered through a 112-μm-pore-size nylon mesh. Peripheral blood, lymph node, and splenic mononuclear cells (PBMC, LNMC, and SPMC) were isolated by density-gradient centrifugation [10] and were either used immediately or cryopreserved. Herpesvirus papiotransformed B–lymphoblastoid cell lines (B-LCL) were established from PBMC by incubation with cell-free supernatant from cell line S594 [10]. Subset-cell separation was done with antibody-coated magnetic beads (Dynabeads M-450; Dynal, Oslo, Norway), as described elsewhere [11]. For flow cytometry, cells in PBS with 1% bovine serum albumin were incubated for 30 min with anti-CD2 (Leu-5b-FITC; Beckton Dickinson, Mountain View, CA) and anti-CD8 (Leu-2a-PE; Beckton Dickinson) or anti-CD4 (OKT4-FITC; Ortho Diagnostic System, Raritan, NJ) monoclonal antibodies, washed twice, fixed in 1% paraformaldehyde (PFA), and analyzed with a FACscan flow cytometer (Beckton Dickinson). Rates of change of $CD4^+$ cell percentages were obtained by linear regression.

Virus detection. Virus load was determined in serial dilutions of freshly isolated PBMC, LNMC, SPMC and in their CD8-depleted fractions, cocultured with the human T-cell line C8166, as described elsewhere [10]; the lowest dilution contained 10^6 cells. SIV p26 core antigenemia was assayed by antigen-capture ELISA (Coulter SIV Core Antigen Assay; Coulter Electronics, Mijdrecht, Netherlands). A nested polymerase chain reaction (PCR), adapted from Chen et al. [12], was used to amplify a 500-bp fragment of SIV Gag. Briefly, DNA was extracted [13] from 10^6 freshly isolated PBMC, LNMC, and SPMC CD4–enriched fractions, and from the same cells after coculture with C8166 cells. After heparinase treatment, DNA was added to a solution containing PCR buffer (50 m*M* KCl, 10 m*M* Tris-HCl pH 9.0, 0.1% Triton X-100), $MgCl_2$ (1.5 m*M*), dNTP (0.2 m*M*), Taq polymerase (1 U), and primers (31 p*M*). Two sets of primers were used, for outer (5′-CATTCACGCAGAAGAGAAAG; 3′-GCATTTTGAATCA-GCAGTG) and inner (5′-ACAAGTAGACCAACAGCACC; 3′-TGAAATGGCTCTTTTGGCC) amplification, respectively. PCR conditions were 1 min, 10 s at 94°C; 1 min, 30 s at 50°C; and 1 min at 72°C for 25 and 30 cycles, with a 10-min final extension. The nested SIV Gag PCR was highly sensitive, and the control plasmid could be diluted 5 log without loss of signal. The higher limit of sensitivity was 1 in 10,000 infected cells. Sequence analyses were conducted both directly and after cloning.

Antibody detection. Anti-Env antibodies were measured by indirect ELISA [10]. Virus-neutralizing antibodies were measured in a yield reduction assay. Briefly, 1 : 50 serum dilutions (20 μL/well) were incubated for 30 min at 37°C with 20 μL of serially diluted (3-fold) SIVmac32H in 8 replicates per dilution. C8166 cells (1.33×10^4/mL) were added to each well in 150 μL. After incubation for 7 days, cells were transferred to poly-L-lysine–coated plates and fixed in methanol. Infected cells were detected by immunoperoxidase staining [10]. The yield reduction was calculated as the ratio of virus titer ($TCID_{50}$/mL) in the absence of serum to the titer in the presence of the sample serum.

Generation of effector cells. Optimal conditions for the detection of SIV-specific CTLps by limiting dilution assay (LDA) were established as described elsewhere [11]. Effector cells were expanded by two 1-week cycles of stimulation with autologous PFA-fixed B-LCL (10^4/well) infected with recombinant vaccinia vectors (rVVs) expressing SIV antigens. The optimal numbers and ranges of dilutions and numbers of replicate wells were predetermined for each monkey in a pilot experiment; cultures included at least 4 and up to 8 cell dilutions; each dilution included at least 24 and up to 48 replicate wells. Cultures also contained autologous irradiated (2500 rad) feeder PBMC (10^4/well), recombinant interleukin-2 (rIL-2, 10 U/mL) from day 3, and 10% supernatant from concanavalin A–stimulated blasts from day 7. The following rVVs were used: SIVmac32H Env [10] and Gag p55 (provided by Prof. A. McMichael, Institute of Molecular Medicine, Oxford, UK); SIVmac32H-J5 Nef [10] and Rev (vv9005; MRC AIDS–Directed Programme, Potters Bar, South Mills, UK); SIVmac-BK28 Pol (ADP263; MRC AIDS–Directed Programme); and SIVmac251 Tat (TG4174; Transgene, Strasbourg, France). The vector 186-poly (Transgene), containing a polycloning site without insert, was used as a control. For peptide-specific stimulation, effector cells were cultured for 14 days with autologous irradiated (5000 rad) peptide-pulsed B-LCL (10^4/well), autologous irradiated feeder PBMC (10^4/well), and rIL-2 (10 U/mL) from day 4. A 9-mer peptide designated p26A.5 [14], covering amino acids 242–250 of p26 (SVDEQIQWM) was synthesized at the European Veterinary Laboratory (Woerden, Netherlands).

Cytotoxicity assay and calculation of CTLp frequencies. Cytotoxicity was measured in standard ^{51}Cr-release assays, as described elsewhere [11, 14]. Target cells were autologous and MHC class I–mismatched allogeneic B-LCL incubated overnight with either SIV rVVs, vaccinia virus control, peptide p26.A5, or medium alone. Individual LDA wells were considered positive when lysis

of targets expressing SIV antigens exceeded that of control targets by 10%, if the latter was <10%, or by 20%, if the latter was >10%. Frequencies were estimated by the maximum-likelihood method with a software package that included a χ^2 goodness-of-fit test statistic [15]. Only estimates with test statistics <10 were accepted. Frequencies were normalized to the number of CTLp/10^6 PBMC, LNMC, or SPMC. Rates of change of CTLp frequencies were calculated by linear regression.

Results

Prospective virological and clinical evaluation of 8 SIV-infected macaques. Eight cynomolgus macaques were infected intravenously with SIVmac32H-J5, a pathogenic molecular clone derived from SIVmac. In monkey K71, PBMC-associated virus load and p26 antigenemia showed a peak at week 2 after infection (>1000 infected cells/10^6 PBMC and 1.10 ng/mL, respectively), followed by a rapid decline. Repeated attempts to isolate virus from PBMC and their CD8-depleted fractions (to remove potential virus-suppressive factors) were unsuccessful between 3 and 6 months after infection (table 1). However, during this time, proviral DNA was detected in PBMC by nested Gag-PCR. Sequence analyses showed that PBMC carried a 520-bp Gag fragment identical to that of the J5 clone used for challenge (not shown). Between 12 and 20 months after infection, virus isolation remained unsuccessful. In addition, repeated attempts failed to detect proviral DNA in PBMC, their CD4-enriched fractions (to increase sensitivity), and the same cells after coculture with C8166 cells. Identical observations were made with monkeys K77 and K80 between 12 and 22 months after infection. In contrast, virus was detected in PBMC of another group of 5 monkeys (K66, K73, K79, K83, and K88) for the entire length of observation (minimum 13 months). Seven of the 8 monkeys remained asymptomatic during their observation periods. Monkey K83 developed symptomatic disease 18 months after infection. The percentages of circulating $CD4^+$ cells (table 1) were stable over time in monkeys K71, K77, and K80 but declined significantly ($P <$ .05) in the other 5 monkeys.

Specificity of circulating CTLps. The optimal conditions for the detection of SIV-specific CTLps in cynomolgus macaques were established as described elsewhere [11]. The frequencies of Env-, Gag-, Pol-, Tat-, Nef- and Rev-specific CTLps were measured by antigen-specific stimulation of PBMC collected from each macaque before and after infection. Frequencies in naive PBMC were always <2/10^6 PBMC (not shown). After infection (table 1), all animals had CTLps against Gag and Tat, and most also had CTLps against Env (5/7), Nef (4/7), or Pol (4/7). Only 3 animals, K71, K77, and K80, showed Rev-specific CTLps at frequencies >5/10^6 PBMC in at least 2 independent experiments. Consistent with previous data reviewed elsewhere [16], even at the peak of response, CTLp frequencies were often <15/10^6 PBMC, but estimates up to 121/10^6 PBMC were found in monkeys K71, K80, and K88.

Kinetics of SIV-specific CTLps. CTLp frequencies were compared in PBMC collected at multiple times during up to 22 months of observation (figure 1). Samples obtained from each macaque at different times were tested simultaneously by use of identical culture and assay conditions, including identical stimulator cells, rIL-2, feeder cells, and ^{51}Cr-labeled targets. Monkey K83 developed a relatively low frequency (up to 13/10^6 PBMC) of multispecific CTLps during the first 6 months of infection and lost detectable CTLps against Env, Tat, and Nef during the subsequent 6 months but maintained anti-Gag CTLps 18 months after infection, the time of onset of symptomatic disease. CTLp frequencies remained either stable or increased over time in monkeys K66, K79, and K88. In contrast, CTLps of monkeys K71, K77, and K80 peaked within the first 7 months of infection but declined significantly thereafter. Although Env- and Gag-specific CTLps showed the most marked decline (figure 2), 20–22 months after infection, all SIV-specific CTLps in the 3 monkeys had declined to levels significantly lower than those measured at 4 months.

Table 1. Prospective virological and immunological evaluation of simian immunodeficiency virus–infected macaques.

	VC/PCR[a] (mo.)					CTLp specificity[b]						
Monkey	1–2	3	4–6	12	18–20	Env	Gag	Pol	Tat	Nef	Rev	CD4 slope[c]
K83	+	+	+	+	+	+	+	+	+	+	−	—0.8 ± 0.2 (.001)
K66	+/+	−/+	−/+	−/+	−/+	+	++	+	++	(+)	(+)	− 0.8 ± 0.2 (.0004)
K73	+/+	+	+	+	ND	ND	+	ND	ND	ND	−	−1.1 ± 0.4 (.004)
K79	+/+	+	+	+	ND	(+)	+	+	+	++	−	−0.7 ± 0.3 (.004)
K88	+	+	+	+	+	+++	+++	−	+++	(+)	−	−0.3 ± 0.1 (.004)
K71	+/+	−/+	−/+	−/−	−/−	−	+++	−	++	−	+	0
K77	+	−/+	+	−/−	−/−	+	+	−	+	+	++	0
K80	+	+	+	−/−	−/−	+++	+	++	+	++	+	0

[a] Virus culture (VC) was done with peripheral blood mononuclear cells (PBMC) and their CD8-depleted fractions; polymerase chain reaction (PCR) was done with DNA from both freshly isolated and cultured PBMC and their CD4-enriched fractions; +, VC positive; +/+, VC positive, PCR positive; −/+, VC negative, PCR positive; −/−, VC negative, PCR negative.

[b] Quantitative data indicate highest cytotoxic T lymphocyte precursor (CTLp) frequency measured during whole observation period and reproduced in at least 2 independent experiments; −, <2; (+), 2–4; +, 5–14; ++, 15–50; +++, >50 CTLp/10^6 PBMC.

[c] Rates of change with SE (and *P* values) of $CD4^+$ cell percentages/month determined from 6 to 9 measurements per monkey (mean 8.1). ND, not done.

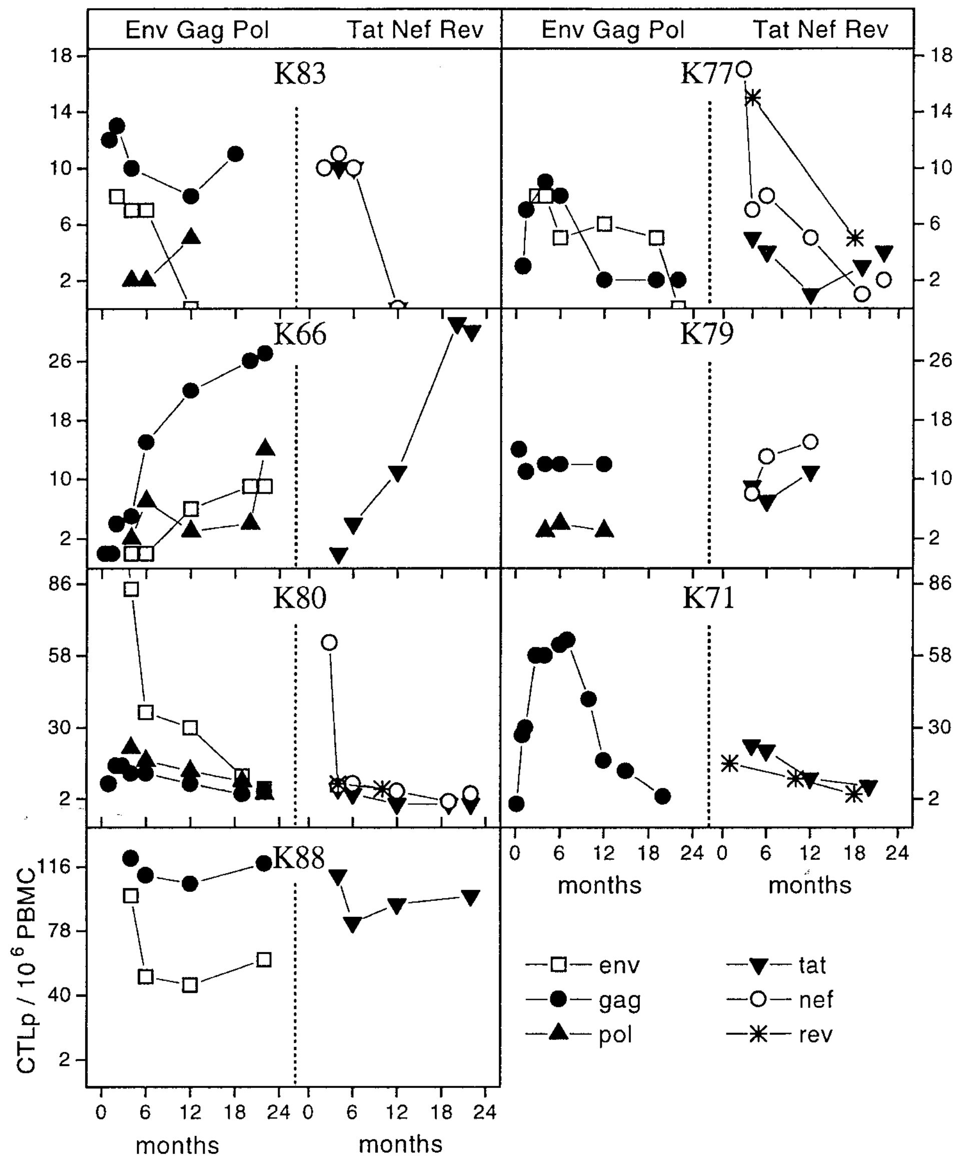

Figure 1. Kinetics of simian immunodeficiency virus (SIV)–specific cytotoxic T-lymphocyte precursors (CTLps) in SIV-infected macaques. Effector cells were expanded with recombinant vaccinia vectors expressing SIV Env, Gag, Pol, Tat, Nef, or Rev. Frequencies were normalized to number of CTLp/10^6 peripheral blood mononuclear cells (PBMC).

SIV protein- and epitope-specific CTLps of monkey K71. To confirm and extend the previous observations, detailed studies were conducted with monkey K71, 1 of the animals showing good virus containment and whose CTLp frequencies were initially >15/10^6 PBMC. By use of both polyclonal and clonal CTL populations, we have shown previously that $CD8^+$ and MHC class I–restricted CTLs of this monkey recognized an immunodominant 9-mer epitope in Gag, designated p26.A5, spanning amino acids 242–250 (SVDEQIQWM) of p26 [14]. Here, we compared the frequencies and kinetics of Gag-specific CTLps with those of p26.A5-specific CTLps. After PBMC stimulation with Gag rVV, 4 aliquots from individual LDA wells were tested in split-well assays against Gag rVV, peptide p26.A5, and 2 control targets. CTLps against p26.A5 represented 77% of Gag-specific CTLps early after infection but only 31%–39% between 4 and 7 months after infection, indicating that Gag recognition was broader at the peak of response (table 2).

The frequencies of Gag-specific CTLps, after reaching a plateau, decreased more rapidly than those of p26.A5-specific CTLps. The rates of decline from 4 months after infection were −4.2 ± 0.6/month ($P = .003$) and −1.1 ± 0.3/month ($P = .01$), for Gag- and p26.A5-specific CTLps, respectively. As a result, between 12 and 20 months after infection, CTLps against

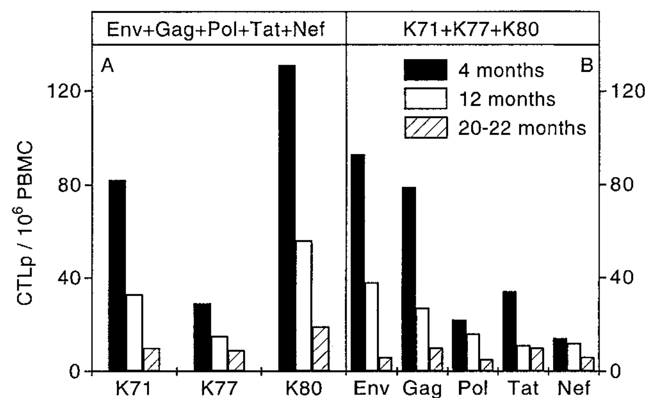

Figure 2. Decline of simian immunodeficiency virus (SIV)–specific cytotoxic T lymphocyte precursors (CTLps) in monkeys K71, K77, and K80. CTLp frequencies were compared at 4, 12, and 20 (K71) or 22 (K77 and K80) months after infection. *A,* For each monkey, total CTLps were obtained by summing individual Env- Gag-, Pol-, Tat-, and Nef-specific CTLps. *B,* For each SIV protein, total CTLps were obtained by summing CTLp frequencies of individual macaques.

p26.A5 constituted 90%–100% of Gag-specific CTLps. The reproducibility of CTLp frequencies was confirmed in independent replicate experiments for each time point (table 2). In addition, the frequencies of p26.A5-specific CTLps measured after stimulation with Gag rVV were reproduced in PBMC expanded by stimulation with peptide p26.A5 (table 2).

Rechallenge of monkey K71. Twenty months after primary infection, monkey K71 was rechallenged intravenously with a high dose of SIVmac32H-J5. Two naive monkeys were infected as controls. Consistent with previous findings [10], a burst of virus replication was observed in the 2 naive monkeys between 2 and 5 weeks after infection (not shown). In monkey K71, a transient and modest increase in virus burden was observed at week 6, coinciding with a transient decrease of circulating $CD4^+$ cells. The numbers and percentages of circulating $CD8^+$ cells and Env-specific antibody titers showed a significant increase (figure 3). Virus-neutralizing antibody titers, however, did not change significantly.

CTLp frequencies were measured simultaneously in all PBMC samples collected during 10 weeks of observation by use of identical culture and assay conditions. Gag-specific CTLps showed a progressive increase after reinfection, and by week 4 their frequency ($22/10^6$ PBMC; range, 15–28; $\chi^2 = 1$) was similar to that measured at week 4 after primary infection ($27/10^6$ PBMC; range, 17–37; $\chi^2 = 4$). In the same cultures tested by split-well assay, p26.A5-specific CTLps also showed a rapid but less pronounced increase after reinfection and by week 10 constituted 50% of circulating Gag-specific CTLps.

Comparison of peripheral blood, lymph nodes, and spleen 22 months after infection. After 22 months of infection, virus isolation and PCR data from peripheral blood of 5 monkeys were compared with those from their spleen and axillary, inguinal, and mesenteric lymph nodes (table 3). By culture, virus was recovered from the blood, lymph nodes, and spleen of monkeys K88 and K66. By use of nested PCR, provirus DNA was detected in the lymph nodes and spleen of all 5 monkeys, including the 3 monkeys (K71, K77, and K80) that lacked detectable virus in peripheral blood. However, in these 3 monkeys, cultures of whole mononuclear cells and their CD8-depleted fractions yielded a virus load either below (lymph nodes) or barely above (spleen) detection levels.

In the reinfected monkey K71, virus load was either similar (spleen) or lower (lymph nodes) than that detected in superficial lymph nodes at weeks 12 and 23 after primary infection (figure 4). CTLps against Gag and p26.A5 were measured simultaneously in cells from the lymph nodes, spleen, and peripheral blood. In lymph nodes, all anti-Gag CTLps also recognized the p26.A5 epitope (figure 4). Their frequency (up to $12/10^6$ mononuclear cells) was lower than that in blood (up to $40/10^6$ PBMC) but similar to that detected in superficial lymph nodes at weeks 12 and 23 after primary infection (up to $13/10^6$ mononuclear cells). Higher Gag-specific CTLp frequencies were found in the spleen (up to $30/10^6$ mononuclear cells), and, as in the blood, these included a proportion of CTLps directed against targets other than p26.A5. These observations were confirmed after adjustment of CTLp frequencies to the initial proportion of $CD8^+$ T cells in PBMC (50%), lymph nodes (36%–40%), and spleen (47%).

Discussion

In the present study, we followed the evolution of SIV-specific CTLps in macaques infected intravenously with a molecular clone of SIVmac. We found that effective virus containment, as indicated by the loss of detectable virus in peripheral blood

Table 2. Gag- and p26.A5-specific cytotoxic T-lymphocyte precursors of monkey K71.

	CTLp specificity[a]			
	SIV Gag		p26.A5	
	Gag-stimulation			
Months	Exp 1	Exp 2	Gag-stimulation	p26.A5-stimulation
1.4	30 (21–29)	ND	23 (16–30)	24 (16–32)
4	58 (44–73)	56 (42–70)	18 (12–25)	ND
6	62 (45–79)	60 (45–74)	21 (15–28)	ND
7	64 (48–80)	ND	25 (17–33)	23 (17–29)
10	41 (30–53)	ND	20 (13–27)	17 (11–23)
12	17 (10–23)	20 (13–26)	16 (9–22)	18 (12–24)
15	13 (9–16)	ND	12 (9–16)	ND
20	3 (1–6)	3 (0.4–6)	3 (1–6)	3 (0.4–6)

[a] Effector cells were generated by stimulation of peripheral blood mononuclear cells (PBMC) with either simian immunodeficiency virus (SIV) Gag recombinant vaccinia vector (rVV) or peptide p26.A5, as indicated. After two 1-week cycles of stimulation, 4 aliquots from each well of limiting dilution cultures were tested for cytotoxicity against autologous target B-LCL infected either with Gag rVV or with vaccinia virus control, sensitized with peptide p26.A5, or incubated with medium alone. Frequencies (with 95% confidence intervals) were normalized to number of cytotoxic T lymphocyte precursors/10^6 PBMC. ND, not done.

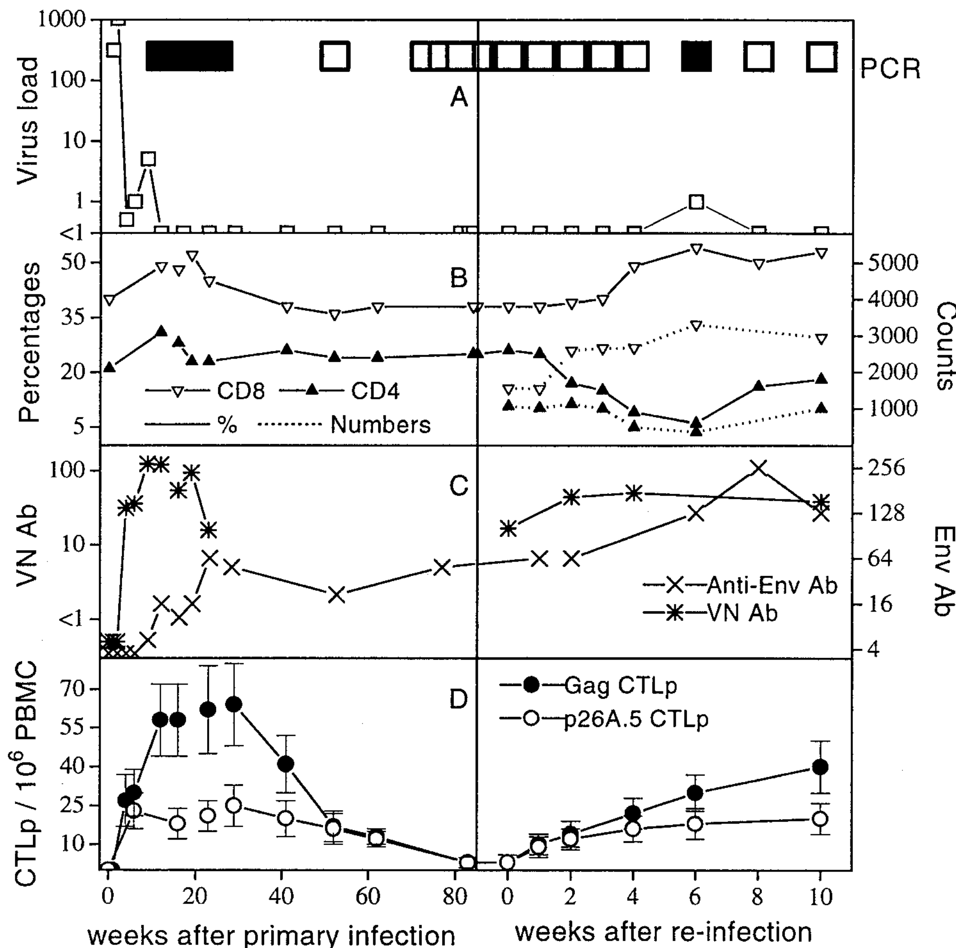

Figure 3. Primary (left) and secondary (right) intravenous simian immunodeficiency virus infection of monkey K71. *A,* Virus load, measured in peripheral blood mononuclear cells (PBMC) and their CD8-depleted fractions, was normalized to number of infected cells/10^6 PBMC. Polymerase chain reaction (PCR) was done with DNA from both freshly isolated and cultured PBMC and their CD4-enriched fractions. □, PCR negative; ■, PCR positive. *B,* Percentages (solid lines) and absolute numbers (dotted lines) of $CD4^+$ and $CD8^+$ cells in blood. *C,* Anti-Env antibody (Ab) titers, determined by ELISA, and virus neutralizing (VN) Ab titers, calculated as yield reduction values. *D,* Frequencies of Gag- (●) and p26.A5- (○) specific cytotoxic T lymphocyte precursors (CTLps), determined by stimulation with SIV Gag and normalized to number of CTLp/10^6 PBMC; error bars indicate 95% confidence intervals.

and detection of low-level virus reservoirs in lymphoid tissues, resulted in declining CTLp frequencies in circulation.

For the detection of SIV-specific CTLps, cells under limiting dilution conditions were restimulated with rVVs expressing SIV antigens. In previous studies of the same macaques, this approach led to the expansion of CTL with a $CD8^+$ and MHC class I–restricted phenotype [11, 14]. We have extensively investigated the conditions for the detection of SIV-specific CTLps in cynomolgus macaques [11] and have found that a reproducible assessment can be obtained by tailoring the LDA settings to individual macaques and by rejecting CTLp frequencies with χ^2-test statistics >10.

CTLps detected by LDA are believed to be correlated with antiviral T-cell memory in vivo [17]. Recently, quantitation of $CD8^+$ T cells by fluorescent staining with tetrameric MHC-peptide complexes has suggested that LDA may underestimate by ~10-fold the actual size of the memory CTL pool. It is noteworthy, however, that despite this potential underestimation, the kinetics and duration of memory CTL responses determined by LDA have been shown to be correct [18]. Our kinetics studies identified 3 patterns of response in SIV-infected macaques. In asymptomatic macaques with persistent virus in PBMC (i.e., K66, K79, and K88; table 1), CTLps were stable or even increased over time (figure 1). In contrast, overall CTLps declined before the onset of symptoms in the monkey that developed overt disease (i.e., K83; figure 1). Although the latter finding agrees with observations made in HIV-infected humans and supports a role for CTLs in preventing disease progression, it is noteworthy that Gag-specific CTLps were maintained in monkey K83 at the time of progression.

Table 3. Virus detection in blood, lymph nodes, and spleen 22 months after infection.

Monkey	Cell type	Virus detection[a]		
		VC	VL	PCR
K88				
	PBMC	+	5	+
	LNMC	+	63	+
	SPMC	+	25	+
K66				
	PBMC	−	−	+
	LNMC	+	25	+
	SPMC	+	>3125	+
K77				
	PBMC	−	−	−
	LNMC	−	−	+
	SPMC	−	−	+
K80				
	PBMC	−	−	−
	LNMC	+	1	+
	SPMC	+	1	+
K71				
	PBMC	−	−	−
	LNMC	−	−	+
	SPMC	+	1	+

[a] Virus culture (VC) was done with peripheral blood, lymph node, and spleen mononuclear cells (PBMC, LNMC, SPMC), and their CD8-depleted fractions virus load (VL) was determined in serial cell dilutions, and polymerase chain reaction (PCR) was done with DNA from both freshly isolated and cultured mononuclear cells and their CD4-enriched fractions.

A remarkable finding was that the frequencies of both SIV protein- (figures 1 and 2) and epitope- (table 2) specific CTLps decreased significantly over time in the 3 animals that lost both culturable virus and PCR-detectable provirus in their PBMC (i.e., K71, K77, and K80; table 1). These findings suggest that SIV-specific memory CTLs decline under conditions of limited antigenic stimulation and bear direct implications for vaccination strategies aimed at inducing protective CTL responses against lentiviruses. The basis of CTL immunological memory and the requirement for antigen persistence, however, are a matter of controversy [19–21]. In fact, evidence has been provided that memory $CD8^+$ CTLs may be maintained in the absence of specific antigen [19]. Nevertheless, our observations are consistent with the finding that CTL responses against multiple HIV antigens disappear under conditions of limited antigenic expression, as did those found in a long-term nonprogressor carrying a defective HIV provirus [8]. Recently, loss of tetramer-positive anti-HIV $CD8^+$ T cells has also been reported in HIV-infected patients receiving highly active antiretroviral therapy [22].

In previous studies, CTLs against a dominant T-cell epitope have been induced by lower antigenic doses and have lasted for a longer time than CTLs against minor epitopes [17]. We found that CTLps against an immunodominant epitope in p26 designated p26.A5 [14] developed more rapidly and lasted longer in circulation than those directed against other putative minor Gag epitopes (table 2). These CTLs may require lower levels of antigenic stimulation [17], be triggered by infected cells displaying very low epitope density [23], be relatively independent of T cell help [24], and ultimately be more effective in virus containment [25]. These observations suggest that qualitative aspects of CTL responses are important determinants of the outcome of the infection. The detection of Rev-specific CTLps in the 3 nonprogressing macaques but not in the other animals (table 1) lends further support to this concept. Because a similar correlation has been observed in HIV-1–infected persons [3], we hope that our preliminary data will encourage further investigation into the contribution of Rev-specific CTLs to protection against lentiviruses.

The CTLp decline observed in macaques that effectively contained the infection prompted a study of the outcome of rechallenge. We chose to reinfect only 1 of the 3 nonprogressing macaques (i.e., K71), so that the other 2 (i.e., K77 and K80) could provide information on virus distribution in lymphoid tissues at the end of the study (table 3). When monkey K71 was reinfected with a high dose of homologous virus, transient viremia with rising CTLp frequencies and antibody titers suggested at least partial susceptibility to reinfection (figure 3). Although the possibility of virus reactivation cannot be excluded, the temporal association of viremia, decreasing $CD4^+$ cells, and increasing $CD8^+$ cells, CTLp and antibody titers with rechallenge makes this possibility less likely. CTLs can be regarded as a very sensitive marker of virus replication. This is indicated by the finding that CTLps against multiple SIV antigens [26], including the nonvirion proteins Rev and Tat (E.G.J.H., unpublished data), can be detected in macaques lacking both detectable virus and antibody responses after challenge.

After reinfection, virus replication in peripheral blood was limited in comparison with the virus burst observed during primary viremia (figure 3). Resistance to superinfection is a well-described phenomenon in macaques, as reviewed elsewhere [27]. Although its mechanisms remain unclear, they are likely to include an immunological component, as reviewed elsewhere [16]. It is tempting to speculate that restimulated CTLps, rather then being a mere reflection of the levels of virus replication, played a role in virus containment after reinfection.

Other mechanisms may be proposed for the observed CTLp decline in the 3 animals that lost detectable virus in peripheral blood. Although progressive immunological dysfunction may result in CTLp decline, the 3 animals showed no signs of disease progression and maintained stable $CD4^+$ cell counts throughout the study (table 1). Their T cell function, as defined by the ability to respond to allogeneic cells in mixed-lymphocyte reactions, was preserved over time (not shown). The increase in CTLp frequencies observed in monkey K71 after reinfection (figure 3) also argues against a compromised immune function.

During primary infection, HIV-specific CTLs accumulate preferentially in peripheral blood rather than in lymph nodes [28]. Consistent with this finding, CTLp frequencies of monkey K71 were lower in lymph nodes than in blood after both pri-

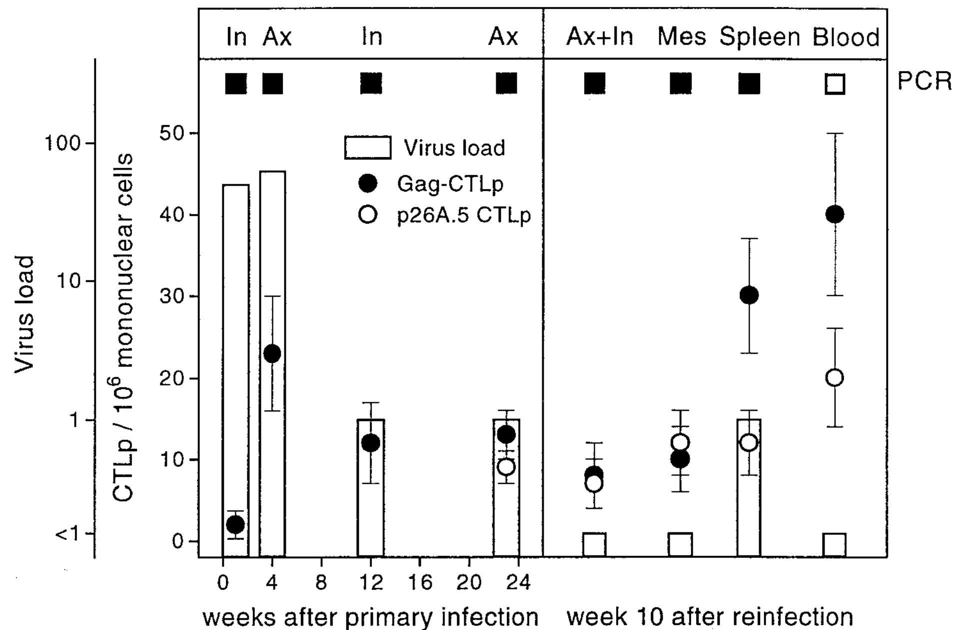

Figure 4. Virus load and cytotoxic T-lymphocyte precursor (CTLp) frequencies in lymphoid tissues of monkey K71, before (left panel) and 10 weeks after (right panel) intravenous rechallenge. Mononuclear cells were obtained from peripheral blood, spleen, inguinal (In), axillary (Ax), and mesenteric (Mes) lymph nodes. Virus load (bars), measured in whole mononuclear cells and their CD8-depleted fractions, was normalized to number of infected cells/10^6 mononuclear cells. Polymerase chain reaction (PCR) was done with DNA from both freshly isolated and cultured mononuclear cells and their CD4-enriched fractions. ■, PCR positive; □, PCR negative. Frequencies of Gag- (●) and p26.A5- (○) specific CTLps, determined by stimulation with simian immunodefieincy virus Gag, were normalized to number of CTLp/10^6 mononuclear cells; error bars indicate 95% confidence intervals.

mary infection and reinfection, whereas splenic CTLp frequencies after reinfection resembled those found in blood (figure 4). As a consequence of virus containment in the periphery, however, CTLs may home to potential sites of ongoing virus replication, such as lymph nodes [29] and spleen. We studied virus distribution in these tissues after 22 months of infection (table 3). Although virus was undetectable in peripheral blood from the 3 nonprogressing macaques, virus reservoirs were found in their lymphoid organs. This is an important observation, because it confirms that loss of virus detection in peripheral blood cannot be regarded as an indicator of virus clearance. In the 3 monkeys, however, virus burden in lymphoid tissues was below or barely above the detection limit. In fact, in monkey K71, it was not dissimilar from that detected in lymph nodes after primary infection (figure 4). Although CTLp sequestration seems unlikely in the presence of such a low virus burden, we aim at extending our study to other macaques and CTLp specificities other than Gag.

In conclusion, our findings add to our understanding of the dynamics of antiviral CTL responses, suggest a role for SIV-specific CTLs in virus containment, and support the concept that the quality of antiviral CTLs is an important determinant of the course of infection.

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