Selective *in vitro* expansion of HLA class I-restricted HIV-1 Gag-specific CD8+ T cells: cytotoxic T-lymphocyte epitopes and precursor frequencies

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Objective: To identify HIV-1 Gag cytotoxic T-lymphocyte (CTL) epitopes and HLA restriction of their recognition, and to define precursor frequencies of HIV-1 Gag-specific CTL in the blood of seropositive individuals.

Methods: B-lymphoblastoid cell lines (B-LCL) infected with recombinant vaccinia viruses (rVV) containing a gene coding for HIV-1 Gag (rVV-Gag) were fixed with paraformaldehyde (PFA) and used as antigen-presenting cells (APC) to stimulate peripheral blood mononuclear cells (PBMC) from asymptomatic HIV-seropositive individuals. Specific CTL activity was determined in ^{51}Cr-release assays using B-LCL as targets after infection with rVV-Gag or after pulsing with partially overlapping peptides spanning the Gag sequence.

Results: *In vitro* stimulation resulted in an increased number of CD8+ T cells and CD45R0+ and HLA-DR+ cells. Gag-specific cytotoxicity, mediated predominantly by HLA class I-restricted CD8+ CTL, was observed in all seven individuals studied. Multiple HLA-restricted CTL epitopes were identified with a single culture from one of the individuals. Gag-expressing APC were successfully used as stimulator cells in limiting dilution analysis to determine CTL precursor (CTLp) frequencies.

Conclusion: PFA-fixed rVV-Gag-infected autologous B-LCL can be used as stimulator cells in bulk PBMC cultures to identify CTL epitopes and to determine CTLp frequencies. This method will facilitate the analysis of HIV-1-specific CTL responses in HIV-infected and vaccinated individuals.

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Keywords: HIV-1, cytotoxic T lymphocytes, antigen-specific stimulation, antigen-presenting cells, HIV-1 Gag.

Introduction

HIV-1-specific CD8+ cytotoxic T lymphocytes (CTL) are generally believed to play a crucial role in controlling HIV-1-related disease progression and to be important for vaccine efficacy. Studies addressing their specificities, HLA restriction and frequencies [1–5] have used non-stimulated lymphocytes [1,6–9] or *in vitro*-stimulated effector cell populations, stimulating either non-specifically, using phytohaemagglutinin (PHA) [8,10] or anti-CD3 monoclonal antibodies (MAb) [11,12], or specifically, using HIV-1-infected PHA-activated lymphoblasts [10,13–20] or HIV-1 peptides [12,18,20,21]. No studies have examined HIV-1 Gag-specific CD8+ CTL by specifically stimulating peripheral blood mononuclear cells (PBMC) with the immunogenic protein. Processing of *de novo* synthesized antigen in antigen-presenting cells (APC) is generally required for stimulation of HLA class I-restricted CTL at the protein level [22]. Hammond *et al.* [23] have recently used stimulation of PBMC with autologous Env-transfected B-lymphoblastoid cell lines (B-LCL) expressing *de novo* synthesized HIV-1 Env to detect HIV-1 Env-specific CD8+ CTL. Here

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we describe the use of autologous B-LCL infected with recombinant vaccinia viruses (rVV) containing a gene encoding for HIV-1 Gag (rVV-Gag) for the expansion of HIV-1 Gag-specific CD8+ CTL in PBMC cultures from seropositive individuals, in order to identify CTL epitopes and to determine CTL precursor (CTLp) frequencies in limiting dilution analysis (LDA).

Materials and methods

Blood samples

Heparinized blood samples were collected from seven asymptomatic HIV-1-seropositive individuals from The Netherlands (subject numbers 008, 038, 157, 665, 617, 8936 and RV). The first five subjects participate in the Amsterdam cohort studies [24,25], and subjects 8936 and RV were monitored by The Academic Hospital of Utrecht and the Academic Medical Centre of Amsterdam, respectively. All individuals were in Centers for Disease Control (CDC) disease stage II or III and had CD4 cell counts of 430–620 $\times 10^6$/l on the day of sampling. PBMC were isolated by Ficoll–Isopaque density gradient centrifugation. Epstein–Barr virus-transformed B-LCL were established and maintained as described elsewhere [26].

Recombinant vaccinia viruses

The rVV used were vsc40 (expressing the *gag–pol* region of HIV-1) [1,27], vsc8 (expressing β-galactosidase), both kindly provided by Dr B. Moss (National Institutes of Health, Bethesda, Maryland, USA), and TG1144 (expressing HIV-1 *gag* p55) [28], kindly provided by Dr M.P. Kiény (Transgène, Strasbourg, France).

Peptides

Sets of overlapping Gag peptides of the HIV-1 SF-2 strain of p17 [ADP703.1–13; 15 mers, five amino-acid (aa) overlap], p24 [ADP788.1–22; 20 mers, 10 aa overlap] and p15 [ADP704.1–13; 15 mers, five aa overlap] were kindly provided by the Medical Research Council's AIDS Directed Programme, England, UK.

Antigen-presenting stimulator cells

APC were obtained by incubating B-LCL for 18 h after infection with rVV at a multiplicity of infection (m.o.i.) of 5–10. These cells were fixed for 15 min with 1.5% (weight/vol) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and treated with 0.2 mol/l glycine in PBS for 15 min. Prepared APC retain their stimulation capacity for at least 1 month when stored at 4°C. Antigen expression in these APC was confirmed by fluorescence-activated cell sorter (FACS)-measured fluoresence (FMF) analysis using a bovine anti-vaccinia serum (RIVM, Bilthoven, The Netherlands) and a murine anti-p24 MAb (CLB14, kindly provided by Dr J.G. Huisman, CLB, Amsterdam, The Netherlands). For HIV-1 Gag staining, fixed APC were permeabilized with n-Octyl β-D-Glucopyranoside (Sigma, St Louis, Missouri, USA), as described elsewhere [29].

Stimulation of PBMC

PBMC were cocultivated with PFA-fixed autologous stimulator cells at cell densities of 5×10^4 and $5–10 \times 10^3$ per well, respectively, and maintained as described previously [26]. Cultures were restimulated on day 7 by addition of fixed autologous stimulator cells in the presence of recombinant interleukin-2 (rIL- 2 10 U/ml; Cetus, Emeryville, California, USA) and autologous feeder cells (γ-irradiated at 3000 rad; 5×10^4 per well).

Target cells

Target cells were infected with rVV as described for APC, and labelled with $Na_2[^{51}Cr]O_4$ (Amersham International, Amersham, England, UK) as described previously [26]. Alternatively, 1×10^6 uninfected ^{51}Cr-labelled B-LCL were incubated with peptide at 10–20 μmol/l for 1 h followed by overnight incubation at a concentration of 1–2 μmol/l.

^{51}Cr-release assay

Stimulated cell cultures were analysed for cytolytic activity in a standard 4-h ^{51}Cr-release assay on days 18–21. Briefly, 5×10^3 ^{51}Cr-labelled target (T) cells were incubated with effector (E) cells at different E : T ratios. After 4 h at 37°C the supernatants were harvested using a Skatron (Oslo, Norway) harvester for counting in a γ-counter (Packard Instruments International, Zurich, Switzerland). In inhibition assays, target cells were pre-incubated for 30 min with anti-HLA class I MAb W6/32 (ascites; final dilution 1 : 200) or anti-HLA class II MAb PDV5.2 or B8.11.2 (both ascites; 1 : 200); or effector cells were pre-incubated for 30 min with anti-CD8 MAb FK18 or WT82 (both ascites; 1 : 200) or anti-CD4 MAb OKT4 or OKT4a (Ortho Diagnostics, Beerse, Belgium; final concentration, 5 μg/ml). MAb W6/32, PDV5.2 and B8.11.2 were a gift from Dr F. Koning (Department of Immunohaematology and Blood Bank, Leiden, The Netherlands). MAb FK18 and WT82 were prepared at our institute (RIVM, Bilthoven, The Netherlands). Percentages of specific release from duplicate cultures were calculated as described previously [26].

FMF analysis of cultured PBMC

At days 0, 7 and 18 of culture, $1–2 \times 10^6$ cells were collected and stained with anti-CD4 or anti-CD8 MAb conjugated with phycoerythrin (Becton Dickinson, Ettenleur, The Netherlands) and/or anti-CD3, anti-CD45RA, anti-CD45R0 or anti-HLA-DR conjugated with fluorescein isothiocyanate (FITC; Becton Dickinson) according to the manufacturer's instructions, and analysed using a FACscan (Becton Dickinson).

Bead sorting

One day before testing, PBMC cultures were separated using anti-CD8 Magnetic Beads (Dynal, Oslo, Norway) and Detachabead (Dynal) according to the manufacturer's instructions. Purity of the fractions was verified by FMF analysis.

Limiting dilution analysis

PBMC were diluted to 3125–50 000 lymphocytes per well in 24 replicate wells of 96-well round-bottomed microtitre plates. Ten thousand rVV-Gag (TG1144)-infected, PFA-fixed, autologous B-LCL were added to each well. rIL-2 (10 U/ml) was added at day 2 and cultures were restimulated as described above at day 7. On day 14 wells were divided and assayed for cytotoxicity on ^{51}Cr-labelled autologous B-LCL infected with TG1144 or control rVV vsc8, as described above. A well was considered positive when the ^{51}Cr release exceeded the mean of the negative control wells by 3 s.d. LDA data were analysed using the methods described by Strijbosch *et al.* [30].

Results

Phenotypic and functional analysis of responder cells

During culture of PBMC from six out of the seven HIV-1-seropositive subjects (subjects 008, 038, 157, 665, 617 and 8936) in the presence of PFA-fixed autologous B-LCL expressing HIV-1 Gag, cell numbers in the cultures increased 10–20-fold (data not shown). For phenotypic analysis of cells responding to this stimulation, cultures from two of the individuals were tested for expression of CD3, CD4, CD8, CD45RA, CD45R0 and HLA-DR markers on days 0, 7 and 18 after the start of the stimulation. This analysis revealed an increased number of predominantly CD8+ cells, together with an increased number of cells with the CD45R0 and HLA-DR markers (data not shown).

PBMC cultures from subject 157 were tested after 18 days in a ^{51}Cr-release assay. High Gag-specific HLA-restricted cytotoxic activity was detected against rVV-Gag-infected B-LCL (Fig. 1 a and d). To identify the phenotype of the effector cells, the culture, consisting of 60% CD8+ cells, was separated into CD8+ cell-enriched and CD8+ cell-depleted fractions. The former fraction, containing more than 80% CD8+ cells, exhibited Gag-specific lysis of autologous, but not of HLA class I-mismatched, targets (Fig. 1 b and e). In contrast, no specific lysis was mediated by the CD8+ cell-depleted fraction, which contained less than 20% CD8+ cells (Figs 1c and f). No Gag-specific HLA-restricted cytotoxicity was observed with PBMC from this subject stimulated with PFA-fixed B-LCL infected with control rVV in the same experiment (data not shown), indicating that Gag-specific CTL numbers increase only if Gag determinants are presented by APC.

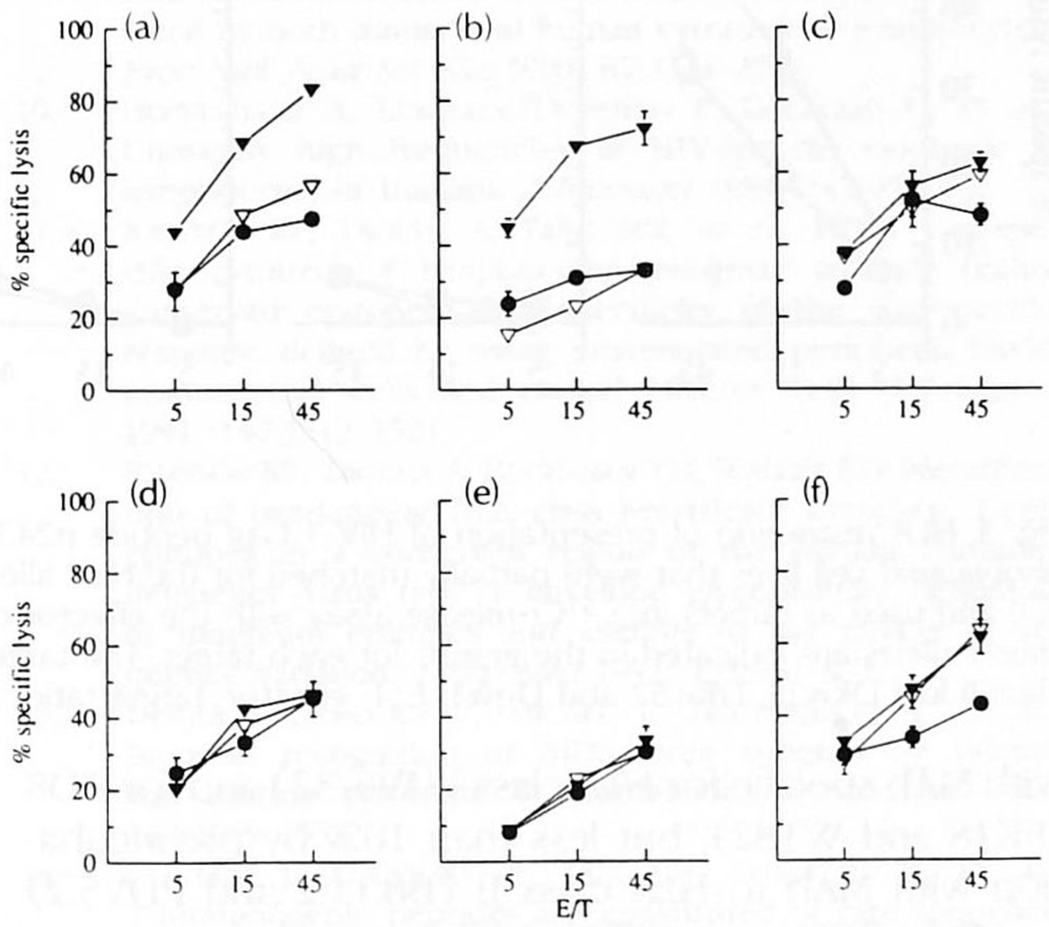

Fig. 1. HLA restriction and phenotype of HIV-1 Gag-specific cytotoxicity in peripheral blood mononuclear cell cultures from subject 157, detected 18 days after protein-specific stimulation. Results are shown as percentage-specific lysis of autologous (a–c) or heterologous HLA class I-mismatched targets (d–f) infected with recombinant vaccinia viruses (rVV) containing a gene encoding for HIV-1 Gag (▼), rVV control (▽) or uninfected (○). Effector cells were tested without separation (a and d), after CD8 enrichment (b and e) or CD8 depletion (c and f), at effector : target (E : T) ratios of 5, 15 and 45.

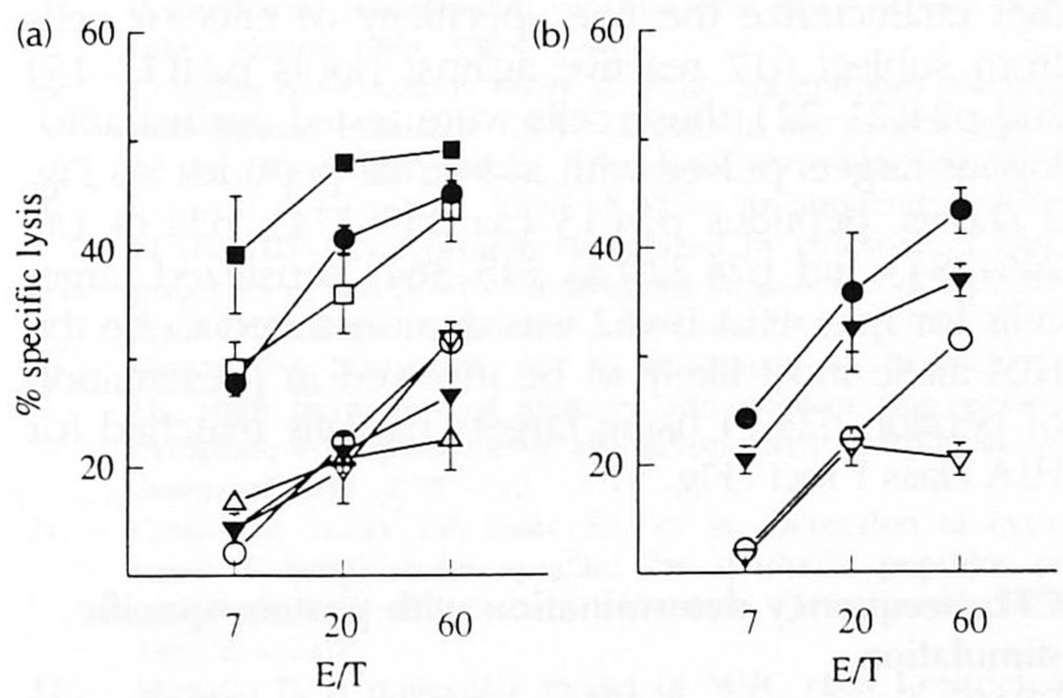

Fig. 2. Sensitization of autologous B-lymphoblastoid cell lines with Gag peptides for lysis by peripheral blood mononuclear cells from subject 617 specifically stimulated with protein. (a) Targets were tested after pre-incubation with peptides p24.11 (▽), p24.12 (▼), p24.13 (□), p24.14 (■) p24.15 (△), with a pool containing p24.11–15 (●), or without peptide (○). (b) Targets were tested after pre-incubation with peptides p24.21 (▽), p24.22 (▼), with a pool containing p24.21–22 (●), or without peptide (○). E : T, effector : target ratio.

The phenotype of the effector cells and the HLA class I restriction of the response were further examined using specific anti-sera and PBMC from subject 617. Gag-specific lysis was inhibited 40–45% by pre-incubation

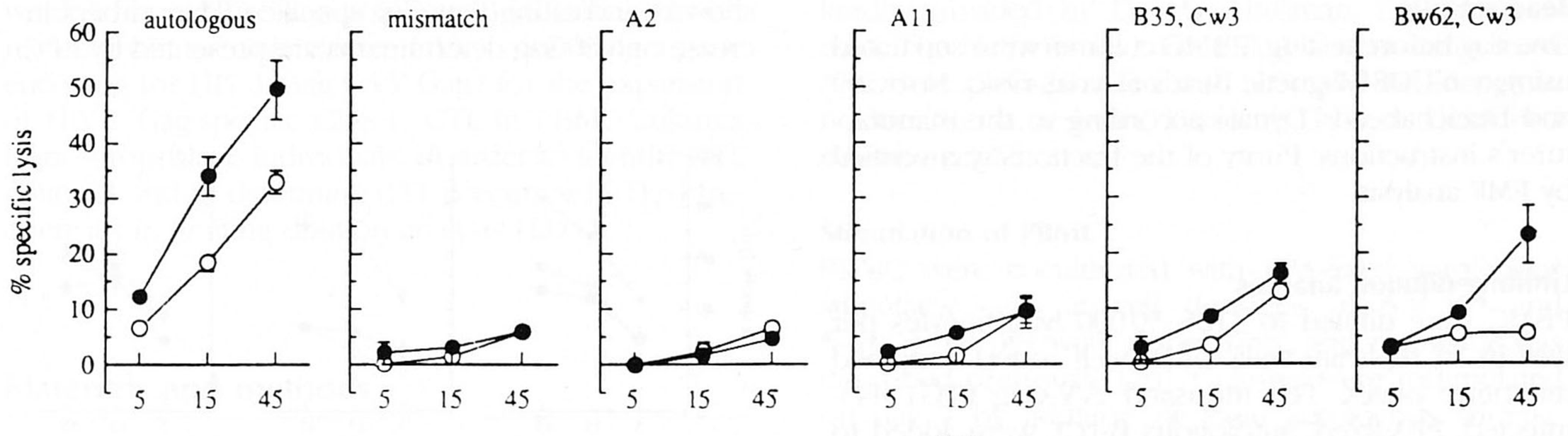

Fig. 3. HLA restriction of presentation of HIV-1 Gag peptide p24.14 to cytotoxic T lymphocytes from subject 617. ^{51}Cr-labelled B-lymphoblastoid cell lines that were partially matched for the HLA alleles of this subject were pre-incubated with peptide p24.14 (●) or not (○) and used as targets in a ^{51}Cr-release assay with the effector cell population used in the experiment shown in Fig. 2. Matching HLA class I alleles are indicated in the graphs for each target. The target that had no match for HLA class I alleles was matched on the HLA class II loci DRw13, DRw52 and Dqw1. E:T, effector:target ratio.

with MAb specific for HLA class I (W6/32) and for CD8 (FK18 and WT82), but less than 10% by pre-incubation with MAb to HLA class II (B8.11.2 and PDV5.2) or CD4 (OKT4 and OKT4a) (data not shown).

Characterization of Gag CTL epitopes

PBMC cultures from subjects 617, 008, 8936, 157, 665 and 038 specifically stimulated with HIV-1 Gag were tested against targets pulsed with pools of two to five overlapping peptides, together spanning the entire Gag sequence. Effector cells from all six individuals recognized two to five pools of peptides in an HLA-restricted manner (data not shown). To further characterize the fine specificity of effector cells from subject 617 reactive against pools p24(11–15) and p24(21–22), these cells were tested against autologous targets pulsed with individual peptides. As Fig. 2 shows, peptides p24.13 (aa 255–274), p24.14 (aa 265–284) and p24.22 (aa 345–364) sensitized target cells for lysis. HLA-Bw62 was demonstrated to be the HLA allele most likely to be involved in presentation of peptide p24.14 using targets partially matched for HLA class I loci (Fig. 3).

CTLp frequency determination with protein-specific stimulation

PBMC from subject RV were tested in LDA using PFA-fixed Gag-expressing APC as stimulator cells. The mean frequency of HIV-1 Gag-specific CTLp in three independent experiments was $338/10^6$ ($267–348/10^6$) PBMC.

Discussion

We have demonstrated the selective *in vitro* expansion of HIV-1 Gag-specific CD8+ T cells from asymptomatic seropositive individuals by stimulation of their PBMC with fixed autologous APC expressing HIV-1 Gag. Stimulated cultures were used to localize CTL epitopes in HIV-1 Gag and to estimate frequencies of Gag-specific CTLp.

HIV-1 Gag expression was achieved by infection of autologous B-LCL with rVV-Gag, which allows *de novo* synthesis of the antigen and processing via a non-endosomal route, generating epitopes that occur naturally and may associate with HLA class I and class II molecules [22,31]. We observed an increase in predominantly CD8+ T cells. Since CD4+ T cells can proliferate in response to stimulation with PFA-fixed rVV-infected APC [32], it is unclear whether the predominance of CD8+ cells responding to stimulation is characteristic of the method. The increase in the number of cells expressing HLA-DR or CD45R0 is consistent with the induction of HLA-DR expression on T cells upon activation, and the expression of CD45R0 on T cells responding to recall antigens [33]. High levels of Gag-specific cytotoxic activity mediated predominantly by CD8+ HLA class I-restricted CTL were observed in these expanded populations. The increased number of Gag-specific CTL was the result of specific stimulation, since culturing of PBMC in the presence of fixed control rVV-infected B-LCL did not reveal Gag-specific cytotoxic activity. The relatively high background cytotoxicity proved not to be primarily mediated by CD8+ T cells, since these backgrounds decreased considerably after CD8+ cell enrichment. These backgrounds may at least partly be the result of the method of stimulation. In addition to specific activity directed against determinants shared by target and stimulator cells, such as Epstein–Barr and vaccinia virus antigens, non-specific lysis mediated by, for example, natural killer cells may have been responsible for these backgrounds.

Three peptides in p24 were found to contain CTL epitopes using effector cells from subject 617, indicating that multiple epitopes can be identified with a single

culture. The assumed Bw62-restricted recognition of p24.14 (aa 265–284) is substantiated by data reported by Johnson *et al.* [11]. However, additional Bw62+ APC should be tested to confirm that this is the restriction element for p24.14-specific CTL from subject 617. The two other peptides, p24.13 (aa 255–274) and p24.22 (aa 345–364), that were recognized by T cells from this subject have not hitherto been recognized in association with any of the HLA class I alleles of this individual. However, both have been reported to contain CTL epitopes [5,11,20]. Since HIV-1 Gag-specific CTL responses were observed in all seven asymptomatic seropositive individuals tested, the majority of individuals at this stage of HIV infection will probably respond in a similar way.

Finally, the fixed APC expressing HIV-1 Gag could also be used for CTLp studies in LDA. The frequency of Gag-specific CTLp in subject RV was in the range reported by Koup *et al.* [34], using anti-CD3 MAb or PHA-stimulated PBMC from healthy seropositive individuals.

The protocol described here will allow the longitudinal retrospective evaluation of the kinetics of epitope recognition and of CTLp directed against HIV-1 Gag, since well-standardized fixed APC presenting naturally occurring HIV-1 Gag epitopes can be prepared in advance and used to stimulate autologous PBMC from infected and vaccinated individuals.

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