Kinetics and Specificities of the T Helper-Cell Response to GP120 in the Asymptomatic Stage of HIV-1 Infection

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Peripheral blood mononuclear cells from 36 asymptomatic HIV-1 seropositive individuals were tested longitudinally for *in vitro* T-cell proliferation and IL-2 production in response to synthetic peptides spanning the entire gp120 of HIV-1. At baseline, significant T-cell proliferation to pooled and individual peptides was observed in 15 of the 36 donors. After 12 months, proliferative responses to peptide pools were lost or decreased significantly in most donors. Responses appeared to fluctuate over time: at 12 months new recognition sites were detected in four of 10 donors showing T-cell proliferation at baseline, as well as in five of 15 donors with no previous proliferative responses. IL-2 production appeared to be a more sensitive and longer preserved parameter of T-helper cell function: at baseline the majority of donors with no T-cell proliferation produced IL-2 in response to pooled peptides. This response was not decreased significantly after 12 months. The overall patterns of response to both pooled and individual peptides were heterogeneous among donors. Multiple recognition sites were detected in both variable and conserved regions of gp120, but no pool or individual peptide was recognized by all responders. Functional T-cell responses were not statistically correlated to CD4$^+$ cell percentile and absolute numbers.

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INTRODUCTION

T-helper lymphocytes are known to play a central role in the generation of protective immunity against infectious agents [1–4]. Although the immune mechanisms which may mediate protection against HIV-1 are still largely unclear, increasing evidence suggests that the generation of strong T helper-cell responses is critical to the prevention or control of HIV-1 infection. T helper-cell responses against gp120 have been demonstrated in individuals exposed repeatedly to HIV-1 but lacking serological or other evidence of HIV-1 infection [5]. Furthermore, HIV-1 infected apes, which do not seem to develop HIV-1-related disease, have vigorous T helper-cell responses towards various HIV-1 antigens [6, 7].

Among HIV-1 antigens, the envelope protein is believed to play a major role in the induction of protective B- and T-cell mediated immunity against the virus [8–10]. The T helper-cell immunostimulatory properties of gp120 have been studied in animal models, vaccinated volunteers, and in both seroposit- ive and seronegative donors [9, 11–21]. Synthetic peptides have been commonly used for the screening of the gp120-specific T helper-cell repertoire. Beside providing information on defined T helper-cell epitopes, the use of discrete peptides rather than whole antigens has been reported to increase the detection level of specific responses [18, 21, 22], and to allow discrimination between putative T-cell activating and suppressive sites [22, 23]. Moreover, this approach circumvents the problem of HIV-1 induced defects of antigen presentation which may interfere with the *in vitro* analysis of T helper-cell responses [24, 25]. Although measurement of peripheral blood mononuclear cell (PBMC) proliferation upon antigen stimulation is widely used to test T helper-cell reactivity *in vitro*, it has been suggested recently that the measurement of IL-2 production is a more sensitive parameter to test for exposure to HIV-1 than T-cell proliferation [5, 15].

The authors measured T-cell proliferation and IL-2 production of PBMC from 36 asymptomatic HIV-1 infected individuals in response to *in vitro* stimulation with synthetic

peptides spanning the entire gp120 of HIV-1IIIB. T helper-cell responses against several conserved and variable sites of gp120 in HIV-1 infected individuals have been described previously. However, since most previous studies have focused on limited numbers of recognition sites [11, 15, 16, 19, 20], to the authors' knowledge the T helper-cell repertoire for the entire protein has not been screened before in seropositive individuals. Although T helper-cell responses observed in asymptomatic HIV-1 infected individuals have been higher than in those who have progressed to symptomatic disease [16, 18, 19], little is known about the longevity of these responses during the asymptomatic phase of infection. The authors' goal was to gain further insight into the specificities and kinetics of T helper-cell responses during the asymptomatic stage of HIV-1 infection, before severe CD4$^+$ cell-count depletion and deterioration of the clinical status occur. The results indicate that the recognition of gp120 is heterogeneous in asymptomatic seropositive individuals and appears to fluctuate over time. Furthermore, the authors showed that in HIV-1-infected individuals IL-2 production is a more sensitive and, longer preserved parameter of T helper-cell function than proliferative T-cell responses.

MATERIALS AND METHODS

Study group. The study included 36 HIV-1 infected individuals 24 males and 12 females), aged 22–52 years, randomly selected from a cohort monitored by the Utrecht University Hospital (Utrecht, The Netherlands) between 1987 and 1991. HIV-1 infection was related to high-risk homosexual or bisexual contacts ($n=21$), heterosexual contacts ($n=10$), or intravenous drug use ($n=5$). To be included in the study, HIV-1-infected individuals had to have baseline circulating CD4$^+$ cell counts above 400 cells/μl, and, throughout the study, had to show no symptoms or signs of HIV-1 related disease (stage II of the Centre for Disease Control (CDC), Atlanta, GA, USA, classification). T helper-cell responses were evaluated retrospectively with PBMC collected at a baseline visit and, depending on availability of blood samples, 6 and 12 months later. Responses were assessed in nine separate experiments, each including five HIV-1 infected donors. Nine of the 36 donors were tested twice in independent experiments. Total leucocyte counts, as well as percentages and absolute numbers of CD4$^+$ cells, were determined by standard methods. PBMC from 11 HIV-seronegative healthy blood donors were used as controls.

Synthetic peptides. A panel of 45 partially overlapping synthetic peptides (20 mers overlapping by 10 amino acids), spanning the entire sequence of gp120 of the IIIB strain of HIV-1, was provided by Dr H. Holmes through the Medical Research Council's AIDS Directed Programme (South Mimms, Potters Bar, UK). Individual peptides or nine pools (A–I) of five contiguous peptides were used for *in vitro* stimulation of PBMC.

Stimulation of PBMC. PBMC were separated from heparinized blood by density gradient centrifugation on Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway), washed three times in RPMI 1640 containing penicillin (100 U/ml), streptomycin (100 μg/ml), 10^{-5} M β-mercapto ethanol and 2 mM L- glutamine (referred to as 'complete medium'), and cryopreserved in liquid nitrogen until use. Cell viability (as assessed by trypan blue exclusion) was preserved after thawing. PBMC (1.5×10^5/well) were cultured in triplicate in flat bottomed 96-well microtitre plates (Greiner, Frickenhausen, Ger-

many) in a final volume of 150 μl of complete medium supplemented with 10% heat inactivated human serum pooled from seronegative donors. Cells were stimulated with either pooled or individual peptides, at a final concentration of 10 μg/ml of each peptide. Phytohaemoagglutinin (PHA-M, Boehringer Mannheim, Mannheim, Germany) at a final concentration of 10 μg/ml, was used to test lymphocyte responsiveness to non-specific stimulation. Unstimulated cultures (cells in medium supplemented with 10% human serum) were used as medium control.

T helper-cell assays. For T-cell proliferation, PBMC were incubated for 6 days at 37°C in a 5% CO$_2$ humidified atmosphere, pulsed for 18 h with 0.5 μCi of (^{3}H)-thymidine, and harvested on glass-fibre filter papers. (^{3}H)-thymidine incorporation was measured in a liquid scintillation β-spectrometer. For measurement of IL-2 production, PBMC were cultured as outlined above in the presence of an anti-IL-2 receptor monoclonal antibody (MoAb) (kindly provided by Dr L. Aarden, CLB, Amsterdam, The Netherlands), at 1:500 final dilution, to prevent IL-2 consumption [15]. Supernatants were harvested 7 days later and frozen at -20°C until use. IL-2 level was defined as the ability to stimulate the proliferation of the IL-2-dependent cell line CTLL-2. For this purpose, 2 days after the last feeding, CTLL cells (0.5×10^4 cells/well) were cultured in round bottomed 96-well microtitre plates, in complete medium supplemented with 10% heat inactivated fetal calf serum (FCS). Supernatants from PBMC cultures were added to the wells at 50 μl/well (dilution 1:2). Following a 24-h incubation at 37°C, CTLL proliferation was determined by measuring overnight incorporation of (^{3}H)-thymidine, as described above. CTLL cells were maintained in 25-cm^2 flasks (Becton Dickinson, UK) in 10 ml of complete medium supplemented with 10% FCS and 10 U/ml recombinant IL-2 (rIL-2, Biotest Diagnostics, Dreieich, Germany).

Results of PBMC and CTLL proliferation assays are given as stimulation indices (SI) according to the following formula:

$$SI = \frac{\text{mean counts per min in stimulated cultures}}{\text{mean counts per min in medium control cultures}}$$

Standard error of mean values was consistently below 27%.

Statistical analysis. Proliferative responses and IL-2 production measurements with PBMC from 11 HIV-1 seronegative blood donors showed a (one-sided) 99% upper confidence limit from the t-distribution of 2.87 and 2.98, respectively. Based on this observation, SI from HIV-1 seropositive donors were considered significant when scoring $\geqslant 3.0$. Linear regression analysis was performed to evaluate the relationship between T helper-cell responses and absolute numbers and percentages of circulating CD4$^+$ cells.

RESULTS

Proliferative responses to gp120 peptide pools at baseline

Baseline proliferative responses of PBMC from 36 HIV-1 seropositive donors stimulated *in vitro* with nine pools (A–I) of five contiguous peptides are shown in Table 1 (left panel). Significant T-cell proliferation to at least one peptide pool was detected in 15 of the 36 donors (42%; referred to as 'responders'), whereas 21 donors (referred to as 'non-responders') did not show significant proliferative responses. The mean number of pools recognized by all responders was 2.9 ± 1.1. Ten of the 15 responders recognized three or more pools. Positive SI values ranged up to 14.3 (mean 5.1 ± 2.7).

Table 1. Proliferative responses of PBMC from HIV-1 seropositive donors to peptide pools A to I. (bold: SI ⩾ 3.0).

Donor	Baseline									Baseline								
	A	B	C	D	E	F	G	H	I	A	B	C	D	E	F	G	H	I
1*	1.7	1.1	2.2	1.2	**8.8**	**5.4**	**5.4**	1.0	**8.2**	2.0	1.9	**5.4**	2.8	**5.8**	**4.1**	**3.0**	2.7	**5.3**
2	**6.6**	1.2	**3.9**	**3.6**	1.7	1.1	2.4	**3.8**	2.0	**3.2**	2.1	1.1	0.7	1.2	1.9	1.9	1.8	0.6
3	1.5	**3.0**	**4.2**	**3.8**	1.7	2.0	1.7	1.0	**6.4**	1.3	1.2	1.6	1.2	2.8	1.1	1.5	2.3	1.3
4*	**5.3**	0.9	**4.0**	0.9	**4.4**	0.5	0.9	0.7	**4.6**	1.0	1.7	**3.9**	1.4	**3.4**	1.3	1.5	**6.1**	**3.5**
5	1.9	**3.6**	**3.2**	1.2	**3.7**	0.9	**3.1**	1.2	1.6	1.4	1.8	1.2	1.0	1.3	1.0	1.9	1.2	1.2
6	2.2	**6.1**	0.4	1.0	0.6	1.3	**14.3**	0.7	**14.1**									
7	1.1	**9.0**	1.8	**3.6**	2.3	**4.3**	2.4	1.3	1.2									
8*	**8.8**	**4.8**	**3.3**	1.3	1.6	1.8	2.0	1.3	1.4	**7.5**	**6.8**	2.2	2.2	1.6	1.2	**3.2**	1.9	1.4
9	**4.0**	1.3	1.7	1.7	**3.2**	1.0	**3.0**	1.2	1.0									
10	1.6	2.1	2.3	2.5	1.9	**3.6**	2.9	**3.2**	**3.6**	1.5	0.9	1.1	1.0	1.9	0.9	1.0	2.6	1.4
11	1.7	1.1	1.2	0.9	**3.2**	1.1	**3.2**	1.2	**3.4**	1.4	0.9	0.9	**3.6**	1.3	**4.5**	**4.8**	0.6	1.9
12	**7.3**	1.7	1.1	1.4	**6.2**	0.8	2.0	0.9	2.4	1.0	1.8	1.0	1.5	1.0	0.8	0.8	2.8	1.0
13*	1.4	1.7	0.6	0.9	0.8	**4.1**	1.1	0.5	0.7									
14	1.9	1.2	2.1	**3.3**	2.7	1.7	2.4	1.1	1.2	1.8	2.7	2.2	1.7	2.3	1.6	**3.0**	2.1	2.1
15	1.5	1.4	**3.1**	2.9	2.9	1.2	1.4	1.8	1.8									
16	1.0	0.9	0.9	1.0	1.5	0.8	0.8	0.9	1.2	1.4	1.0	1.0	1.0	1.6	1.9	1.8	1.0	1.7
17	1.8	0.8	1.0	1.7	2.1	1.3	0.9	1.0	1.0	0.7	1.1	0.8	0.8	1.6	0.9	0.8	0.8	1.5
18	1.8	1.3	1.0	1.2	0.8	1.2	1.2	0.4	1.6	2.8	1.9	2.5	2.0	**4.2**	2.9	1.9	**8.2**	1.2
19	1.0	1.1	1.0	0.5	1.7	1.0	1.2	1.4	0.9	1.1	0.9	1.3	1.1	1.0	0.8	0.9	1.0	0.9
20	2.3	0.9	1.0	1.1	1.1	1.1	1.0	1.0	1.0									
21*	0.9	0.6	1.5	1.8	1.5	2.2	0.8	1.2	0.9	1.1	1.1	1.2	1.8	1.1	1.3	1.3	1.3	1.4
22*	1.7	1.3	1.2	2.2	1.8	1.3	2.3	1.9	2.3	1.4	1.2	1.5	1.7	1.5	1.7	2.3	1.8	1.1
23	0.8	0.8	1.2	1.2	1.4	1.5	1.2	1.4	1.4									
24	1.6	0.9	1.0	1.1	1.5	0.9	1.2	1.4	1.0	0.7	0.5	0.6	0.8	1.2	1.0	1.3	1.6	0.9
25	2.2	1.5	1.2	2.4	1.2	1.9	2.1	2.2	1.8	2.0	0.9	**5.4**	1.2	1.3	1.4	2.3	**3.3**	2.5
26	1.7	1.9	1.9	1.8	1.7	1.9	1.6	1.6	2.2	2.3	1.0	1.3	1.1	1.0	1.4	2.0	1.8	1.4
27	1.1	0.5	1.0	1.0	1.4	0.8	0.7	0.8	1.0									
28	0.8	2.1	1.3	1.0	0.8	1.6	1.5	1.5	1.8	1.7	1.3	1.2	1.9	1.3	1.0	2.2	1.2	1.1
29	1.0	0.7	0.8	0.5	1.1	1.1	1.4	0.9	1.6	0.8	0.9	2.4	1.8	1.5	2.1	1.1	1.5	1.0
30	0.7	0.9	1.5	0.7	1.7	0.6	1.0	1.0	0.6									
31*	1.6	0.8	0.7	1.0	2.5	0.8	0.8	0.8	1.2	0.8	1.0	0.6	2.1	1.3	1.3	1.1	1.6	0.6
32*	1.1	1.4	1.5	1.2	1.0	0.7	1.1	1.3	2.1	**4.1**	1.2	1.2	1.4	1.5	1.1	1.0	1.4	1.6
33	1.4	1.2	1.6	1.1	2.7	2.8	2.0	1.7	1.5									
34	1.0	0.7	1.2	1.4	1.6	0.9	0.7	1.0	1.5	1.1	1.0	0.7	1.1	**3.6**	1.2	0.7	1.6	1.1
35	1.1	0.4	1.3	1.0	1.1	0.5	1.1	0.8	2.6									
36*	0.9	1.1	0.7	1.3	1.5	1.3	1.0	0.8	1.0	**4.1**	1.2	1.2	1.4	1.5	1.1	1.0	1.4	1.6

* Results are mean SI values from two independent experiments (SD < 10%).

PBMC from 11 HIV-1 seronegative healthy controls did not proliferate to any of the peptide pools (data not shown).

Proliferative responses to gp120 peptide pools 12 months after baseline

Proliferative responses could be evaluated 12 months after baseline determination with PBMC from 10 responders and 15 non-responders. As shown in Table 1 (right panel), four of the 10 responders lost their proliferative responses (individuals 3, 5, 10 and 12), whereas six retained their responses to at least one pool of peptides. Although in most cases proliferative responses were directed towards the same pools recognized at baseline, new responses to previously unrecog-

nized pools were demonstrated in four responders (individuals 4, 8, 11 and 14). New reactivities were observed also with PBMC from five of the 15 non-responders towards one (individuals 32, 34 and 36) or two (individuals 18 and 25) peptide pools. The mean number of pools recognized by all donors was 2.2 ± 1.4. Positive SI values ranged up to 8.2 (mean 4.6 ± 1.4). Patterns of pool recognition were still heterogeneous 12 months after baseline.

The overall response of the PBMC from these 25 donors to non-specific stimulation with PHA remained intact during these 12 months. Differences in peptide pool-specific responses did not correlate with differences in PHA-induced responses: 18 of the donors showed a higher SI to PHA at 12

Table 2. Proliferative responses to individual peptides of PBMC from 15 HIV-1 seropositive donors responding to one or more peptide pools.

Peptide number*	Amino-acid sequence	Region	Number of responders	Highest SI	Mean SI of all responders
A1	30–51		2	6.3	4.6
A2	42–61		2	8.7	6.6
A3	52–71		3	5.3	4.3
A4	62–80		1	3.5	3.5
A5	72–91		—	—	—
B1	81–101		2	5.4	5.1
B2	92–111		—	—	—
B3	102–121		1	5.9	5.9
B4	112–130		3	6.0	4.4
B5	122–141		1	3.1	3.1
C1	131–151	V1	3	4.6	3.9
C2	142–161		4	6.5	4.3
C3	152–171		4	6.7	4.4
C4	162–182	V2	1	3.3	3.3
C5	172–191		4	7.4	5.4
D1	182–201		—	—	—
D2	192–211		1	3.6	3.6
D3	202–221		—	—	—
D4	212–231		3	5.5	4.2
D5	222–241		2	5.0	4.8
E1	232–251		3	4.3	3.9
E2	242–261		1	3.4	3.4
E3	252–271		1	7.4	7.4
E4	262–281		2	3.2	3.1
E5	272–291		4	8.3	5.0
F1	292–310		1	9.9	9.9
F2	302–321	V3	1	5.6	5.6
F3	311–329		—	—	—
F4	322–341		1	7.6	7.6
F5	330–349		3	7.8	5.5
G1	342–361		2	9.1	6.0
G2	350–370		1	3.2	3.2
G3	362–381		—	—	—
G4	380–393		4	5.8	4.4
G5	392–411	V4	1	9.3	9.3
H1	402–420		—	—	—
H2	412–431		1	5.7	5.7
H3	421–440		—	—	—
H4	432–451		1	6.3	6.3
H5	441–461		—	—	—
I1	452–471	V5	2	3.5	3.5
I2	462–481		—	—	—
I3	472–491		2	9.8	7.2
I4	482–501		3	8.2	6.0
I5	492–512		1	4.9	4.9

* Individual peptides are indicated by respective pool (A–I) and by position inside the pool (1–5).
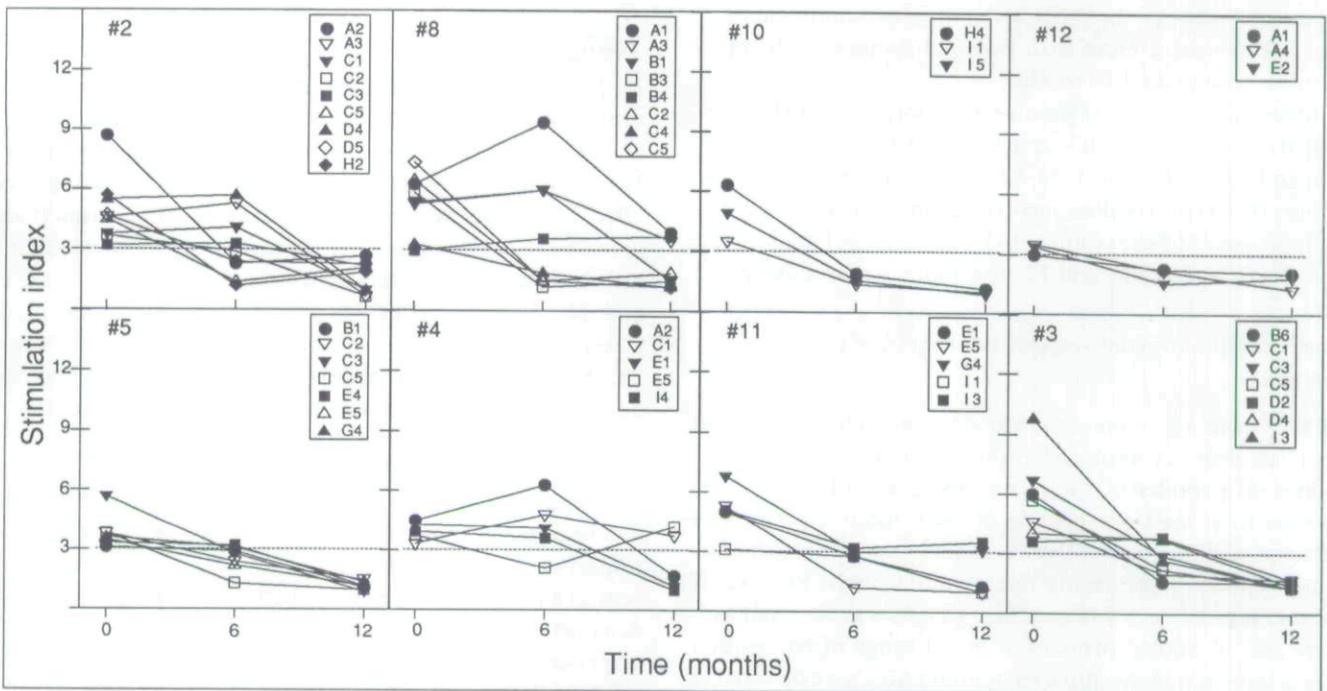
Fig. 1. Proliferative responses to individual peptides determined at baseline, and after 6 and 12 months in eight HIV-1 seropositive individuals. Symbols indicate individual peptides used for cell stimulation.

Table 3. IL-2 production defined as SI of CTLL proliferation to culture supernatants of PBMC from HIV-1 seropositive donors stimulated with peptide pools A–I. (bold: SI ⩾ 3.0)

Donor	Baseline									12 months								
	A	B	C	D	E	F	G	H	I	A	B	C	D	E	F	G	H	I
6	2.5	**3.0**	2.5	2.1	**3.8**	1.6	**3.9**	2.0	**3.5**									
8*	**3.6**	**3.6**	**4.2**	2.8	2.7	2.1	1.7	2.8	1.8									
13*	**4.5**	**3.1**	2.1	2.1	**5.6**	**7.5**	**4.0**	2.2	**4.2**									
15	**9.7**	1.7	**3.6**	1.7	2.4	1.6	**3.2**	1.5	**9.2**									
17	**4.8**	1.4	**7.4**	**4.2**	**3.5**	2.4	1.3	0.6	**7.5**	2.8	1.3	**4.9**	**3.3**	2.7	2.3	1.4	1.1	**3.3**
18	**5.6**	2.1	2.3	**3.0**	**4.1**	1.8	2.4	1.9	**4.5**	**3.3**	0.9	2.2	**8.4**	**8.7**	0.8	0.9	2.9	0.6
19	1.8	**7.7**	1.7	1.5	2.3	1.5	**15.0**	1.1	**3.0**	0.7	1.5	1.2	1.1	0.5	1.0	2.3	0.8	**3.1**
20	**3.7**	2.4	**3.5**	1.6	**3.4**	1.7	2.0	1.7	1.9									
21*	1.0	0.9	**3.0**	1.8	**3.3**	2.2	1.8	1.1	**3.7**	1.1	1.1	**8.1**	1.4	**9.5**	1.2	2.0	1.0	**19.0**
22*	1.6	0.9	1.5	**4.4**	**9.7**	1.2	2.0	1.2	1.0									
23	0.5	1.2	1.0	0.5	**4.5**	0.8	0.5	1.5	2.6									
24	**3.1**	2.0	**3.6**	1.0	1.1	0.6	0.9	1.4	0.7	**3.3**	1.7	1.9	2.2	2.5	1.3	1.4	1.6	1.8
25	0.7	**3.0**	**3.2**	2.8	0.4	0.7	1.0	1.2	1.7	1.3	0.9	**3.6**	0.6	0.5	1.0	0.9	**3.3**	0.7
26	2.5	1.4	**8.5**	1.3	1.3	0.9	2.5	1.0	2.8	**5.0**	0.9	2.9	1.4	1.2	0.9	**12.3**	1.3	**6.6**
27	1.3	0.7	1.6	2.1	**4.5**	2.1	2.1	2.6	1.7									
28	0.6	**3.2**	1.1	**3.7**	1.1	0.9	0.6	0.3	1.2	0.9	0.7	**3.2**	1.1	2.2	**6.1**	2.8	1.2	2.6
29	0.8	0.8	1.1	1.4	1.1	1.4	0.8	1.3	0.8	1.0	0.8	1.4	0.7	1.2	2.1	1.6	0.8	1.0
30	1.1	0.7	1.5	0.9	1.0	0.9	0.9	0.7	2.3									
31*	0.8	1.0	1.2	1.2	2.9	1.9	1.0	1.0	0.7	1.5	1.2	0.9	0.7	0.6	0.6	0.7	1.5	2.1
32*	0.5	0.4	2.9	0.7	0.6	2.5	0.7	0.3	0.3									
33	1.4	1.4	1.2	1.2	1.8	1.3	1.4	1.5	1.9									

* Results are mean SI values of CTLL proliferation to culture supernatants obtained from two independent experiments (SD < 10%).

months whereas of the other seven only two (individuals 12 and 34) showed a more than two-fold decrease in SI at 12 months as compared to baseline.

Mean values of (^{3}H)-thymidine incorporation in PHA-stimulated cultures were 16,064 cpm (range: 4508–32,208) at baseline and 19,583 (range: 4374–67,073) 12 months later. Mean values of (^{3}H)-thymidine incorporation in medium control cultures were 665 cpm (range: 143–1690) and 461 cpm (range: 138–1155), at baseline and 12 months later, respectively.

Kinetics of proliferative responses to individual gp120 peptides

PBMC from 15 seropositive donors responding to pooled peptides were assayed for proliferative responses to the individual peptides of the recognized pools (Table 2). Responses to at least one peptide of the respective pools were detected in all 15 donors. Twelve donors responded to three or more peptides representing one or more pools. Positive SI values ranged up to 9.9 (mean 5.1 ± 1.1). As was observed with responses to pooled peptides, a broad range of recognition was demonstrated. Multiple recognition sites were detected in both variable and conserved regions of gp120, but no single peptide was able to stimulate PBMC from all responders. Five peptides covering amino-acid sequences 142–161, 152–171, 172–191, 272–291, and 380–393, and designated C2, C3, C5, E5, and G4, respectively, induced responses more frequently. Nine of the 15 responders (67%) recognized at least one of these peptides (data not shown). As a control, proliferative responses to the individual peptides of non-recognized pools were measured with PBMC from six responders and four non-responders. No proliferative responses were observed in these assays (data not shown). The kinetics of peptide recognition at 6 and 12 months after baseline could be evaluated in eight responders. As shown in Fig. 1, most donors showed a general decrease in reactivity after 6 months, which was more pronounced after 12 months.

IL-2 production to gp120 peptide pools at baseline

The authors assayed the baseline pool-induced IL-2 production of PBMC from four responders and 17 non-responders from whom samples could be obtained (Table 3, left panel). PBMC from all the four responders (individuals 6, 8, 13 and 15) were shown to produce IL-2 in response to the pools that also induced proliferation. The numbers of pools stimulating IL-2 production were equal to (individual 8) or higher than (individuals 6, 13 and 15) those inducing proliferative responses. Among the 17 non-responders, IL-2 production to at least one pool of peptides was demonstrated in 12 cases (71%). PBMC from nine of these 12 donors produced IL-2 in response to three or more pools. The mean number of pools recognized by all donors was 2.9 ± 1.4. In the healthy control group, stimulation with pooled peptides did not induce significant IL-2 production (data not shown).

IL-2 production to gp120 peptide pools 12 months after baseline

IL-2 production in response to pooled peptides could be evaluated 12 months after baseline determination in 10 donors (Table 3, right panel). Among eight donors who had shown IL-2 responses at baseline, the number of pools recognized after 12 months remained unchanged in three cases (individuals 21, 25 and 28), increased in one case (individual 26), and decreased in the other four cases (individuals 17, 18, 19 and 24). New reactivities to previously unrecognized pools emerged in three of the eight donors (individuals 25, 26 and 28). No new reactivities emerged in the two donors who had failed to produce IL-2 at baseline (individuals 29 and 31).

T helper-cell responses in relation to circulating CD4$^+$ cells

Absolute numbers and percentages of circulating CD4$^+$ cells had baseline mean values of 786 ± 291 cells/μl and $36 \pm 9.5\%$, respectively. After 12 months, mean values were 656 ± 262 cells/μl and $30 \pm 10\%$, respectively. Linear regression analysis demonstrated the absence of significant association between T helper-cell responses and absolute or percentile numbers of CD4$^+$ cells, at baseline or 12 months later (data not shown). Moreover, baseline CD4$^+$ cell parameters proved not to be predictive for loss of T-helper responses 12 months later.

DISCUSSION

In this paper the authors have shown that T-cell proliferative responses to partially overlapping synthetic peptides covering the entire amino-acid sequence of gp120 are heterogeneous in asymptomatic HIV-1 infected individuals and decline significantly during a 12-month observation period. As compared to T-cell proliferation, measurement of IL-2 production upon specific stimulation appears to be a more informative and longer preserved parameter of T helper-cell function (Table 3). This is in line with earlier observations by Clerici et al. [5], suggesting that IL-2 production by T cells in response to synthetic peptides is a more sensitive indicator of exposure to HIV-1 than the presence of serum antibody, T-cell proliferation, or PCR assessment.

Proliferative responses obtained with individual peptides were concordant with those obtained with pools of five contiguous peptides. This allowed the authors to economize on the use of PBMC in in vitro assays. The patterns of response towards both pooled and individual peptides were heterogeneous among donors, and multiple sites were recognized in both variable and conserved regions of gp120 (Table 2). This is likely to reflect, at least in part, MHC polymorphism which is probably high in this group of individuals who were selected randomly from a large panel of asymptomatic seropositive donors with CD4$^+$ counts above 400. A similar heterogeneous response to synthetic peptides covering the entire amino-acid sequence of SIV gp120 was observed recently using T-cell clones from immunized macaques [26]. In HIV-1 infected individuals, most previous studies on T helper-cell responses to gp120 synthetic peptides have focused on limited numbers

of recognition sites [11, 15, 16, 19, 20], often predicted as immunodominant T helper-cell epitopes on the basis of their amphipathic α-helic structure [27]. However, in a comprehensive analysis of the COOH-terminal of gp120 Wahren *et al.* [18] showed that, T helper-cell responses can be elicited not only by peptides with the predicted α-helical structure, but e.g. also by peptides with a β-turn configuration. Our data showed the presence of T-helper determinants scattered throughout gp120 (Table 2). Comparison of these results with those obtained in earlier reports is complicated by the fact that in many cases lower SI threshold values have been considered positive. The authors chose to consider positive, values above 3.0, on the basis of results obtained with PBMC from 11 seronegative donors, whose SI-value t-distribution showed a 99% upper confidence limit of 2.87 for proliferative responses, and 2.98 for IL-2 production. It is noteworthy that proliferation to the T helper-cell epitope envT2 (amino acids 112–124) described previously [28] was observed in three of the 15 responders, whereas none of the donors showed T-cell proliferation to the other two T-helper epitopes envT1 (amino acids 428–443) [29] and p18 (amino acids 315–319) [30] (Table 2). In the study described here none of the pooled or individual peptides were recognized by all or most responding donors and, consequently, no immunodominant site could be identified. The authors were able to map five recognition sites to amino-acid sequences 142–161 (peptide C2), 152–171 (peptide C3), 172–191 (peptide C5), 272–291 (peptide E5), and 380–393 (peptide G4), which induced blastogenic responses with higher frequency: nine of the 15 responding donors (67%) proliferated to at least one of these five peptides (Table 2). One possible explanation for the lack of detectable T helper-cell responses in some of the individuals studied, could be the use of synthetic peptides spanning the IIIB sequence of gp120 in donors infected with HIV-1 strains of different geographical origin. This approach was chosen intentionally to obtain information on the recognition of gp120 determinants whose immunostimulatory properties are conserved among different viral strains and may therefore have particular relevance to vaccine development.

Results of earlier studies indicate that individuals in the advanced stage of HIV-1 infection have weaker antigen-specific T helper-cell responses than asymptomatic individuals [16, 18, 19]. Moreover, a sequential loss of T helper-cell reactivity, to recall first antigens and then alloantigens and mitogens, has been described in HIV-1 infected individuals by Clerici *et al.* [31]. The authors' goal in the study described here was to evaluate the kinetics of the gp120-specific T-helper responses in the early phase of infection, before severe depletion of CD4$^+$ cell and development of HIV-1-related disease occured. One interesting aspect in the longitudinal evaluation of both T-cell proliferation and IL-2 production to pooled peptides was the emergence, after 12 months, of new reactivities not detected at baseline, which in several cases compensated for the loss of determinants recognized previously (Tables 1 and 3). A fluctuation of gp120-specific T helper-cell responses over time was suggested also by the

kinetics of responses to individual peptides. In three of the eight donors studied (individuals 2, 4 and 11), proliferative responses to individual peptides were lost after 6 or 12 months, whereas those to the respective pools were still detectable. Although the identification of the new sites of recognition might have brought interesting information, lack of cell samples prevented further investigation.

In the authors' study T helper-cell functional responses were shown not to be correlated to absolute or percentual numbers of circulating CD4$^+$ cells, both at baseline and 12 months later. Moreover, baseline CD4$^+$ cell parameters were not found to be predictive for the persistence or loss of T helper-cell responsiveness 12 months later. This is not surprising in view of earlier observations that the T helper-cell functionality of seropositive individuals is affected very early in the course of HIV-1 infection and may be predictive for the onset of decline in CD4$^+$ cell numbers, rather than a consequence of such decline [31–35].

The authors' cohort of 36 seropositive individuals remained asymptomatic throughout the study. Subsequent clinical follow-up showed that all donors remained asymptomatic up to 24 months after the baseline visit. The only exception was individual 24, who showed no specific proliferative responses and low levels of IL-2 production both at baseline and 12 months later, and has in the meantime progressed to symptomatic disease (CDCIV-C1).

Collectively, the authors' results suggest that a progressive deterioration of HIV-1-specific T helper-cell function occurs during the early stage of HIV-1 infection. The antigen-specific T-cell proliferation declines first, whereas the ability to produce IL-2 upon specific stimulation is preserved for longer. This tendency towards progressive decline appears to develop with a fluctuating pattern, so that the loss of reactivities is compensated initially for by the emergence of newly acquired recognition sites. Further prospective evaluation of the cohort studied, may reveal a possible relationship between the gp120-specific T helper-cell function and progression of HIV-1-related disease.

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