Kinetics of Gag-specific Cytotoxic T Lymphocyte Responses during the Clinical Course of HIV-1 Infection: A Longitudinal Analysis of Rapid Progressors and Long-term Asymptomatics

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Summary

To gain more insight into the role of HIV-1–specific cytotoxic T lymphocytes (CTL) in the pathogenesis of AIDS, we investigated temporal relations between HIV-1 Gag-specific precursor CTL (CTLp), HIV-1 viral load, $CD4^+$ T cell counts, and T cell function. Six HIV-1–infected subjects, who were asymptomatic for more than 8 yr with $CD4^+$ counts >500 cells/mm^3, were compared with six subjects who progressed to AIDS within 5 yr after HIV-1 seroconversion. In the long-term asymptomatics, persistent HIV-1 Gag-specific CTL responses and very low numbers of HIV-1–infected $CD4^+$ T cells coincided with normal and stable $CD4^+$ counts and preserved CD3 mAb-induced T cell reactivity for more than 8 yr. In five out of six rapid progressors Gag-specific CTLp were also detected. However, early in infection the number of circulating HIV-1–infected $CD4^+$ T cells increased despite strong and mounting Gag-specific CTL responses. During subsequent clinical progression to AIDS, loss of Gag-specific CTLp coincided with precipitating $CD4^+$ counts and severe deterioration of T cell function. The possible relationships of HIV-1 Gag-specific CTLp to disease progression are discussed.

During progressive HIV-1 infection immune responses deteriorate with subsequent development of AIDS. Although several correlates for progression to AIDS have been identified, the exact mechanisms underlying immune dysfunction remain to be elucidated (1, 2). The clinical course of HIV-1 infection is determined by complex interactions between viral parameters, host properties, and cofactors. Virus-specific CTL that kill virus-infected cells are thought to be a major host defense against viral infections (3). Therefore, HIV-1–specific CTL may be important for controlling viral spread during acute HIV-1 infection (4) and for maintaining viral load at low levels during the asymptomatic phase (5). Observations from cross-sectional studies have shown absent or severely depressed HIV-1–specific CTL responses during advanced stages of HIV-1 infection (6–8). These studies however, have not resolved whether rapid progressors are non-responders to HIV-1 or whether HIV-1–specific CTL responses are elicited which subsequently diminish during progression to AIDS. In contrast, strong HIV-1–specific CTL responses have been proposed to cause immunosuppression in HIV-1 infection rather than being beneficial (9).

To gain more insight in the role of HIV-1–specific CTL in the pathogenesis of AIDS, we analyzed long-term asymptomatics (LTA)[1] and rapid progressors for precursor CTL (CTLp) specific for Gag, the protein of HIV-1 which is most predominantly recognized by CTL during asymptomatic HIV-1 infection (10–16). Longitudinal studies were undertaken to investigate temporal relations between Gag-specific CTLp, HIV-1 viral load, immune status, and clinical course of HIV-1 infection.

[1] *Abbreviations used in this paper:* B-LCL, B-lymphoblastoid cell line; CDC, Centers for Disease Control; CI, confidence interval; CTLp, precursor CTL; LTA, long-term asymptomatics; (N)SI, (non-)syncytium inducing; rVV, recombinant vaccinia virus; TCID, tissue culture infectious dose.

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Materials and Methods

Study Population. The Amsterdam Cohort Studies on AIDS were initiated in October 1984 (17). Data about cohort participants were collected in three monthly visits that consisted of a standardized medical history and collection of blood samples for HIV-1 serology and cellular immunology. Date of entry for participants already HIV-1 seropositive at enrollment, or documented HIV-1 seroconversion, taken as the midpoint between the last seronegative and first seropositive visit, were used as reference points for clinical follow-up. Previously it was shown that participants who were HIV-1 seropositive at entry in the study, seroconverted within 1.5 yr before enrollment (18). By December 1991 there were 106 cohort–participants with documented HIV-1 seroconversion. By December 1992, 34 of these seroconvertors were diagnosed with AIDS (19, 20) according to 1987 Centers for Disease Control (CDC) classifications (21). For this study, six cohort participants were studied, who progressed to AIDS within 5 yr after HIV-1 seroconversion: P159, P186, P187, P224, P450, and P748. From 273 cohort–participants who either entered the study as HIV-1 seropositive or seroconverted before January 1986, 61 participants remained asymptomatic (CDC II/III) for more than 7 yr (22). For this study, six LTA were selected, who had at least 8 yr of asymptomatic follow-up and $CD4^+$ T cell counts >500 cells/mm^3: L008, L067, L090, L617, L709, and L206. Subject L206 is a patient who is monitored at The Academic Medical Centre in Amsterdam.

Immunological Markers. T lymphocyte immunophenotyping for CD4 and CD8 membrane markers was carried out at three monthly intervals by flow cytofluorometry. PBMC were stained with CD4 mAb (Leu-3a-PE; Becton Dickinson, Mountain View, CA) or CD8 mAb (Leu-2a-PE; Becton Dickinson) according to the manufacturer's protocols. Polyclonal T cell functions were measured in real time after May 1987 as previously described (23), by measuring the CD3-mAb (CLB-T3/4E; CLB, Amsterdam, The Netherlands) induced proliferative capacity of PBMC in whole-blood cultures. T cell reactivity is expressed as counts per minute.

Recombinant Vaccinia Viruses (rVV). rVV used in these studies were constructed from the Copenhagen strain of Vaccinia virus, and include rVV TG.1144 expressing Gag of $HIV\text{-}1_{LAI}$ (24, 25) and control-rVV 186-poly containing no insert; kindly provided by Dr. Y. Rivière (Institut Pasteur, Paris, France) and Dr. M.P. Kieny (Transgène S.A., Strasbourg, France).

Induction of HIV-1–specific CTL Responses. HIV-1–specific CTLp were expanded in vitro by Ag-specific stimulation as previously described (15). Frequencies of Gag-specific CTLp were determined using standard methods of limiting dilution analysis (26). Briefly, PBMC isolated and cryopreserved at different time points during the study were thawed and resuspended in IMDM supplemented with antibiotics and 10% pooled human serum. Eight serial dilutions of PBMC ranging from 20,000 to 745 cells/well were seeded in 24-fold in 96-well round-bottom microtiter plates. Stimulator cells were autologous EBV-transformed B-lymphoblastoid cell lines (B-LCL) infected with rVV-TG.1144 and subsequently inactivated with paraformaldehyde. To each well, 10^4 fixed stimulator cells and 10^4 autologous PBMC (30 Gy irradiated) were added, and microcultures were maintained for 15 d at 37°C and 5% CO_2. At day 2 and 9 cultures were fed with medium containing rIL-2 (10 U/ml; Cetus Corp., Emeryville, CA), and at day 7 they were restimulated with 10^4 fixed stimulator cells and rIL-2 (10 U/ml). On day 15, wells were split and effector cells tested for cytotoxicity.

Cytotoxicity Assays. Standard ^{51}Chromium-release assays were performed as previously described (15). Briefly, autologous B-LCL were infected with 5 MOI rVV-TG.1144 or rVV 186-poly and labeled with $Na_2{}^{51}CrO_4$ (Amersham Intl., Amersham, Bucks, UK) for 16 h. After three additional washings, 4×10^3 target cells were added to each well. After 4 h, supernatants were harvested and radioactivity was counted on γ-counter (Cobra II; Packard Instr. Co., Inc., Meriden, CT). Spontaneous ^{51}Cr-release was always <15% of maximum release. Specific lysis was calculated with the formula: 100× ([experimental release − spontaneous release]/[maximum release − spontaneous release]). Wells were considered positive when the ^{51}Cr-release exceeded 10% specific lysis. Statistical analysis was performed using methods as previously has been described by Strijbosch et al. (27). CTLp frequencies are expressed as number of CTLp/10^6 PBMC. Gag-CTLp frequencies were computed as differences between CTLp frequencies determined on Gag-expressing versus control targets. The average CTLp-frequency on control targets was <25/10^6 PBMC.

Virological Markers. Viral load in peripheral blood samples was determined using clonal virus isolation procedures as previously described (28). Briefly, 12,500–25,000 PBMC of HIV-1–infected patients were cocultivated with 10^5 2-d PHA–stimulated PBMC from HIV-1 seronegative blood donors. HIV-1 replication was monitored by screening culture supernatants for p24 production using a p24 capture ELISA. Statistical analysis of positive wells was performed using methods as previously has been described by Strijbosch et al. (27). Viral burden was expressed as tissue culture infectious dose (TCID)/10^6 $CD4^+$ T cells, representing the number of cells productively infected with HIV-1 in the peripheral blood. Biological phenotype of HIV-1 viruses was determined as previously described (29). Briefly, 10^6 PBMC of HIV-1–infected patients were cocultivated with MT2 cells, and cultures were monitored microscopically several times per week to check for syncytium formation to determine the viral phenotype.

Results

Natural History of HIV-1 Infection in Long-term Asymptomatics and Rapid Progressors. Six LTA who were selected for this study remained asymptomatic for >8.0 yr with $CD4^+$ counts >500/mm^3. Total follow-up period until October 1994 was 9.6 ± 0.4 yr. In addition, six cohort-participants who progressed to AIDS within 5 yr after HIV-1 seroconversion were also longitudinally studied. Mean time between HIV-1 seroconversion and AIDS diagnosis was 3.8 ± 1.2 yr. Clinical and laboratory findings of all studied subjects are presented in Table 1. Except for P186, all progressors suffered from severe to mild influenza-like disease in the 3 mo preceding HIV-1 seroconversion indicative for symptomatic acute HIV-1 infection (19), whereas none of the LTA reported history of primary HIV-1 infection in the months preceding seroconversion or enrollment in the cohort study.

$CD4^+$ T cell numbers in LTA were in the range of values found in healthy uninfected controls (90% confidence interval (CI): 560–1,550/mm^3). $CD4^+$ T cell counts of L090, L617, and L709 remained stable, whereas in subjects L008, L067, and L206, $CD4^+$ counts tended to decline towards the end of the study. $CD8^+$ T cell numbers of L617 and L206 were increased, whereas in other LTA $CD8^+$ T cell counts remained within normal range (90% CI: 310–1,000/mm^3). $CD4^+/CD8^+$ ratios were clearly reversed in L617; while in subjects L008, L067, and L206, ratios inverted after ~5–6 yr of follow up (Fig. 1 *A*). Upon HIV-1 seroconversion $CD4^+$ counts rapidly declined in five out of six progressors with

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Table 1. *Clinical and Laboratory Data of LTA and Rapid Progressors*

Subjects	HLA Class I*	Seroconversion status‡	Age§	Virus phenotype‖	AIDS diagnosis¶	Follow-up**
LTA						
L008	A2,26;B27,44;Cw1,6	II	38	SI (95)	NA	>119
L067	A26,28;B7,57;Cw7	II	35	NSI	NA	>119
L090	A1,2;B41,57;Cw6	I	41	NSI	NA	116
L617	A2,11;B35,62;Cw3	II	28	NSI	NA	>115
L709	A1,69;B14,57;Cw6	I	29	NSI	NA	108
L206	A3,25;B18,51	II	31	NSI	NA	>111
Progressors						
P159	A1;B8;Cw7	I	46	SI (18)	CAO, PCP	32
P186	A3,24;B60,Cw3,4	I	30	NSI	PCP	42
P187	A1;B8;Cw7	I	34	SI (1)	HSV, TXP	28
P224	A3;B44,51;Cw4,7	I	28	SI (45)	PCP	60
P450	A24,28;B39,44	I	29	SI (52)	KS	65
P748	A1;B8;Cw7	I	29	SI (31)	CD4 <200	47

* HLA class-I typings were performed at Department Transplantation Immunology, CLB, Amsterdam, using standard serological typing methods.
‡ Known date of HIV-1 seroconversion (I) or seropositive upon entry in the cohort study (II).
§ Age (yr) at HIV-1 seroconversion or first seropositive visit.
‖ Biological virus phenotype: NSI vs. SI; number of months after seroconversion or seropositive entry at which NSI to SI switch occurred indicated in parentheses.
¶ AIDS diagnosis according to CDC classifications (21): PCP, *pneumocystis carinii* pneumonia; HSV, Herpes Simplex virus infection; TXP, Toxoplasmosis; CAO, *Candida albicans* oesophagitis; KS, Kaposi's sarcoma or $CD4^+$ T cell numbers $<200/mm^3$; NA, not applicable for LTA.
** Time (mo) between HIV-1 seroconversion or seropositive entry and AIDS diagnosis for progressors or October 1994 for LTA.

>140 cells/mm^3 per year. In subject P224 $CD4^+$ counts initially remained quite stable, but dropped precipitously after 45 mo of infection. In general, $CD4^+/CD8^+$ ratios were inverted after seroconversion, which in patients P159 and P186 was also due to elevated $CD8^+$ T cell numbers (>1,000/mm^3) (Fig. 1 *D*). T cell function of LTA, measured by CD3 mAb-induced proliferation, was stable and within the range of normal values (90% CI: 1,100–10,100 cpm), whereas T cell function in all progressors gradually diminished to below normal values (Fig. 2). We have shown this to be predictive for progression to AIDS (23).

Subject P748 remained asymptomatic during follow-up but $CD4^+$ counts dropped <200 cells/mm^3 within 4 yr after seroconversion. Currently P748 is being treated with AZT and *pneumocystis carinii* pneumonia (PCP) prophylaxis. Subject P187 became infected with HIV-1 after unprotected sexual intercourse with an AIDS patient. Six months after seroconversion he was lost for the cohort study, but kept monitored by a local general practitioner who also conducted AZT antiretroviral therapy and PCP prophylaxis. Subject P450 suffered from multiple allergic complaints during all stages of HIV-1 infection. General skin rash, eczema, erythema, and dermatomycosis were observed, as well as allergic reactions to rubber and allergic skin rash after treatment with erythromycin, cotrimoxazol, and ciprofloxacin. Except for L008 who started AZT treatment at 109 mo after entry, none of the LTA was subjected to anti-retroviral therapy.

Longitudinal Analysis of HIV-1 Gag-specific CTL Responses. During the entire follow-up period of the study Gag-specific CTL responses could be detected in all LTA. The observed CTLp frequencies were between 1/300–1/21,000; in the same range as previously has been reported by other investigators (8, 10, 13). In subjects L067 and L206, strong (average of >300 CTLp/10^6 PBMC) persistent Gag-specific CTL responses were detectable although they tended to decline at later time points (Fig. 1 *B*). Subjects L008, L617, and L090 had stable intermediate (average of 100–300 CTLp/10^6 PBMC) Gag-specific CTL activity during follow-up. Finally, subject L709 showed persistent but lower (average 20–100 CTLp/10^6 PBMC) Gag-specific CTL responses (Fig. 1 *B*). In addition, CTL responses from subjects L206, L008, and L617 were analyzed in greater detail, using series of overlapping peptides spanning the entire Gag sequence of HIV-1_{LAI}. Multiple CTL epitopes were identified mainly localized in Gag-p24 (15). In addition, after 7 yr of follow-up, CTLp frequencies for Nef and Env in subject L206 were only 53 and 32/10^6 PBMC, respectively; 7–10-fold lower than the Gag-specific CTLp frequency. In blood sampled from subject L090 5.7 and 7.7 yr after seroconversion no Env-specific CTL directed against the HIV-1_{LAI} sequence could be detected.

All tested progressors, except for patient P450, showed distinct Gag-specific CTL responses after seroconversion albeit with different kinetics (Fig. 1 *E*). Participants P159, P186, and P748 initially showed strong though transient Gag-specific

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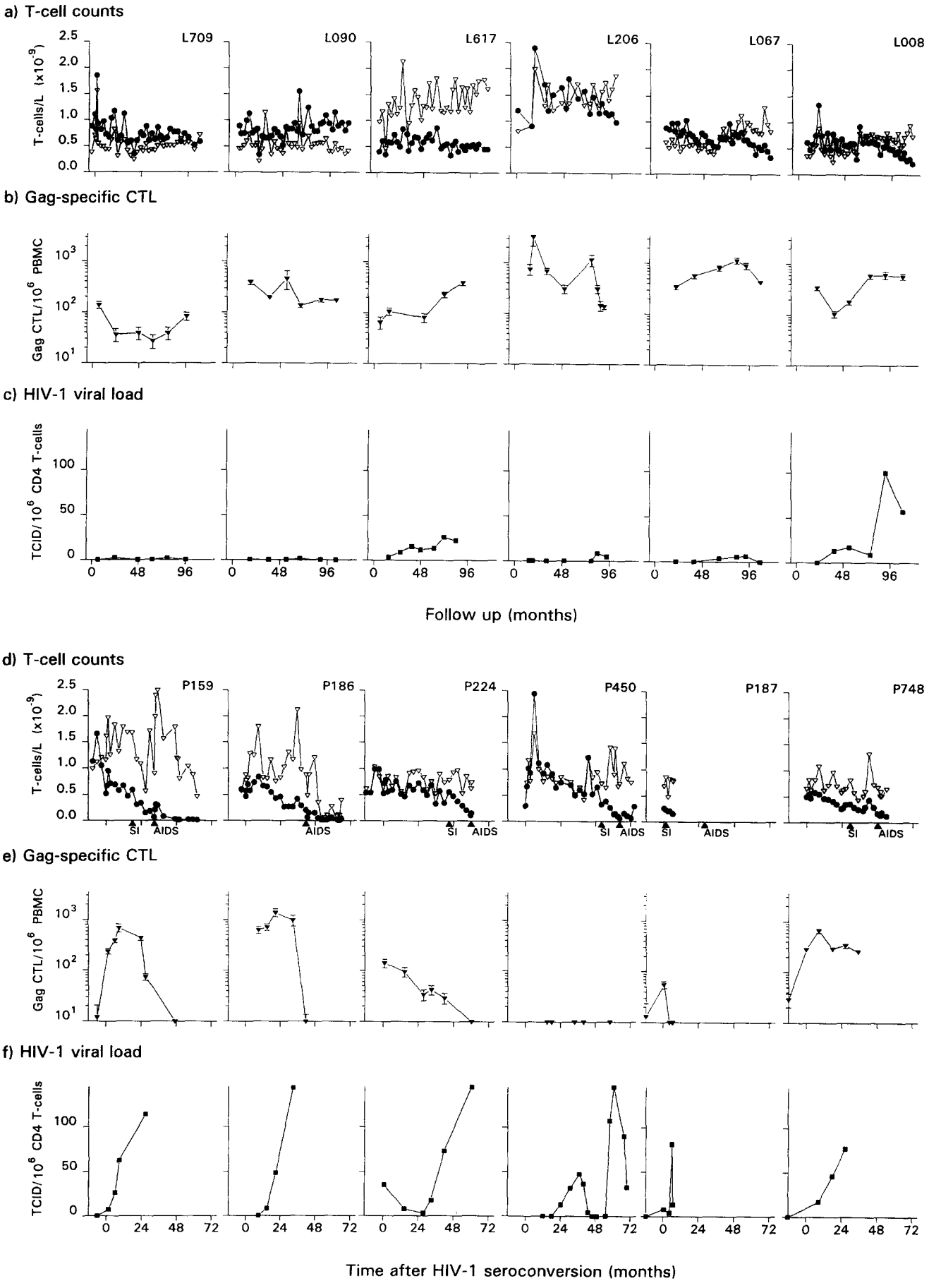

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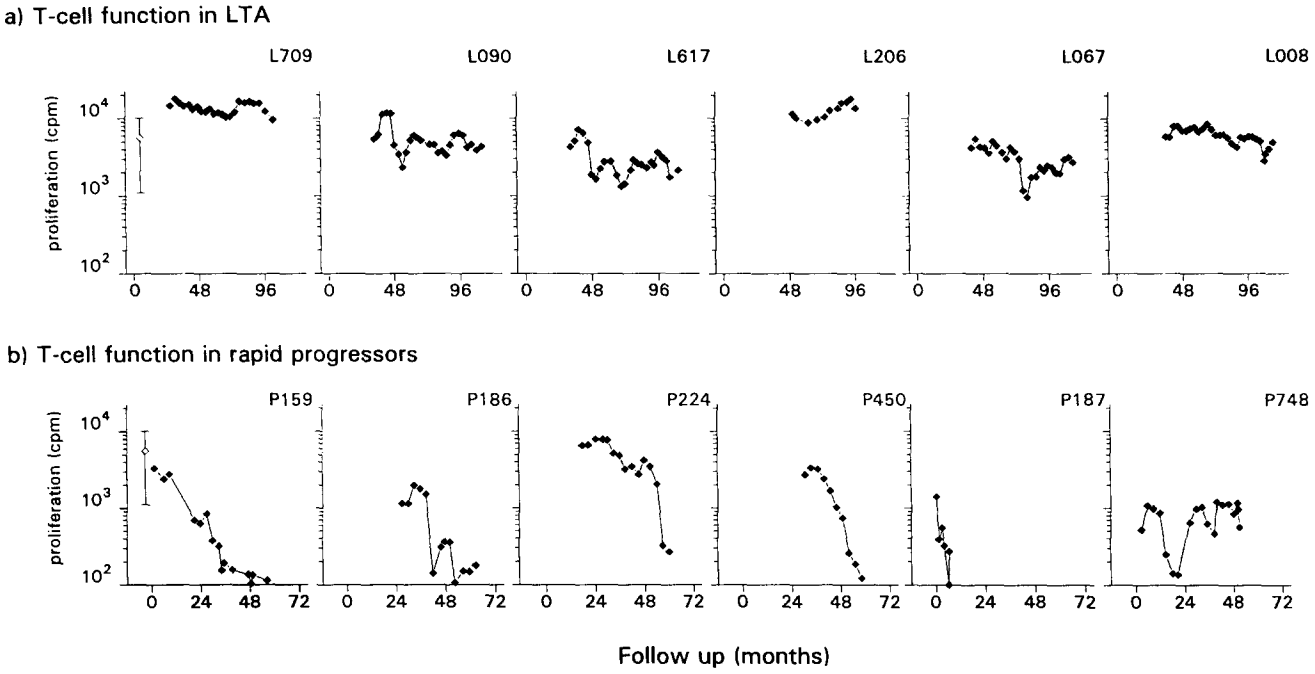

Figure 2. Longitudinal analysis of CD3-mAb induced T cell reactivity. Follow up on x-axis indicates time (mo) after HIV-1 seroconversion or HIV-1-seropositive entry in the study. In vitro T cell function (◆) of LTA (*A*) and progressors (*B*) is measured by whole blood proliferation assays using CD3 mAb, and is expressed as cpm. 90% CI of normal values are indicated by ◇.

CTL responses during the first 2 yr after seroconversion, with frequencies up to 700–1,400/10^6 PBMC. Gag-specific CTL responses in patient P224 gradually subsided over time. In patient P450 repeatedly no Gag-specific CTL responses could be detected in blood samples from 10 different time points (Fig. 1 *E*).

Virological Characteristics during the Clinical Course of HIV-1 Infection. In general, the number of circulating HIV-1–infected $CD4^+$ T cells was low in LTA. From blood samples of subjects L090 and L709 only 2, respectively, 3 virus clones were isolated at 65, respectively, 24 and 78 mo. From subjects L067 and L206, virus could only be isolated at later time points. On average, viral load was <5 TCID/10^6 $CD4^+$ T cells. From L617, virus could be isolated at all time points tested; the frequency of infected cells was low, although it tended to increase towards the end of the study (<25 TCID/10^6 $CD4^+$ T cells). In subject L008, there was a sudden increase of viral load up to 100 TCID/10^6 $CD4^+$ T cells after 91 mo of follow-up, coinciding with a change in viral phenotype (Fig. 1 *C* and Table 1). In all patients studied, viral load increased during progression to AIDS, with the number of infected cells up to 81–415 TCID/10^6 $CD4^+$ T cells. Except for patient P186, all progressors studied here developed AIDS with SI HIV-1 variants. Viral load in patient P450 showed a biphasic course, and the emergence of SI-variants coincided with a high number of HIV-1–infected $CD4^+$ T cells 4 mo before AIDS diagnosis. Increased decline of $CD4^+$ T cells and accelerated progression to AIDS is strongly associated with emergence of SI variants (30), which was most obvious in patient P224 (Fig. 1 *F*).

Discussion

We evaluated HIV-1 Gag-specific CTLp, HIV-1 viral load and general immune status in relation to clinical course of HIV-1 infection, to gain more insight in temporal relations between HIV-1 replication and host immune responses.

Persistent Gag-specific CTL responses and low numbers

Figure 1. Natural history of HIV-1 infection in LTA and rapid progressors. Follow up on x-axis indicates time (mo) after HIV-1 seroconversion or HIV-1–seropositive entry in the study. (*A* and *D*) Longitudinal analysis of CD4 (●) and CD8 (▽) T lymphocyte subsets. Reference values (90% CI) for $CD4^+$ and $CD8^+$ subsets were determined in a group of healthy HIV-1–seronegative volunteers (n = 430), and ranged from 0.56–1.55 and 0.31–1.00*10^9 cells/L, respectively. Arrows (▲) indicate time points when NSI to SI-phenotype switch occurred or date of AIDS diagnosis. (*B* and *E*) Longitudinal analysis of HIV-1 Gag-specific CTL responses in cryopreserved blood samples. Ag-specific CTL effectors were tested in split-well ^{51}Chromium-release assay on autologous B-LCL infected with rVV TG.1144 expressing *gag* or control targets infected with rVV 186 poly, containing no insert. Gag-CTLp frequencies (▼) were computed as differences between CTLp frequencies determined on *gag* versus control targets and normalized to the number of CTLp per 10^6 PBMC. Error bars indicate standard error of calculated frequencies. (*C* and *F*) Longitudinal analysis of HIV-1 viral load in peripheral blood samples. HIV-1 viral load was determined with clonal virus isolation procedures. The number of cells productively infected with HIV-1 is expressed as TCID/10^6 $CD4^+$ T cells (■).

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of circulating HIV-1–infected CD4+ T cells were observed in LTA, together with stable and normal CD4+ counts and preserved T cell functions for more than 8 yr. This may indicate that HIV-1 Gag-specific CTL contribute to maintenance of the asymptomatic state by effectively controlling HIV-1 replication. However, in four out of six progressors, a rise of Gag-specific CTLp frequencies early in infection was paralleled by increasing numbers of HIV-1–infected CD4+ T cells. During subsequent progression, Gag-CTLp frequencies decreased severely in three out of four progressors. Subject P748 with CD4+ counts dropping below 200 cells/mm^3, impaired T cell function, SI viruses, and increasing viral load, all predictive for rapid progression to AIDS (2, 31), remained asymptomatic during follow-up. In this patient, Gag-specific CTLp remained relatively stable during the study period, which may be related to anti-retroviral treatment (32). In subject P224, Gag-specific CTL responses gradually decreased during progression to AIDS. At the time when CTLp frequencies were very low, an increase in viral load, change in biological viral phenotype and subsequent progressive depletion of CD4+ T cells was observed (1, 2). In progressor P450, no Gag-specific CTLp were detected at all. However, it could be that CTL recognizing strain-specific sequences of autologous HIV-1 variants are present that are not detected using prototype HIV-1 sequences. Furthermore, the presence of efficacious CTL responses directed against other antigens of HIV-1 can also not be excluded at the present time.

This longitudinal analysis revealed that five out of six rapid progressors were able to mount substantial Gag-specific CTL responses early in infection, with magnitudes comparable to those observed in LTA. In contrast to observations in LTA however, Gag-specific CTL responses were only transient and disappeared during progression to AIDS, apparently failing to contain viral replication and spread. Increase of viral load in the face of mounting Gag-specific CTL responses might be due to expanding HIV-1 variants which have escaped from CTL recognition (33), but a clear demonstration that these escape variants have selective advantage in vivo is still lacking (34, 35). Another explanation may be that, although CTL can be detected in vitro, they may not be able to execute effector functions in vivo. For example, IL-10, an immunosuppressive cytokine, which has been reported to induce a state of tolerance by downregulating allogeneic CTL responses in human long-term chimeric patients that received HLA-mismatched bone marrow transplants (36), may have frustrated in vivo CTL function in rapid progressors (37). In addition, as has been shown for mice infected with lymphocytic choriomeningitis virus, persistent viral infections may exhaust virus-specific effector CTL resulting in loss of immune surveillance (38).

Zinkernagel and Hengartner (9), have suggested that strong CTL responses in fact could be instrumental in deteriorating the immune system by depleting HIV-1–infected CD4+ T cells and APC. In LTA however, vigourous Gag-specific responses were not detrimental per se, since little loss of CD4+ T cells and well preserved T cell function were observed for more than 8 yr. In patients P224 and 450, precipitous loss of CD4+ T cells and T cell function were observed, only when changes in viral phenotype and viral load occurred, pointing to a role for HIV-1 next to cellular immunity in determining kinetics of clinical progression (28, 30).

In conclusion, our results show that long-term asymptomatic HIV-1 infection is characterized by sustained HIV-1 Gag-specific CTL responses and low numbers of circulating HIV-1–infected CD4+ T cells. Rapid progressors, however, were not protected from disease progression despite high Gag-specific CTLp frequencies early in HIV-1 infection. Besides quantitative aspects of Gag-specific CTL as analyzed here, repertoire differences and phenotypical and functional differences in CTL may contribute to control of HIV-1 infection (39).

Alternatively, based on these data, one could argue that HIV-1–specific CTL responses do not play a critical role in determining the rate of progression to AIDS. Sustained HIV-1–specific CTL activity may merely be a reflection of preserved cellular immunity as observed during long-term asymptomatic HIV-1 infection (22). Loss of HIV-1–specific CTL may be a reflection of progressive immunodeficiency induced by HIV-1 infection (1, 2). Our observations in the progressors suggest that HIV-1–induced perturbation of the immune system, rather than loss of HIV-1–specific CTL, could be the critical event. Clinical outcome of HIV-1 infection may be determined by host genetics (20), virulence of HIV-1 variants (40, 41), as well as by virus–host interactions already at the time of primary HIV-1 infection (42). Thus, our results warrant more detailed studies into underlying pathogenic mechanisms causing immune dysfunction to better understand differences between long-term asymptomatic HIV-1 infection and rapid progression to AIDS.

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