ELSEVIER
PII: S0264-410X(97)00029-7
Vaccine, Vol. 15, No. 11, pp. 1269–1275, 1997

Printed in Great Britain
0264–410X/97 $17+0.00

Simian immunodeficiency virus (SIV)-specific CD8$^+$ cytotoxic T lymphocyte responses of naive and vaccinated cynomolgus macaques infected with SIVmac32H(J5): quantitative analysis by *in vitro* antigenic stimulation

Anna-Maria Geretti*, †, Ellen G.J. Hulskotte*, Marlinda E.M. Dings*, Carel A. van Baalen*, Geert van Amerongen‡ and Albert D.M.E. Osterhaus*§

*Detailed analyses of simian immunodeficiency virus (SIV)-specific cytotoxic T lymphocyte (CTL) responses in vaccinated and infected macaques may help to clarify the role of CTL immunity in protection against lentiviruses. Here, the optimal conditions for the measurement of SIV Gag-specific CTL were investigated by bulk and limiting dilution assays of peripheral blood mononuclear cells (PBMC) from naive and vaccinated cynomolgus macaques (*Macaca fascicularis*) infected with SIVmac32H(J5).* In vitro *restimulation was generally required for CTL detection. Selective activation of CD8$^+$ and MHC-restricted SIV Gag-specific CTL was induced by stimulation with autologous* para*-formaldehyde-fixed B-lymphoblastoid cell lines infected with a recombinant vaccinia virus expressing SIV Gag. Applied to limiting dilution assays, antigenic stimulation reproducibly demonstrated SIV Gag-specific CTL precursors (CTLp) in PBMC of all animals studied, including those lacking significant responses in standard bulk CTL assays. © 1997 Elsevier Science Ltd.*

Keywords: animal models, protective immunity, recombinant antigens

Even though cytotoxic T lymphocytes (CTL) are believed to contribute to the control of human and animal viral infections, their role in the host defense against lentiviruses has not been firmly established[1]. Infection of macaques with several strains or clones of simian immunodeficiency virus (SIV)mac shows remarkable similarities with human immunodeficiency virus (HIV) infection of humans[2]. Following SIV inoculation, a burst of virus replication is observed, which rapidly subsides in coincidence with the development of antiviral immune responses[3]. The subsequent course of the infection varies considerably among macaques: some animals rapidly develop an AIDS-like disease and die within few weeks or months, whereas others may remain asymptomatic for >3 years. This individual variability, combined with the opportunity to define parameters of infection such as strain, dose and route of virus inoculation, may prove valuable for clarifying the role of CTL immunity in the control of lentiviral infections.

We[4] and others[5,6] have reported recently that vaccine-induced SIV Env-, Gag-, or Nef-specific CTL failed to protect macaques upon challenge with cell-free SIVmac. In the same model, however, Gallimore *et al.*[7] found an inverse correlation between the frequency of vaccine-induced SIV Nef-specific CTL precursors (CTLp) and peak virus load measured after challenge. These findings indicate that CTL may not be able to prevent or control HIV or SIV infection, unless stringent qualitative and quantitative requirements are met. Detailed analyses of CTL responses in both vaccinated and infected macaques may help to clarify these

*Institute of Virology, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. †Present address: Department of Virology, Division of Pathology and Communicable Diseases, Rowland Hill Street, London NW3 2PF, UK. ‡Central Animal Laboratory, National Institute of Public Health and Environmental Protection, P.O.Box 1, 3720 BA Bilthoven, The Netherlands. §Author to whom all correspondence should be addressed. (Received 21 October 1996; revised version received 2 January 1997; accepted 13 Janauary 1997)

requirements, thereby facilitating the design of immunotherapeutic interventions[8]. However, initial reports have indicated that CTLp frequencies of immunized and naive rhesus[6,9] or cynomolgus[4,7,10] macaques infected with SIVmac may be relatively low by comparison with those often measured in HIV-1 infected humans[11], implying that sensitive and reproducible methods are required to evaluate CTL responses in macaques. Extending observations previously made in HIV-1 infected humans[12,13], we report here that *in vitro* expansion of effector cells under limiting dilution conditions by stimulation with SIV Gag recombinant vaccinia virus (rVV)-infected and *para*-formaldehyde (pfa)-fixed B-lymphoblastoid cell lines (B-LCL), significantly enhances CTL detection in both immunized and naive cynomolgus macaques infected with SIVmac32H(J5).

MATERIALS AND METHODS

Animals

The study included eight colony-bred juvenile (1.5–3.5 years) cynomolgus macaques seronegative for SIV, type D simian retrovirus (SRV) and simian T cell leukaemia virus-I (STLV-I). Four monkeys (designated K77*, K80*, K83* and K88*) were immunized with an SIV Env-Iscoms, Gag-Iscoms and Nef-lipopeptides subunit vaccine, as described[4]. The other four monkeys (K73, K79, K66 and K71) were naive at the time of SIV infection. Animals were inoculated intravenously with 50 monkey median infectious doses of cell-free SIVmacJ5, a pathogenic molecular clone derived from SIVmac32H and grown on rhesus peripheral blood mononuclear cells (PBMC)[14]. Infection was confirmed by virus isolation[4], antibody detection in ELISA[4] and nested-Gag PCR[15].

PBMC preparation

Blood samples were drawn from the femoral vein into tubes containing heparin. PBMC were separated by density gradient centrifugation[4], washed three times in complete RPMI 1640 medium (containing 100 U ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin, 10^{-5} M β-mercapto ethanol and 2 mM L-glutamine), and either used immediately or cryopreserved. Cell viability (as assessed by Trypan Blue exclusion) exceeded 95% after thawing. Cell-associated virus load was determined in serial dilutions of freshly isolated PBMC co-cultured with the human T cell line C8166; the lowest dilution contained 10^6 PBMC[4]. Herpes papio-transformed B-LCL were established by incubation of PBMC with cell-free supernatant from the Herpes papio-producing cell line S594, and maintained in complete medium containing 10% foetal calf serum (FCS; R-10). Serological MHC typing was performed by Dr R. Bontrop (BPRC, Rijswijk, The Netherlands). SIV p26 core antigen in plasma was assayed by antigen-capture ELISA (Coulter SIV Core Antigen Assay, Coulter Electronics, Mijdrecht, The Netherlands).

Immunomagnetic cell fractionation

PBMC in complete medium with 2% FCS were mixed with magnetic beads coated with anti-CD8 monoclonal antibodies (mAb) (Dynabeads M-450, Dynal, Oslo, Norway) at a 1:10 target cell-to-bead ratio[16]. After incubation for 60 min at 4°C, fractions were separated on a magnetic separation device (MPC-6, Dynal). Positively selected $CD8^+$ cells were detached from magnetic beads using a goat anti-mouse-Fab polyclonal antiserum (DETACHaBEAD, Dynal). This separation procedure yields highly pure cell populations, without interfering with either CD8 expression or cytotoxic function of effector cells[16]. By immunofluorescence analysis positively selected fractions typically consisted of $>99\%$ $CD8^+$ cells, whereas $CD8^+$ contamination of depleted fractions was consistently $<2\%$ (data not shown).

Immunofluorescence analysis

Cell samples in PBS with 0.1% BSA were incubated for 30 min at room temperature with anti-CD2 (Leu-5b-fluorescein isothiocyanate, Becton Dickinson, Mountan View, CA) and anti-CD8 (Leu-2a-phycoerythrin, Becton Dickinson) or anti-CD4 (OKT4-FITC, Ortho Diagnostic System, Raritan, NJ) mAb. Samples were washed twice with PBS, fixed in 1.5% pfa and analysed with a FACScan (Becton Dickinson).

Preparation of antigen presenting cells (APC)

Autologous B-LCL were infected overnight with rVV (10 m.o.i.) expressing either the Gag p56 gene of SIVmac32H (kindly provided by Professor A. McMichel, Institute of Molecular Medicine, Oxford, UK) or the Tat gene of SIVmac251 (TG4174, kindly provided by Dr M.P. Kieny, Transgene, Strasbourg, France), and subsequently fixed in 1.5% pfa, as described[12]. Autologous irradiated (8500 rad) SIV Gag rVV-infected B-LCL were used as APC in a limited number of experiments. Cultures stimulated with these APC showed no signs of vaccinia virus-induced cytopathic effects. Antigen expression in SIV Gag APC was confirmed by immunofluorescence analysis using a polyclonal bovine anti-vaccinia serum (RIVM, Bilthoven, The Netherlands) and the murine anti-Gag mAb CLB14 (CLB, Amsterdam, The Netherlands).

Bulk culture conditions

Bulk assays were done with either unstimulated or restimulated PBMC. For antigenic stimulation, PBMC (2.5×10^4 per well) in R-10 were cultured for 14 days in 96-well round-bottomed plates with SIV Gag or Tat APC (10^4 per well) and autologous irradiated (2500 rad) feeder PBMC (10^4 per well). Cultures were supplemented with 10 U ml^{-1} recombinant interleukin (IL)-2 (rIL-2) from day 3, and restimulated with 10^4 per well APC on day 7. During the second cycle of stimulation the culture medium was enriched with 10% supernatant from concanavalin A (ConA)-stimulated blasts. In some experiments, PBMC ($10^6\ ml^{-1}$) were cultured for 10–14 days with autologous irradiated PHA-blasts infected with SIVmac32H(J5) ($10^5\ ml^{-1}$), as described by Gotch *et al.*[17]. For mitogenic stimulation, PBMC ($10^6\ ml^{-1}$) were cultured for 3 days with ConA (5 $\mu g\ ml^{-1}$), washed, and expanded with rIL-2 (20 U ml^{-1}) for 4–11 days. Effector cells were assayed for cytotoxicity in duplicate or triplicate wells at indicated effector-to-target cell (*E*:*T*) ratios.

Limiting dilution culture conditions

Limiting dilution assays were done with either unstimulated or restimulated PBMC, to estimate CTL[18] and CTLp frequencies respectively. PBMC restimulation was performed as described for bulk assays. The optimal limiting dilution assay parameters, including numbers and ranges of dilutions, and numbers of replicate wells, were predetermined for each monkey in a pilot experiment. All assays included at least four and up to eight PBMC dilutions, and each dilution included at least 24 and up to 48 replicate wells of 96-well round-bottomed plates. Three or four aliquots from each well were tested in split-well cytotoxicity assays.

Cytotoxicity assay

Cytotoxicity was measured in standard ^{51}Cr-release assays against autologous and allogenic MHC class I mismatched B-LCL infected overnight with 10 m.o.i. of SIV Gag or Tat rVV. Autologous B-LCL either infected with 186-poly rVV (containing a polycloning site without insert and kindly provided by Dr M.P. Kieny, Transgene) or incubated with medium alone served as control. Targets were labelled for 1 h with 100 μCi of ^{51}Cr, washed three times, resuspended in R-10, and added to effector cells at 5×10^3 cells per well in 96-well round-bottomed plates (Costar, Cambridge, UK). After a 5 h incubation, supernatants were harvested (Skatron Harvester, Skatron, Oslo, Norway), and the release of ^{51}Cr was measured in a gamma counter. Maximum ^{51}Cr release was determined by detergent (5% Triton X-100) lysis of target cells. Spontaneous release was determined by incubation of target cells in R-10 alone. Spontaneous release was $<30\%$ of maximum release.

Calculation of results

The percentage of lysis of specific and control targets was calculated for duplicate or triplicate wells of bulk cultures and for each well of limiting dilution cultures according to the formula: %lysis = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Individual wells of limiting dilution cultures were considered positive when lysis of specific targets exceeded by 10% that of control targets if the latter was $<10\%$, or by 20% if the latter was $>10\%$. In all positive wells the experimental release exceeded the spontaneous release by at least three standard deviations (S.D.). CTLp frequencies were estimated by the maximum likelihood method using the statistical software package described by Strijbosch *et al.*[19], which included a χ^2 goodness-of-fit test statistic. Frequencies were normalized to the number of CTLp per 10^6 PBMC. Rates of change/month of follow-up (slopes), and correlation coefficients (r) of virus load, CTLp frequencies and CD4$^+$ cell percentages were calculated by linear regression analysis.

RESULTS

Measurement of SIV Gag-specific CTL in bulk assays

SIV Gag-specific CTL responses were studied in eight cynomolgus macaques during the first 4 months of infection with SIVmac32H(J5). Four monkeys (indicated by an asterisk) had been immunized before infection with an SIV Env-Iscoms, Gag-Iscoms and Nef-lipopetides subunit vaccine[4], whereas the other four animals were naive at time of virus inoculation. To establish optimal conditions for CTL measurement, we first compared in bulk assays the direct SIV Gag-specific CTL responses of unstimulated PBMC with those mediated by the same PBMC following either mitogenic or antigenic *in vitro* restimulation. As shown in the examples of *Figure 1*A, no significant CTL responses against SIV Gag were mediated by unstimulated PBMC in *E:T* ratios up to 200:1. After mitogenic expansion with ConA (*Figure 1*B), effector cells specifically reactive against SIV Gag were detected in monkey K71, whereas relatively high levels of non-specific background lysis hindered reliable CTL measurement in monkey K88*. In parallel, the specific lysis of SIV Gag rVV-infected targets was markedly enhanced by two 1-week cycles of antigenic stimulation, using as APC autologous pfa-fixed B-LCL infected with SIV Gag rVV (*Figure 1*C).

Previously we[13] and others[20] have shown that after pfa-fixation, Epstein–Barr virus-transformed B-LCL retain their ability to induce CTL activation, but lose their release of helper-like soluble factors. These factors are known to stimulate the growth of both MHC restricted and unrestricted cytotoxic cells reactive against the B-LCL[13,20]. Extending these observations to Herpes papio-transformed B-LCL, we observed that stimulation of PBMC from monkey K88* with pfa-fixed SIV Gag APC elicited SIV Gag-specific

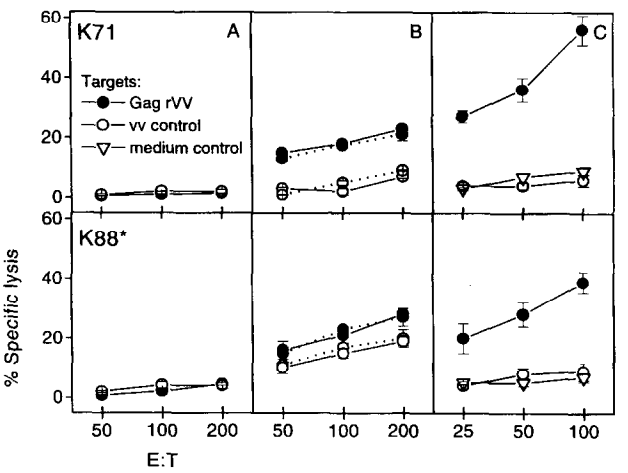

Figure 1 *In vitro* restimulation is required to detect SIV Gag-specific CTL in PBMC of immunized and non-immunized cynomolgus macaques infected with SIVmac32H(J5). Data are shown for two monkeys. Monkey K88* had been immunized with an SIV Env-Iscoms, Gag-Iscoms and Nef-lipopeptides subunit vaccine before infection. Monkey K71 was naive at the time of virus inoculation. Effector cells were: (A) unstimulated PBMC isolated either 12 weeks (monkey K71) or four months (monkey K88*) after infection; (B) the same PBMC expanded by mitogenic stimulation with ConA, and assayed twice, either on days 10 (· · ·) and 14 (—) of culture (monkey K71), or on days 7 (· · ·) and 10 (—) of culture (monkey K88*); (C) the same PBMC expanded by two 1-week cycles of antigenic stimulation, using as APC autologous pfa-fixed B-LCL infected with SIV Gag rVV. Targets were autologous B-LCL either infected with SIV Gag rVV or with vaccinia virus (vv) control, or uninfected (medium control). Results are expressed as mean specific lysis ± SD from triplicate (A and B) or duplicate (C) well estimations

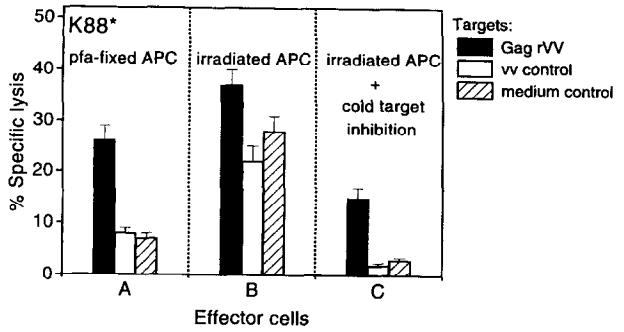

Figure 2 Stimulation efficiency of SIV Gag APC. Effector cells were generated by stimulation of PBMC from monkey K88* with either pfa-fixed (A) or irradiated (B and C) SIV Gag APC. Targets were autologous B-LCL either infected with SIV Gag rVV or with vaccinia virus (vv) control, or uninfected (medium control). For cold target inhibition (C), non-labelled uninfected B-LCL were mixed with the ^{51}Cr-labelled targets at a ratio of 5:1 before the CTL assay was initiated. Results are expressed as mean specific lysis with S.D. from duplicate well estimations at an E:T ratio of 40:1

CTL without significant expansion of cytotoxic cells reactive against control targets (*Figure 2*A). In contrast, after stimulation of the same PBMC with irradiated SIV Gag APC (*Figure 2*B and C), relatively high numbers of cold (non-^{51}Cr-labelled) uninfected targets were required to inhibit non-specific background lysis and measure SIV Gag-specific CTL.

In monkey K71, effector cells generated by stimulation with either SIV Gag (*Figure 3*A) or Tat (*Figure 3*B) APC mediated significant lysis of targets infected with the inducing rVV, but not of targets infected with either the discordant rVV or with vaccinia virus control. This finding confirmed that antigenic stimulation induces selective CTL activation. Furthermore, immunofluorescence analyses indicated an expansion of CD8$^+$ cells at the end of the two cycles of SIV Gag-specific stimulation (data not shown). Depletion studies confirmed that SIV Gag-specific CTL were indeed CD8$^+$ cells: as shown in *Figure 4* for monkey K71, SIV Gag-specific CTL responses mediated by total effector cells were preserved in positively isolated CD8$^+$ cells, but were abolished by CD8-depletion. In addition, these responses were restricted to autologous targets, whereas allogenic MHC class-I mismatched targets infected with SIV Gag rVV were not recognized.

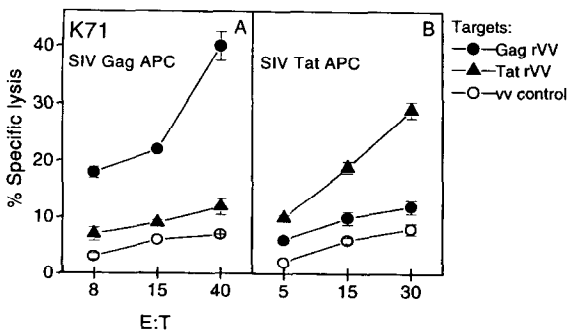

Figure 3 Selective expansion of antigen-specific CTL. Effector cells were generated by stimulation of PBMC from monkey K71 with either SIV Gag (A) or Tat (B) APC. Targets were autologous B-LCL infected with either SIV Gag or Tat rVV or with vaccinia virus (vv) control. Results are expressed as mean specific lysis with S.D. from duplicate well estimations

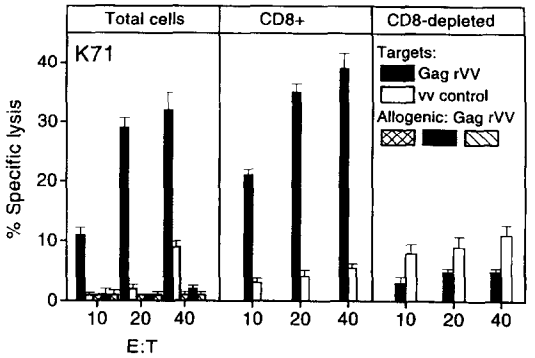

Figure 4 CD8$^+$ cell-mediated and MHC-restricted cytotoxic responses against SIV Gag. Effector cells generated by stimulation of PBMC from monkey K71 with SIV Gag APC were assayed as total cell population, positively isolated CD8$^+$ cells (>99% CD8$^+$), and CD8-depleted cell population (<2% CD8$^+$). Targets were autologous B-LCL infected with either SIV Gag rVV or with vaccinia virus (vv) control, and allogenic SIV Gag rVV-infected B-LCL from three MHC class-I mismatched monkeys

Measurement of SIV Gag-specific CTL and CTLp in limiting dilution assays

The frequencies of SIV Gag-specific CTL and CTLp were estimated by limiting dilution assays of unstimulated and restimulated PBMC, respectively. The optimal assay parameters (i.e. numbers and ranges of dilutions and numbers of replicate wells) yielding χ^2 goodness-of-fit test statistic <10 were predetermined in a pilot experiment for each monkeys. Single-well cytotoxic responses of restimulated PBMC from monkey K71 are shown in *Figure 5*. In agreement with the results of bulk CTL assays (*Figure 1*C), after two 1-week cycles of SIV Gag-specific stimulation, effector cells in most wells mediated killing of SIV Gag rVV-infected targets (*Figure 5*A), with negligible killing of control targets (*Figure 5*B). The relationship between the percentage of negative wells and the initial cell number was consistent with the single-hit Poisson model and indicated a CTLp frequency of 58 (95% confidence interval: 44–72; χ^2:1) per 10^6 PBMC (*Figure 5*C, filled circles). In contrast, in the absence of PBMC restimulation, SIV Gag-specific CTL were detected at a frequency of only two (0.3–3; χ^2:1) per 10^6 PBMC (*Figure 5*C, filled triangles). Similarly, 4 months after infection, the frequency of SIV Gag-specific CTL in unstimulated PBMC of monkey K88* was only 2 (0.4–4; χ^2:4) per 10^6 PBMC, whereas no CTL could be detected in unstimulated PBMC of other monkeys (data not shown). These findings confirmed that *in vitro* restimulation greatly amplifies SIV Gag-specific CTL responses.

Cell culture under limiting dilution conditions further increased the sensitivity of CTL measurement. Even after SIV Gag-specific PBMC restimulation, monkeys with low, but still measurable CTLp frequencies (<10 per 10^6 PBMC), showed no consistent CTL responses in standard bulk CTL assays, using *E:T* ratios up to 100:1. As an example, a comparison of bulk and limiting dilution assays of PBMC from monkey K66 is shown in *Figure 6*. In bulk assays (*Figure 6*A), no significant lysis of SIV Gag rVV-infected targets was mediated by PBMC that were unstimulated or restimulated with either ConA or SIV-infected blasts. Lysis increased after SIV Gag-specific stimula-

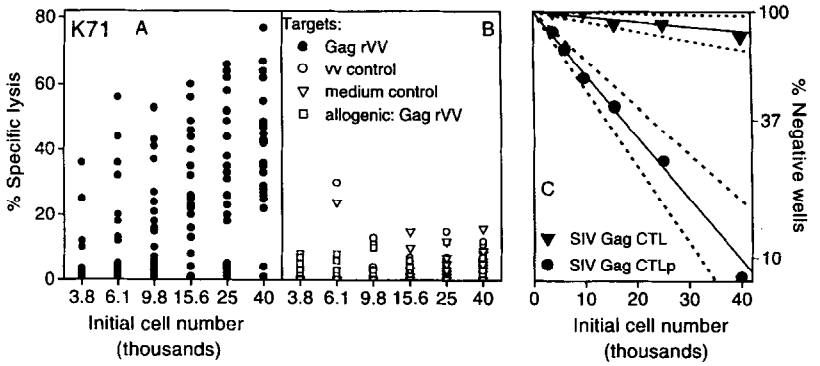

Figure 5 Limiting dilution analysis of cytotoxic responses against SIV Gag. PBMC isolated from monkey K71 at week 12 after infection were cultured in six dilutions (range: 40000 to 3815 cells per well), each including 24 replicate wells. After two 1-week cycles of SIV Gag-specific stimulation, individual wells were tested against (A) autologous B-LCL infected with SIV Gag rVV, and (B) autologous B-LCL either infected with vaccinia virus (vv) control or uninfected (medium control), and allogenic SIV Gag rVV-infected B-LCL from one MHC class-I mismatched monkey. (C) Frequency analysis of SIV Gag-specific CTL and CTLp in unstimulated and restimulated PBMC, respectively. The relationship between the percentage of negative wells and the initial cell number was consistent with the single-hit Poisson model. Frequencies were estimated by the maximum likelihood method and normalized to number of CTL or CTLp per 10^6 PBMC. Dotted lines indicate 95% confidence intervals. The frequency of SIV Gag-specific CTL was 2 (0.3–3; χ^2: 1) per 10^6 PBMC. The frequency of SIV Gag-specific CTLp was 58 (44–72; χ^2: 1) per 10^6 PBMC

tion, but it was still <10% after subtraction of non-specific background lysis. In limiting dilution assays, SIV Gag-specific CTLp were detected at a frequency of four (2–6; χ^2: 1) per 10^6 PBMC (*Figure 6*B).

The reproducibility of CTLp frequency estimates was verified in 10 replicate assays of cryopreserved PBMC, which showed a mean coefficient of variation (as 100 × the S.D. of the residuals/mean CTLp frequency estimate) of 9.5% (data not shown). We also investigated the ability of SIV Gag APC to stimulate unprimed CTLp in naive PBMC isolated from the eight monkeys before exposure to either whole SIV or SIV antigens. These PBMC were assayed in parallel with those obtained 4 months after infection, using identical culture and assay conditions. The individual wells of limiting dilution assays were never or rarely positive in naive PBMC (CTLp frequencies <1 per 10^6 PBMC, data not shown), indicating that only primed CTLp were responsive to SIV Gag-specific stimulation.

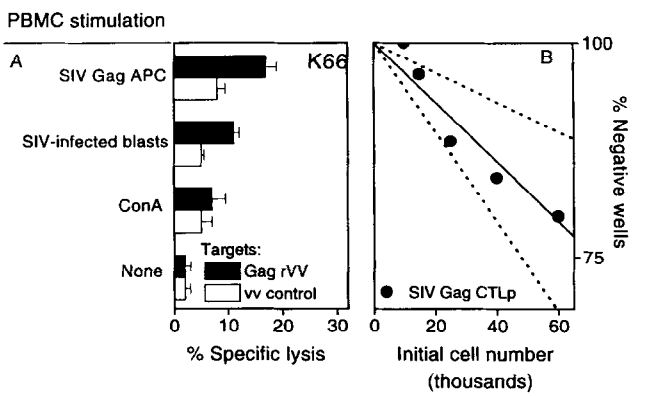

Figure 6 Bulk and limiting dilution assays of PBMC isolated from monkey K66 at week 12 after infection. In bulk assays (A), PBMC were unstimulated or restimulated with either ConA, or SIV-infected blasts, or SIV Gag APC, and assayed in duplicate at an E:T ratio of 80:1. In limiting dilution assays (B), PBMC were restimulated with SIV Gag APC. Target cells were autologous B-LCL either infected with SIV Gag rVV or with vaccinia virus (vv) control (A and B) or uninfected (medium control, B). The frequency of SIV Gag-specific CTLp was 4 (2–6; χ^2:1) per 10^6 PBMC

Kinetics of SIV Gag-specific CTLp

In kinetic studies of the four monkeys that were naive at the time of virus inoculation, SIV Gag-specific CTLp were first detected between week 1 and 4 (K71, K73, K79) or 9 (K66) after infection (*Table 1*). CTLp detection in circulation coincided therefore with the containment of PBMC-associated virus load and p26 antigenemia which followed the initial virus burst observed at week 2 or 4 after infection[4]. The four vaccinated monkeys (K77*, K80*, K83* and K88*) had developed SIV Gag-specific CTLp upon immunization, at frequencies ranging from 2 to 13 per 10^6 PBMC[4]. After infection, their CTLp frequencies were still similar to those found in the non-immunized animals (*Table 1*). In all eight monkeys, frequencies were generally maintained between week 12 and 4 months after infection, but a marked increase was observed in monkey K88*. Overall, no clear correlation emerged between CTLp frequencies and PBMC-associated virus load. In fact, 4 months after infection statistical analysis seemed to suggest a strong positive correlation between virus load and CTLp frequencies ($r = 0.89$, $P = 0.003$, not shown); however, this relationship was lost if monkey K88* was excluded from analysis. All eight monkeys remained asymptomatic during the first 4 months of infection. Six animals showed a progressive decline of $CD4^+$ cell percentages, which was pronounced in monkeys K73 and K79 (*Table 1*).

DISCUSSION

In the present study, we have studied the CTL responses of immunized and naive cynomolgus monkeys after infection with SIVmac32H(J5). We focused our attention on responses directed against the relatively conserved Gag protein, which has been described as a major target of CTL immunity against lentiviruses[3,11,21]. Our findings demonstrate that antigenic stimulation of effector cells under limiting dilu-

Table 1 Evaluation of virological and immunological parameters in vaccinated and naive cynomolgus macaques infected with SIVmac32H(J5)[a]

Monkey	Virus load[b]		CTLp[c]			$CD4^+$ cell slope[e]
	2–4 week (peak)	4 months	1–4 weeks[d]	6–12 weeks[d]	4 months	
K66	503	<1	0	4 (2–6; 1)	5 (3–7; 3)	−1.7±1
K71	>1000	<1	27 (17–37; 4)	58 (44–72; 1)	56 (42–70; 3)	+1±1.5
K73	625	5	12 (8–17; 2)	9 (6–13; 1.5)	7 (5–9; 3)	−4.3±0.5 ($P<0.05$)
K79	>1000	6	14 (8–20; 1)	11 (15–26; 2.5)	12 (8–15; 4)	−3.2±0.8 ($P<0.05$)
K77*	208	25	3 (1–5; 3)	7 (4–9; 2)	9 (6–12; 4)	−0.6±0.6
K80*	208	3	8 (4–12; 2)	15 (10–21; 1)	12 (8–16; 2.5)	−0.6±0.3
K83*	>1000	3	12 (9–16; 1)	13 (9–17; 1)	10 (7–14; 1)	−1.3±0.9
K88*	>1000	156	N.D.	18 (12–24; 1)	121 (95–147; 1)	−2.4±1

[a]Data are presented for eight animals. Four monkeys (denoted by an asterisk) had been immunized with an SIV Env-Iscoms, Gag-Iscoms, Nef-lipopetides subunit vaccine before infection, whereas the other four animals were naive at the time of virus inoculation

[b]Cell-associated virus load was measured in co-cultures of PBMC with C8166 cells and normalized to the number of infected cells per 10^6 PBMC

[c]Effector cells were generated under limiting dilution culture conditions by stimulation with autologous pfa-fixed B-LCL infected with SIV Gag rVV. Split-well ^{51}Cr-release assays were performed on day 14 of culture. Frequencies were estimated by the maximum likelihood method and normalized to the number of CTLp per 10^6 PBMC; 95% confidence intervals and χ^2 goodness-of-fit test statistics are given in parentheses

[d]Results are presented from one of at least two experiments, with coefficients of variation < 10%

[e]Rates of change (slopes) in $CD4^+$ cell percentages $month^{-1}$ were determined by linear regression analyses of longitudinal data obtained at minimum three time points (mean = 3.75) during 4 months of observation. Positive slopes indicate increase, negative slopes correspond to a decrease

tion conditions provides a sensitive and reproducible approach to characterize CTL responses against SIV. With this approach we were able to detect SIV Gag-specific CTLp in PBMC of all animals studied, including those lacking significant CTL responses in standard bulk CTL assays (*Figure 6*).

In vitro restimulation was shown to be required to amplify SIV Gag-specific CTL responses to detectable levels (*Figure 1*). In contrast, Gag-specific CTL responses of asymptomatic HIV-1-infected adults have often been of sufficient magnitude to allow detection in primary assays, in the absence of *in vitro* restimulation[11,21]. In line with these observations, and in agreement with previous studies of SIVmac-infected rhesus[6,9] and cynomolgus[10] macaques, SIV Gag-specific CTLp were detected at frequencies generally lower than those measured in asymptomatic HIV-1-infected adults[13,21]. This discrepancy may reflect a relatively low degree of antigenic stimulation in macaques, due to rapid downregulation of SIV replication after infection. Our kinetic studies appear to support this hypothesis. Immediately after infection, we found that CTLp detection in the naive macaques coincided with the initial decline of cell-associated virus load and p26 antigenemia. Subsequently, we found no significant differences in the CTLp frequencies of immunized and naive monkeys, whereas a positive correlation seemed to develop between virus load and the frequencies of SIV Gag-specific CTLp in the monkey showing relatively poor virus containment (*Table 1*). Taken together, these observations support the hypothesis that the rapid development of CTL responses targeting certain critical viral proteins or epitopes may indeed contribute towards the initial virus containment[22,23]. However, as also suggested by Ferbas *et al.*[24], in cases of poor virus control, CTLp frequencies may become a reflection of persistently high levels of antigenic stimulation.

To stimulate the growth of SIV Gag-specific CTLp we used autologous B-LCL infected with SIV Gag rVV and fixed in pfa. With a similar approach, we have been able to demonstrate Gag-specific CTLp in PBMC from HIV-1-infected persons, even after the onset of overt disease[13]. In the macaques, *in vitro* antigenic stimulation was shown to promote the selective expansion of SIV Gag-specific CTL of the $CD8^+$ MHC class-I restricted phenotype (*Figure 3Figure 4Figure 5*). As a result, CTL detection was enhanced compared with results obtained after mitogenic stimulation with ConA (*Figure 1*), a well-established method for CTL expansion[3,9]. Other authors have also reported the use of rVV to expand CTL against HIV[25] or SIV[26] antigens. In the study by Lubaki *et al.*[25], the use of irradiated rVV-infected B-LCL as APC caused high levels of non-specific background lysis, and cold target inhibition was required to detect HIV-specific CTL. In our study, PBMC stimulation with irradiated SIV Gag APC also allowed CTL detection in bulk assays when combined with cold target inhibition (*Figure 2*). However, this method did not allow a reproducible measurement of CTLp frequencies in limiting dilution assays (data not shown). Along the same line, Kent *et al.*[26] had to replace irradiated rVV-infected B-LCL with irradiated rVV-infected PBMC as APC to reduce the growth of non-specific cytotoxic cells and measure SIV-specific CTL. The improved ratio of specific to non-specific lysis we obtained with pfa-fixed APC (*Figure 2*), probably resulted from an adequate preservation of their stimulation efficiency, together with a reduced release of T-helper like soluble factors that may stimulate the growth of non-SIV-specific cytotoxic cells[13,20].

Interestingly, a previous study by Venet *et al.*[9] described an earlier development of SIV-induced disease in those monkeys which lacked detectable CTL responses. All our monkeys remained asymptomatic during the first 6 months of observation. Among the six animals with relatively low CTLp frequencies, five showed a progressive decrease of $CD4^+$ cell percentages, which was pronounced in only two cases (*Table 1*). Nonetheless, a longer follow-up may clarify whether the generation of higher SIV Gag-specific CTLp frequencies may be associated with a better long-term control of the infection in some monkeys. Studies are in progress to address this hypothesis, which may contribute to the design of effective vaccina-

tion strategies for the prevention and control of lentiviral infections.

ACKNOWLEDGEMENTS

The authors would like to thank M.P. Cranage and E.W. Rud for providing the SIVmac32H(J5).

REFERENCES

1 Zinkernagel, R.M. Are HIV-specific CTL responses salutary or pathogenic? *Curr. Opin. Immunol.* 1995, **7,** 462–470.
2 Desrosiers, R. The simian immunodeficiency viruses. *A. Rev. Immunol.* 1990, **8,** 557–578.
3 Letvin, N.L., Reimann, K.A., Yasutomi, Y., Ringler, D.J. and Yamamoto, H. The SIVmac specific cytotoxic T lymphocyte response in the acutely infected rhesus monkey. *Curr. Top. Microbiol. Immunol.* 1994, **188,** 175–184.
4 Hulskotte, E.G.J., Geretti, A.M. and Siebelink, K.H.J. *et al.* Vaccine-induced virus-neutralizing antibodies and cytotoxic T cells do not protect macaques from experimental infection with simian immunodeficiency virus SIVmac 32H (J5). *J. Virol.* 1995, **69,** 6289–6296.
5 Newman, M.J., Munroe, K.J. and Anderson, C.A. *et al.* Induction of antigen-specific killer T lymphocyte responses using subunit SIVmac251 gag and env vaccines containing QS-21 saponin adjuvant. *AIDS Res. Hum. Retroviruses* 1994, **10,** 853–861.
6 Yasutomi, Y., Koenig, S. and Woods, R.M. *et al.* A vaccine-elicited, single viral epitope-specific cytotoxic T lymphocyte response does not protect against cell-free simian immunodeficiency virus challenge. *J. Virol.* 1995, **69,** 2279–2284.
7 Gallimore, A.M., Cranage, M. and Cook, N. *et al.* Early suppression of SIV replication by $CD8^+$ nef-specific cytotoxic T cells in vaccinated macaques. *Nature Med.* 1995, **1,** 1167–1173.
8 Berzofsky, J.A. Epitope selection and design of synthetic vaccines: molecular approaches to enhancing immunogenicity and crossreactivity of engineered vaccines. *Ann. N.Y. Acad. Sci.* 1993, **690,** 256–264.
9 Venet, A., Bourgault, I., Aubertin, A.M., Kieny, M.P. and Levy, J.P. Cytotoxic T-lymphocyte response against multiple simian immunodeficiency virus (SIV) proteins in SIV-infected macaques. *J. Immunol.* 1992, **148,** 2899–2908.
10 Kent, S.J., Hu, S.L., Corey, L., Morton, W.R. and Greenberg, P.D. Detection of simian immunodeficiency virus (SIV)-specific $CD8^+$ T cells in macaques protected from SIV challenge by prior subunit vaccination. *J. Virol.* 1996, **70,** 4941–4947.
11 Cease, K.B. and Berzofsky, J.A. Toward a vaccine for AIDS: the emergence of immunobiology-based vaccine development. *A. Rev. Immunol.* 1994, **12,** 923–989.
12 Van Baalen, C.A., Klein, M.R. and Geretti, A.M. *et al.* Selective *in vitro* expansion of HLA class I-restricted HIV-1 Gag-specific T cells: cytotoxic T-lymphocyte epitopes and precursor frequencies. *AIDS* 1993, **7,** 781–786.
13 Geretti, A.M., Dings, M.E.M. and Van Els, C.A.C.M. *et al.* HIV-1 and EBV specific cytotoxic T lymphocyte precursors exhibit different kinetics in HIV-1 infected persons. *J. Infect. Dis.* 1996, **174,** 34–45.
14 Rud, E.W., Cranage, M. and Yon, J. *et al.* Molecular and biological characterization of simian immunodeficiency virus macaque strain 32H proviral clones containing nef size variants. *J. Gen. Virol.* 1994, **75,** 529–543.
15 Chen, Z.W., Shen, L., Miller, M.D., Ghim, S.H., Hughes, A.L. and Letvin, N.L. Cytotoxic T lymphocytes do not appear to select for mutations in an immunodominant epitope of simian immunodeficiency virus gag. *J. Immunol.* 1992, **149,** 4060–4066.
16 Geretti, A.M., Van Els, C.A.C.M., Van Baalen, C.A., Poelen, M.C.M. and Osterhaus, A.D.M.E. Preservation of phenotype and function of positively selected virus-specific $CD8^+$ T lymphocytes following anti-Fab detachment from immunomagnetic beads. *J. Immunol. Methods* 1993, **161,** 129–133.
17 Gotch, F., Hovell, R., Delchambre, M., Silvera, P. and McMichael, A. CTL responses to simian immunodeficiency virus by cynomolgus macaques immunised with recombinant vaccinia virus. *AIDS* 1991, **5,** 317–320.
18 Koup, R.A., Pikora, C.A. and Luzuriaga, K. *et al.* Limiting dilution analysis of cytotoxic T lymphocytes to human immunodeficiency virus gag antigens in infected persons: *in vitro* quantitation of effector cell populations with p17 and p24 specificities. *J. Exp. Med.* 1991, **174,** 1593–1600.
19 Strijbosch, L.W.G., Does, R.J.M.M. and Buurman, W.A. Computer aided design and evaluation of limiting and serial dilution experiments. *Int. J. Biomed. Comput.* 1988, **23,** 279–290.
20 Fishwild, D.M., Benike, C.J. and Engleman, E.G. Activation of HLA-restricted EBV-specific cytotoxic T cells does not require $CD4^+$ (helper) T cells or erogenous cytokines. *J. Immunol.* 1988, **140,** 1994–1998.
21 Johnson, R.P. and Walker, B.D. Cytotoxic T lymphocytes in human immunodeficiency virus infection: responses to structural proteins. *Curr. Top. Microbiol. Immunol.* 1994, **189,** 35–63.
22 Koup, R.A., Safrit, J.T. and Cao, Y. *et al.* Temporal association of cellular immune response with the initial control of viremia in primary HIV-1 syndrome. *J. Virol.* 1994, **68,** 4650–4655.
23 Nowak, M.A., May, R.M. and Phillips, R.E. *et al.* Antigenic oscillations and shifting immunodominance in HIV-1 infections. *Nature* 1995, **375,** 606–611.
24 Ferbas, J., Kaplan, A.H. and Hausner, M.A. *et al.* Virus burden in long-term survivors of human immunodeficiency virus (HIV) infection is a determinant of anti-HIV $CD8^+$ lymphocyte activity. *J. Infect. Dis.* 1995, **172,** 329–339.
25 Lubaki, M.N., Egan, M.A., Siliciano, R.F., Weinhold, K.J. and Bollinger, R.C. A novel method for detection and *ex vivo* expansion of HIV type 1-specific cytolytic T lymphocytes. *AIDS Res. Hum. Retroviruses* 1994, **10,** 1427–1431.
26 Kent, S.J., Stallard, V. and Corey, L. *et al.* Analysis of cytotoxic T lymphocyte responses to SIV proteins in SIV-infected macaques using antigen-specific stimulation with recombinant vaccinia and Fowl poxviruses. *AIDS Res. Hum. Retroviruses* 1994, **10,** 551–560.