Human immunodeficiency virus type 1 Rev- and Tat-specific cytotoxic T lymphocyte frequencies inversely correlate with rapid progression to Aids

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Human immunodeficiency virus type 1 Rev- and Tat-specific cytotoxic T lymphocyte frequencies inversely correlate with rapid progression to AIDS

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Immunological correlates of AIDS-free survival after human immunodeficiency virus type 1 (HIV-1) infection are largely unknown. Cytotoxic T lymphocyte (CTL) responses are generally believed to be a major component of protective immunity against viral infections. However, the relationship between HIV-1-specific CTL responses and disease progression rate is presently unclear. Here we show in twelve HIV-1-infected individuals that detection of Rev-specific CTL precursors (CTLp) early in the asymptomatic stage, as well as detection of Rev- and Tat-specific CTLp later during follow-up, inversely correlate with rapid disease progression. No such correlation was found for detection of CTLp against Gag, RT or Nef. Further studies are required to determine whether a protective mechanism is indeed the basis of the observed correlation. The data presented are in agreement with the hypothesis that CTL against proteins that are important for early viral transcription and translation are of particular importance in protection from rapid disease progression.

The duration of the asymptomatic period after human immunodeficiency virus type 1 (HIV-1) infection varies considerably (Klein & Miedema, 1995; Haynes *et al.*, 1996), and is inversely related to plasma RNA levels following initial viraemia (Jurriaans *et al.*, 1994; Mellors *et al.*, 1996). These parameters may be determined by viral characteristics, host genetics and immunological factors (Klein & Miedema, 1995; Bollinger *et al.*, 1996; Haynes *et al.*, 1996). Cytotoxic T lymphocyte (CTL) responses are associated with initial control of viraemia, persist in asymptomatic individuals, and eventually decline with disease progression (Carmichael *et al.*, 1993; Borrow *et al.*, 1994; Koup *et al.*, 1994; Klein *et al.*, 1995; Rinaldo *et al.*, 1995; Bollinger *et al.*, 1996; Geretti *et al.*, 1996). This decline coincides with HIV-1-induced $CD4^+$ T cell loss and perturbation of T cell function (Klein *et al.*, 1995; Geretti *et al.*, 1996).

Recent reports on virus escape from HIV-1-specific CTL responses clearly indicate that CTL exert pressure on virus replication *in vivo*, but that the overall CTL efficacy may be dependent on the (lack of) conservation in the epitope sequences (Borrow *et al.*, 1997; Goulder *et al.*, 1997). In addition, it has been suggested that CTL responses against early expressed epitopes may be more efficacious than those against late expressed epitopes (Ranki *et al.*, 1994; Riviere *et al.*, 1994). Qualitative and quantitative analyses of specific CTL responses, before the immune status has deteriorated, may identify the requirements for a CTL response to be protective. In asymptomatic HIV-1-infected individuals the early expressed proteins Rev and Tat are generally less frequently recognized than the late expressed structural proteins Gag and RT (Johnson & Walker, 1994; Riviere *et al.*, 1994; Lamhamedi-Cherradi *et al.*, 1995).

Here, we investigated whether differences between CTL responses against HIV-1 Gag, RT, Nef, Rev and Tat are related to differences in disease progression rates. CTL precursor (CTLp) frequencies were determined in twelve participants of the Amsterdam Cohort Studies on AIDS, who were selected on the basis of their disease progression rates and HLA phenotypes (Table 1). Seven of these individuals remained AIDS-free for more than a decade (median 129 months, range 110–140) after seroconversion (L090, L658, L709 and L434) or

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Table 1. Characteristics of the seven LTA and five progressors selected for this study

Individual*	HLA phenotype A	HLA phenotype B	Serostatus at entry†	Seroconversion interval (months)	Clinical status (months after seroconversion or entry) AIDS	CD4 < 200‡	Asymptomatic Follow-up	CD4 slope (cells/μl per month)	Virus load§ (RNA copies/ml)	Sampling for CTL assays (months after seroconversion or entry)	Corresponding CD4 cell count (cells/μl)
L090	1,2	41,57	I	2·6	NA	NA	> 129	+1·1	$< 10^3$	24	860
										103	1160
L658	1,2	8,61	I	3·3	NA	NA	> 110	−1·3	$8·3 \times 10^4$	4	950
										69	690
L211	1,2	8,57	II	NA	NA	NA	> 139	−3·1	NT	24	730
										74	710
L709	1,69	14,57	I	3·4	NA	NA	> 122	−3·5	$2·5 \times 10^3$	8	850
										84	770
L434	2,28	7,27	I	3·0	NA	NA	> 129	−3·7	$7·4 \times 10^3$	8	630
L008	2,26	27,44	II	NA	NA	132	> 140	−4·5	NT	97	490
L157	3,28	13,14	II	NA	NA	130	> 139	−5·6	NT	88	710
P493	1,2	8,35	I	3·0	40	28		−3·1	$4·7 \times 10^5$	5	420
										9	450
P1215	1,2	7,8	I	3·0	72	50		−4·4	$3·2 \times 10^5$	21	310
										62	280
P356	2,28	27,38	I	3·0	41	38		−7	$1·9 \times 10^4$	13	420
P424	1,2	8,61	I	10·6	43	46		−14	$4·0 \times 10^4$	15	510
P039	1,2	8,44	I	3·4	39	39		−19	$7·4 \times 10^4$	4	870
										15	730

* L, LTA; P, progressor. The number following L or P indicates the number of the participant in the Amsterdam Cohort studies on AIDS.
† I, seronegative; II, seropositive. Seroconversion interval is time between last seronegative and first seropositive visit.
‡ First time-point at which $CD4^+$ T cell count was below 200 cells/μl.
§ Mean serum viral RNA load in first year after seroconversion.
NA, Not applicable.
NT, Not tested.

study entry (L211, L008 and L157). The other five individuals developed AIDS within 3–6 years (median 47 months, range 39–72) after seroconversion (P493, P1215, P356, P424 and P039). In accordance with our previous studies (Van Baalen *et al.*, 1993, 1996; Klein & Miedema, 1995; Klein *et al.*, 1995), and to highlight the profound difference in progression rates, these individuals are referred to as LTA and progressors, respectively. AIDS-defining symptoms of the progressors were: Kaposi's sarcoma (P493); *Candida albicans* oesophagitis (P1215, P424 and P039); *Pneumocystis carinii* pneumonia (P356). Rates of $CD4^+$ T cell decline (slopes) were calculated from $CD4^+$ T cell counts measured at regular 3 monthly intervals during the entire follow-up period (Table 1). Only two of the twelve individuals received antiviral therapy during follow-up. For L008 and P1215 AZT therapy was started at 109 and 51 months after entry, respectively, and DDC therapy was started at 126 and 69 months, respectively. No CTLp frequencies were determined after the start of antiviral therapy. HLA-A and -B phenotypes of the individuals were serologically determined at the Department of Transplantation Immunology, CLB, Amsterdam (Table 1).

Because CTL responses may be impaired as a result of disease progression, we decided to test samples collected before deterioration of the immune status had become evident. Retrospective CTLp frequency analyses were performed on the earliest available PBMC samples. Most of the individuals could also be tested during follow-up. The PBMC were cultured *in vitro* in RPMI 1640 containing 10% human pooled serum and recombinant IL-2 under limiting dilution conditions for 14–20 days, as described previously (Van Baalen *et al.*, 1993; Klein *et al.*, 1995). On day 0 and day 7, cultures were stimulated by addition of paraformaldehyde-fixed autologous B lymphoblastoid cell lines infected with recombinant vaccinia viruses VVTG1144 (Gag), VVTG4163 (RT), VVTG1147 (Nef), VVTG4113 (Rev) or VVTG3196 (Tat), kindly provided by M. P. Kieny (Transgène, Strasbourg, France). Cytotoxic activity was measured by standard 4 h ^{51}Cr-release assays (Van Baalen *et al.*, 1993; Klein *et al.*, 1995). CTLp frequency calculations were performed as described previously (Geretti *et al.*, 1996).

The most striking finding of these studies was that Rev- and Tat-specific CTLp were predominantly detected in the LTA (Fig. 1): their frequencies in LTA and progressors proved to be

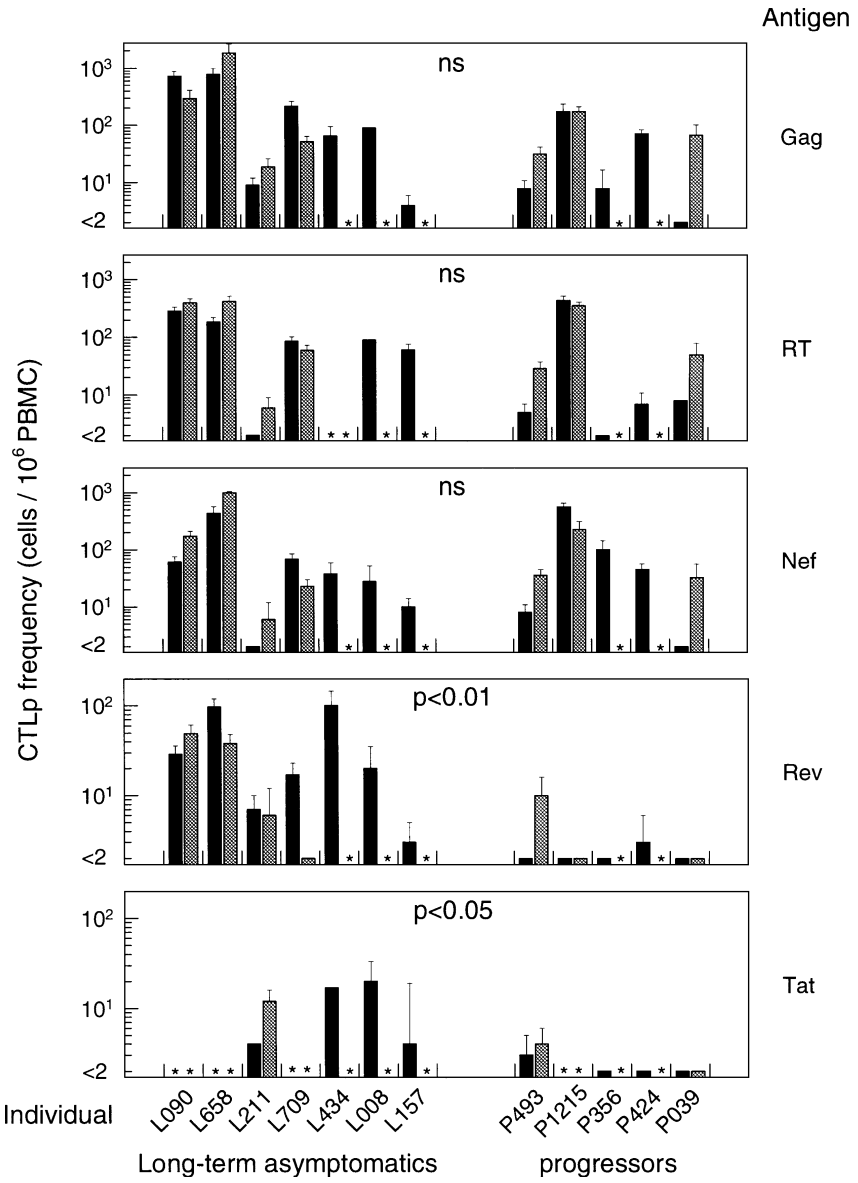

Fig. 1. Frequencies of CTLp against HIV-1 Gag, RT, Nef, Rev and Tat detected in the asymptomatic stage of seven LTA and five progressors. Cultures were established using cryopreserved PBMC sampled from LTA and progressors at time-points indicated in Table 1. Progressor samples were tested in parallel with those of LTA sharing at least three HLA-A and -B alleles. Solid bars, first available sampling time-points; hatched bars, second sampling time points. Error bars indicate standard error of calculated frequencies. *P* values result from the Mann–Whitney analyses in which the CTLp frequencies of the LTA were compared to those of the progressors. ns, not significant; * not tested.

significantly different (Mann–Whitney $P < 0{\cdot}01$ and $P < 0{\cdot}05$, respectively). In contrast, CTLp directed against Gag, RT or Nef were found at similar frequencies in individuals of both groups.

Since all the progressors and four of the seven LTA were seronegative at study entry, the data could be analysed in relation to the time elapsed after infection. During the first 24 months after seroconversion, Rev-specific CTLp, but not Gag-, RT- or Nef-specific CTLp, proved to be significantly more prevalent in these LTA (Mann–Whitney $P < 0{\cdot}02$). Due to limited numbers of PBMC, this analysis could not be made for Tat-specific CTLp. Collectively, these data show that the detection of Rev-specific CTLp early in the asymptomatic stage, as well as the detection of Rev- and Tat-specific CTLp during follow-up, are inversely correlated with rapid disease progression.

The failure to detect responses against Rev and Tat in the progressors could have resulted from an overall decline in CTLp frequencies or from the early impairment of $CD4^+$ T cell function. However, as for the LTA, frequencies of CTLp against Gag, RT and Nef remained stable or even increased early after infection. This indicates that the absence of detectable Rev- and Tat-specific CTL activity in progressors was not due to a general failure of CTL responses *in vivo*.

To explain the specific absence of detectable Rev- and Tat-specific CTL responses in the progressors, a number of hypotheses may be considered. These include the absence of functional HLA–epitope complexes due to either host genetic (Klein *et al.*, 1994; Kaslow *et al.*, 1996) or viral characteristics (Phillips *et al.*, 1991; Couillin *et al.*, 1994; Koenig *et al.*, 1995), and the mobilization of a restricted T-cell receptor repertoire (Pantaleo *et al.*, 1994; Kalams *et al.*, 1996). It may be expected

Table 2. HLA class I peptide-binding motifs of Rev sequences obtained from non-cultured PBMC of L658 and P424

The individuals L658 and P424 share HLA-A1, 2; -B8, 40, 61; -C2, 7; -DR3, 6, 13; -DR52; -DQ1, 2. Sequences were determined for 20 and 19 individual recombinant PCR clones generated from PCR amplification products of the individuals, respectively. Sequences were analysed for the presence of HLA-A1, 2 and -B8, 61 peptide-binding motifs (Rammensee *et al.*, 1995). Anchor residues are underlined. Motifs of HIV-1_{Lai} Rev, which was used for CTL detection, are indicated for reference purposes.

	HLA-A1 (X[ST]XXXXXXY)		HLA-A2 (X[LM]XXXXXX[VL])				HLA-B8(XX[KR]X[KR]XXX[IL])	
	Sequence	Frequency	Sequence	Frequency	Sequence	Frequency	Sequence	Frequency
Lai	ISERILSTY		YLGRSAEPV		ILVESPTVL		RWRERQRQI	
L658	L.GWL...T	16/20	.T.....T	20/20	.T.....T	19/20	..T.T...T	20/20
	L.GWLI..T	4/20			.T.....A.	1/20		
P424	.TGW...TS	15/19	S....K.T	19/19	.T....E.T	18/19	..TQ...T	19/19
	.TGW...NS	4/19			.T.G..E.T	1/19		

on the basis of protein size that the number of CTL epitopes on Rev and Tat is smaller than that on Gag and RT. This may also explain the overall lower levels of CTLp against Rev and Tat in the LTA as compared to those against Gag, RT and Nef.

Given the number of matching HLA class I alleles between progressors and LTA, it may be considered that variation in viral sequences has had an impact on the generation of HLA–epitope complexes in these individuals. In support of this hypothesis, we found differences in the Rev sequences of viruses obtained from LTA L658 and progressor P424, who differed markedly in their CTL response to Rev but were identical for the HLA class I and class II alleles tested (Table 2). High-molecular-mass DNA was isolated from PBMC using cell lite beads (Boom *et al.*, 1991). A nested PCR was used to amplify the second exon of *rev*. Outer primers were AAATGTCAGCACAGTACAATGT and CATTGGTCTTAAAGGTACCTG, inner primers were GTACTTTCTATAGTGAATAGAGTTAGGC and CCTATCTGTCCCCTCAGCTACT. PCR conditions were: 5 min 95 °C, 1 min 55 °C, 1 min 30 s 72 °C for one cycle then 50 s 95 °C, 50 s 55 °C, 1 min 30 s 72 °C for 30 cycles and 7 min 72 °C extension for both the outer and inner amplification. Amplified fragments were cloned with a pCR2 kit according to the manufacturer's protocol (Invitrogen). Clones were sequenced with a Taq Dye Deoxy Terminator sequencing kit on an Applied Biosystems 373A sequencing system. All clones were sequenced on both strands with the inner primers. Sequences were analysed with Geneworks (IntelliGenetics). In viral sequences from L658, who developed Rev-specific CTL, the anchor residues of a previously described HLA-A1 peptide-binding motif were present. One of these anchor residues was not present in all viral sequences obtained from P424, who did not show Rev-specific CTL responses. Considerable variation was found in HLA-A2 and HLA-B8 peptide-binding motifs of Rev. The influence of this variation on the antigenicity of these sequences remains to be elucidated. In addition, the presence of HLA class I alleles unique to the LTA may also, at least in part, explain the observed differences in CTL responses between LTA and progressors. Three of the seven LTA, but none of the progressors, were positive for HLA-B57. This allele has been suggested to be associated with prolonged survival (Klein *et al.*, 1994; Kaslow *et al.*, 1996). To address this point, a larger number of HLA-matched individuals would have to be studied. Whether Rev and Tat epitopes may be presented in the context of HLA-B57 is presently being studied.

To investigate the relationship between viral loads and disease progression rates in these individuals, serum viral RNA loads were determined in the individuals with a known seroconversion time-point (Table 1). Mean HIV-1 RNA loads in the first year after infection were quantified using a nucleic acid sequence-based amplification assay (NASBA HIV-1 RNA QT; Organon Teknika), according to the manufacturer's instructions. As expected, these mean viral loads were relatively low in most LTA and high in the progressors. These differences could be due to viral characteristics. However, the *in vitro* replication of viruses isolated early after infection from these groups showed similar kinetics (H. Schuitemaker and others, unpublished), which is in line with the observation that predominantly slowly replicating NSI variants are transmitted (Connor & Ho, 1994).

Rev- and Tat-specific CTL may influence viral load more efficiently than CTL against structural proteins in different ways. Firstly, in the asymptomatic stage, many infected cells, both in circulation and in lymph nodes, do not actively produce virus, but do express multiple spliced mRNAs that encode the regulatory proteins (Seshamma *et al.*, 1992; Embretson *et al.*, 1993). This renders these cells targets for CTL against regulatory, but not against structural proteins. Secondly, in the replicative cycle, Rev and Tat are expressed earlier than the structural proteins. This may allow specific CTL to kill infected cells well before release of progeny virus (Ranki *et al.*, 1994; Riviere *et al.*, 1994). Although both considerations would also hold true for Nef, specific CTL responses to this protein were observed in most progressors. This may be

related to a lack of major structural constraints on Nef (Couillin *et al.*, 1994; Koenig *et al.*, 1995).

Our data which indicate that Rev- and Tat-specific CTL are involved in protection from rapid disease progression are in line with earlier suggestions that CTL responses against conserved and early proteins of HIV-1 would be protective (Johnson & Walker, 1994; Riviere *et al.*, 1994, 1995; Harrer *et al.*, 1996; Van Baalen *et al.*, 1996). Recent data obtained from studies in SIV_{mac}-infected macaques (Hulskotte *et al.*, 1995) indicate that in these animals also, Rev-specific CTL responses inversely correlate with disease progression (A. M. Geretti, unpublished). Analysis of the efficacy of CTL specific for the early and regulatory proteins Rev and Tat in containing HIV-1 infection in *in vitro* and *in vivo* models, which are prompted by the results of the present study, will contribute to our understanding of the pathogenesis of human and animal lentivirus infections. This will facilitate the development of rational strategies for vaccination and specific immunotherapy.

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