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Detection of Human Immunodeficiency Virus Type 1-Specific Memory Cytotoxic T Lymphocytes in Freshly Donated and Frozen-Thawed Peripheral Blood Mononuclear Cells

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Loss of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte (CTLm) responses is associated with disease progression in HIV-1 infection. In this study, nonspecific stimulation of peripheral blood mononuclear cells (PBMC) from HIV-1-infected homosexual men with anti-CD3 monoclonal antibody (MAb) was compared with antigen-specific stimulation with inactivated, autologous B lymphoblastoid cells (B-LCL) infected with a vaccinia virus vector encoding HIV-1 IIIb Gag, Pol, and Env (VV-GPE) for activation of HIV-1-specific CTLm responses in a bulk lysis assay and by precursor frequency analysis. The results show that VV-GPE-infected B-LCL stimulated on average 10-fold greater anti-HIV-1 CTLm activity, as detected in the bulk lysis assay, and 55-fold-greater CTLm precursor frequencies specific for the three HIV-1 structural proteins than did stimulation with anti-CD3 MAb. This effect was noted with both freshly donated and frozen-thawed PBMC. The lysis was mediated by $CD8^+$ T cells and was restricted by the major histocompatibility class I complex. These data indicate that antigen-specific stimulation with VV-GPE-infected B-LCL is a highly efficient method for detection of anti-HIV-1 CTLm responses that is applicable to noncurrent prospective studies with frozen PBMC.

Cytotoxic T lymphocytes (CTL) specific for human immunodeficiency virus type 1 (HIV-1) are considered important in resistance to progression of disease during HIV-1 infection (13). It is not clear, however, which assessment of CTL function is the most sensitive parameter of the anti-HIV-1 host response relative to disease progression. We (16, 17) and others (2) have reported that HIV-1-specific CTL effectors, which are directly detectable in the blood of most HIV-1-infected subjects without prestimulation in vitro (23), are not related to progression of disease. In contrast, high precursor frequencies of anti-HIV-1 memory CTL (CTLm) induced by in vitro stimulation with anti-CD3 monoclonal antibody (MAb) are related to decreases in primary HIV-1 viremia (1, 11) and lack of disease in long-term nonprogressors (17).

Recent studies have shown that HIV-1-specific CTLm stimulated with HIV-1 antigen can be detected in bulk lysis assays with freshly donated peripheral blood mononuclear cells (PBMC) from HIV-1-infected subjects with high or low $CD4^+$ T-cell numbers (12, 22). Moreover, others have reported that lower precursor frequencies of anti-HIV-1 CTLm stimulated with HIV-1 antigen in vitro are related to decreased $CD4^+$ T-cell counts in HIV-1-infected individuals (2, 9). Finally, in preliminary studies, we found that nonspecific stimulation of frozen-thawed PBMC with anti-CD3 MAb gave inconsistent and relatively lower anti-HIV-1 CTLm precursor frequencies than freshly donated PBMC. The use of frozen-thawed PBMC is essential for conducting longitudinal studies in a nonconcurrent, prospective manner that would allow a detailed analysis of the role of anti-HIV-1 CTLm in disease progression.

We therefore compared nonspecific and antigen-specific stimulation of anti-HIV-1 CTLm responses in both freshly donated and frozen-thawed PBMC from HIV-1-infected subjects. The results show that antigen-specific stimulation with inactivated, autologous B lymphoblastoid cells (B-LCL) infected with a vaccinia virus (VV) vector encoding HIV-1 IIIb Gag, Pol, and Env (VV-GPE) is consistently superior to nonspecific stimulation with anti-CD3 MAb for detection of anti-HIV-1 CTLm responses by either bulk lysis assay or precursor frequency analysis. The precursor frequency method, however, has the advantage of being a more precise quantitative measure of CTLm activity. Of importance is that this method also proved highly sensitive for measurement of anti-HIV-1 CTLm levels in frozen-thawed PBMC and is thus applicable to nonconcurrent prospective investigations.

MATERIALS AND METHODS

Study subjects. Seven HIV-1-seropositive homosexual men (A to G) from the Pittsburgh, Pa., portion of the Multicenter AIDS Cohort Study, an investigation of the natural history of HIV infection, were studied (8). They were asymptomatic, with only two subjects (C and D) receiving anti-HIV-1 therapy (azidothymidine and either dideoxyinosine or dideoxycytidine) during the study period. The median (range) $CD4^+$ T-cell count of the seven subjects was 409 (16–784) per mm^3. HIV-1-seronegative heterosexual individuals were included as controls.

Effector cells. Venous blood anticoagulated with heparin (10 U/ml) was separated on Ficoll-Hypaque gradients to obtain PBMC (17). PBMC were prepared for freezing by suspension in cold freezing solution containing RPMI 1640 medium (GIBCO, Grand Island, N.Y.), 100 U of penicillin per ml, 100 μg of streptomycin per ml, 25% (vol/vol) heat-inactivated fetal calf serum (FCS; Hyclone, Logan, Utah), and 10% dimethyl sulfoxide. The cells were frozen at a rate of −1°C per min in a controlled-rate freezer (Gordinier Electronics, Roseville, Mich.) and stored at −135°C. The cells were stored frozen for a median of 1 month (range, 1 day to 16 months) prior to thawing for use in the CTLm assays. The frozen PBMC were quickly thawed at 37°C, diluted slowly with RPMI 1640 medium supplemented with penicillin, streptomycin, and 10% heat-inactivated FCS (complete medium) at 22°C, centrifuged at 250 × *g* for 10 min, washed with Hanks' balanced salt solution (HBSS), centrifuged, and resuspended in complete medium prior to nonspecific or antigen-specific stimulation. The cell viability of freshly isolated PBMC and frozen-thawed PBMC was monitored by trypan blue dye exclusion.

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TABLE 1. Anti-HIV-1 CTLm precursor frequency in freshly donated PBMC stimulated with either anti-CD3 MAb or VV-GPE-infected, autologous B-LCL

Subject ($CD4^+$ T cells/mm^3)	PBMC stimulation	No. of precursors/10^6 PBMC (95% confidence interval)				
		Vac	Gag	Pol	Env	GPE
B (684)	Anti-CD3 MAb	17 (10–29)	6 (2–14)	15 (9–27)	3 (1–9)	1 (0–8)
	VV-GPE B-LCL	293 (221–388)	1,389 (1,022–1,887)	755 (559–1,019)	260 (197–343)	1,596 (1,174–2,169)
C (261)	Anti-CD3 MAb	21 (13–34)	52 (37–75)	29 (18–45)	11 (5–22)	48 (33–70)
	VV-GPE B-LCL	108 (81–146)	134 (100–178)	348 (258–469)	97 (72–131)	214 (157–292)

Target cells and recombinant VV. The target cells were autologous B-LCL immortalized by infection with Epstein-Barr virus (EBV) (filtered supernatant from the EBV-transformed B95-8 marmoset cell line; American Type Culture Collection, Rockville, Md.). The VV constructs used to infect B-LCL for the detection of CTL were vAbT 141, containing the *gag* coding sequence for p55 (VV-Gag); vAbT 204, containing the full-length *pol* gene, including the reverse transcriptase, protease, and integrase (VV-Pol); and vAbT 408, containing the combined *gag/pol* and *env* coding sequences (VV-GPE). These vectors contain coding sequences from the BH10 and HxB2 strains of HIV-1 (Therion Biologics, Cambridge, Mass.). Other vectors were vT23, encoding *nef* of the NL43 strain of HIV-1; VV tat, encoding the *tat* exon 1 of BH10; and vPE11, expressing the *env* coding region of HIV-1 strain BH10 minus the signal sequence (VV-Env) (B. Moss, National Institutes of Health) (14). Infection with the last construct does not lead to expression of surface gp160 that is essential for antibody-dependent cell cytotoxicity mediated by $CD16^+$ cells. The NYCBH strain of VV (VV-Vac; Therion) was used as the control virus. Expression of the VV vectors in B-LCL to be used as CTL targets was determined by detection of the β-galactosidase gene product regulated by the same VV promoters as HIV-1 genes, as previously described (21); additionally, *gag* expression was directly determined by anti-p24 immunofluorescence (MAb K57; Coulter Immunology, Hialeah, Fla.). B-LCL from human leukocyte antigen (HLA)-mismatched donors were used as targets in certain experiments. All B-LCL and VV pools were negative for mycoplasmas, as determined by use of a nucleic acid probe (Genprobe, San Diego, Calif.).

Preparation of stimulator cells. Autologous B-LCL (2×10^6) were infected with VV-GPE at an input virus-to-cell multiplicity of 4 to 1 in a 15-ml round-bottomed polypropylene tube, spun at $700 \times g$ for 30 min at room temperature, and incubated for 16 h at 37°C in a 5% CO_2 atmosphere. The infected cells were washed three times with complete medium, treated with 10 μg of psoralen (Calbiochem, La Jolla, Calif.) per ml, and exposed to long-wave UV light irradiation (40 mW/cm^2) for 5 min. The cells were then washed three times and suspended to 32,000 cells per ml in complete medium; 50 μl of this cell suspension was added to each well of 96-well plates containing PBMC, i.e., 1,600 stimulator cells per well.

CTLm limiting-dilution analysis. The precursor frequencies of anti-HIV-1 CTLm were determined by limiting-dilution assay of freshly isolated and frozen-thawed PBMC. The freshly donated or frozen-thawed PBMC were seeded in RPMI 1640 medium supplemented with antibiotics and 15% FCS (culture medium) to 250, 500, 1,000, 3,000, 6,000, 12,000, and 16,000 cells per well in 24 replicate wells of 96-well round-bottomed microtiter plates (10, 17). To each well were added 2.5×10^4 gamma-irradiated allogeneic PBMC from an HIV-1-seronegative normal donor as feeder cells (Central Blood Bank, Pittsburgh, Pa.), 100 U of recombinant interleukin-2 (rIL-2; Chiron, Emeryville, Calif.), and either stimulator cells (1,600 stimulator cells per well) for antigen-specific stimulation or 0.1 μg of anti-CD3 murine MAb (12F6 [24]; courtesy of J. Wong, Boston, Mass.) per ml for nonspecific stimulation. The cells were cultured for 14 days at 37°C in 5% CO_2, with fresh culture medium and rIL-2 added every 5 days. On day 14, the cells in culture were divided, transferred to two new wells, and adjusted to 100 μl with complete medium.

For target cells, B-LCL (3×10^6) were infected with VV at an input multiplicity of 4 to 1 in a 15-ml round-bottomed polypropylene tube and spun at 700 $\times g$ for 30 min at room temperature. The VV-infected B-LCL were then washed twice with 10 ml of RPMI 1640 medium supplemented with antibiotics and 5% FCS and labeled with 150 μCi of $Na_2{}^{51}CrO_4$ in 1 ml of culture medium for 16 h at 37°C in a 5% CO_2 atmosphere. The B-LCL were then washed three times with RPMI 1640 medium with 5% FCS at 4°C.

VV-infected, ^{51}Cr-labeled B-LCL targets were added at 10^4 cells per well to 24 replicate wells of effector cells in 100 μl and assayed for cytotoxicity in a standard chromium release assay (21). The fraction of nonresponding wells was the number of wells in which the ^{51}Cr release did not exceed the mean spontaneous release plus 10% of the incorporation (total ^{51}Cr release − spontaneous ^{51}Cr release) divided by the number of wells assayed (2). The precursor cell frequency was estimated by the maximum-likelihood method (5) with a statistical program kindly provided by S. Kalams (Boston, Mass.); CTLm activity (precursor frequency) and 95% confidence intervals were expressed per 10^6 PBMC.

CTLm bulk lysis assay. Effector cells were freshly donated or frozen-thawed PBMC stimulated for 14 days with VV-GPE-infected, inactivated, autologous B-LCL or with anti-CD3 MAb by the same procedures as in the limiting-dilution assays except that the cell suspensions were incubated at 8×10^4 cells per ml in round-bottomed 96-well culture plates. On day 14, the effector cells were adjusted to a concentration of 10^6 in 1 ml of complete medium and assayed at an effector-to-target cell ratio of 10:1 in triplicate wells. The target cells were prepared as described above and assayed for cytotoxicity as in the standard chromium release assay.

PBMC viability and phenotyping. The cell viability of freshly isolated and frozen-thawed PBMC, 14-day-cultured PBMC, and feeder cells was monitored by trypan blue dye exclusion. Fresh or frozen-thawed PBMC and 14-day-cultured PBMC were phenotyped by staining with fluorescent dye-conjugated MAb anti-Leu4 ($CD3^+$ T cells), anti-Leu3a ($CD4^+$ T cells), anti-Leu2a ($CD8^+$ T cells), anti-Leu11a and -Leu19 ($CD16^+$ and $CD56^+$ natural killer [NK] cells), and anti-HLA DR (activation marker) (Becton Dickinson, Mountain View, Calif.) and analyzed by flow cytometry (Profile II; Coulter) by previously described methods (6). Major histocompatibility complex (MHC) class I typing was done by a standard microlymphocytotoxicity assay with typing antisera.

Effector cell enrichment and depletion. For $CD8^+$ cell enrichment, freshly isolated PBMC were treated with murine anti-CD16 MAb (Becton Dickinson) (10 μl per 3×10^6 PBMC) and incubated for 15 min at 4°C. The cells were washed and treated with biotinylated goat anti-mouse immunoglobulin (Antigenix, Franklin Square, N.Y.), washed and mixed with streptavidin-conjugated microbeads and microbeads directly conjugated to anti-CD4, -CD14, and -CD19 MAb (Miltenyi Biotec, Sunnyvale, Calif.) at 10 μl per 10^7 cells at 4°C, and then loaded onto a magnetic separation column (Miltenyi). The nonadherent cells eluted through the column were stained with fluorescent MAb specific for CD4, CD8, CD14 (monocyte), CD16, and CD20 (B cell) and analyzed in a flow cytometer. A purity of 97% $CD8^+$ cells with <1% $CD4^+$, $CD14^+$, $CD16^+$, and $CD20^+$ cells was achieved. $CD8^-$ cells were enriched from PBMC by removal of >99% $CD8^+$ cells in anti-$CD8^+$ MAb-coated flasks (Applied Immune Sciences, Menlo Park, Calif.) (21).

RESULTS

Detection of HIV-1-specific CTLm reactivity of PBMC from HIV-1-infected subjects. Freshly donated PBMC from HIV-1-seropositive homosexual men were nonspecifically stimulated with anti-CD3 MAb or specifically stimulated with VV-GPE-infected B-LCL. After 2 weeks of culture, lytic activity was assessed by limiting-dilution analysis and bulk lysis assays. The results show an average 55-fold-greater HIV-1-specific CTLm precursor frequency against Gag, Pol, Env, and GPE after stimulation with psoralen- and UV light-inactivated, VV-GPE-infected B-LCL than after nonspecific stimulation. The representative results in Table 1 show that anti-CD3 MAb stimulated low levels of CTLm precursors. In contrast, GPE stimulation elicited relatively high CTLm responses specific for Gag, Pol, and GPE in subject B and Pol and GPE in subject C. Similarly, an average 10-fold-greater CTLm activity was detectable with GPE stimulation in the bulk lysis assay at a low effector-to-target ratio of 10:1 (representative results shown in Table 2). Anti-CD3 MAb-stimulated PBMC from <10% of the HIV-1-infected subjects lysed Nef- or Tat-expressing targets (17), whereas stimulation with VV-GPE-infected B-LCL did not result in lytic activity against Nef or Tat (data not shown).

In most experiments, Vac-expressing B-LCL were also lysed, particularly by PBMC that were stimulated with VV-GPE-infected B-LCL (Tables 1 and 2). However, there was at least

TABLE 2. Anti-HIV-1 bulk lysis mediated by freshly donated PBMC stimulated with either anti-CD3 MAb or VV-GPE-infected, autologous B-LCL

Subject ($CD4^+$ T cells/mm^3)	PBMC stimulation	% Bulk lysis[a]				
		Vac	Gag	Pol	Env	GPE
B (684)	Anti-CD3 MAb	0	0	0	0	0
	VV-GPE B-LCL	6	13	5	2	9
C (261)	Anti-CD3 MAb	0	3	0	0	10
	VV-GPE B-LCL	7	7	29	3	23

[a] Bulk lysis at an effector-to-target cell ratio of 10:1.

TABLE 3. Lack of anti-HIV-1 CTLm activity in PBMC from HIV-1-seronegative donors stimulated with VV-GPE-infected, autologous B-LCL

Donor	No. of precursors/10^6 PBMC (95% confidence interval)			% Bulk lysis[a]		
	None[b]	Vac	GPE	None	Vac	GPE
1	92 (69–121)	94 (71–124)	109 (83–143)	10	17	14
2	48 (34–67)	85 (64–113)	63 (46–85)	0	1	1

[a] Effector-to-target cell ratio, 10:1.
[b] Uninfected, EBV-transformed, autologous B-LCL as targets.

a twofold-greater response against ≥1 HIV-1 gene-expressing target compared with the Vac-expressing targets in every assay of PBMC stimulated with VV-GPE-infected B-LCL (representative results shown in Tables 1 and 2). PBMC stimulated by either method also mediated low levels of lysis of EBV-transformed, uninfected, autologous B-LCL in separate experiments (data not shown). Lysis of Vac-expressing targets by PBMC stimulated with VV-GPE-infected B-LCL, however, was always greater than lysis of uninfected B-LCL (data not shown). These data suggest that CTLm precursors specific for Vac and EBV are activated together with HIV-1-specific CTLm in these assays.

There was a strong correlation between $CD4^+$ T-cell counts and GPE-specific CTLm precursor frequency induced by antigen-specific stimulation of PBMC from the six HIV-1-infected subjects tested ($r = 0.96$, $P < 0.01$, Pearson correlation coefficient test [Fig. 1]). There was no correlation of GPE-specific CTLm precursor frequency stimulated by anti-CD3 MAb and $CD4^+$ T-cell numbers ($r = 0.36$, $P > 0.05$).

There was no detectable HIV-1-specific CTLm reactivity in PBMC from two HIV-1-seronegative controls following either anti-CD3 MAb stimulation (data not shown) or HIV-1 antigen-specific stimulation (Table 3) by limiting-dilution analysis or bulk lysis assay. The levels of lytic activity mediated by the normal donor PBMC against either uninfected B-LCL or Vac-expressing B-LCL were comparable to those mediated by PBMC from the HIV-1-seropositive subjects against uninfected B-LCL (data not shown) or Vac-expressing B-LCL (Tables 1 and 2).

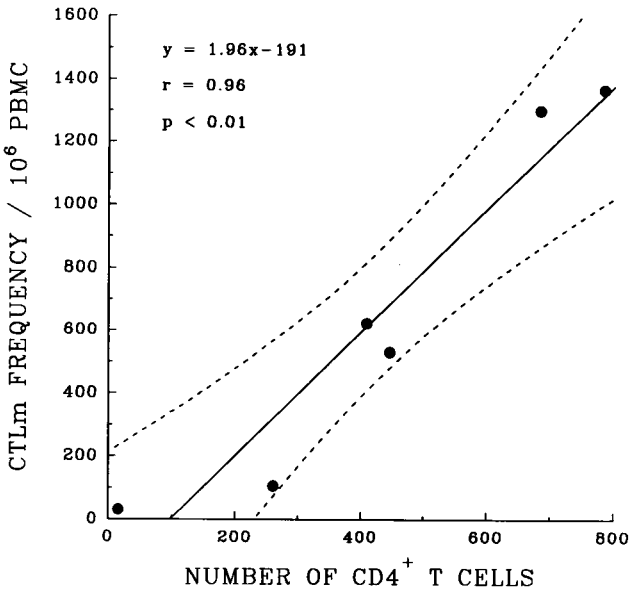

FIG. 1. Regression and correlation of anti-HIV-1 CTLm precursor frequency against GPE-expressing targets in PBMC stimulated with VV-GPE-infected, autologous B-LCL with $CD4^+$ T-cell numbers in six HIV-1-seropositive subjects (A to F).

HIV-1-specific CTLm reactivity in frozen-thawed PBMC from HIV-1-infected subjects. An aliquot of freshly donated PBMC was frozen at the time of the CTLm assays for later testing. Frozen-thawed PBMC from HIV-1-infected individuals were subjected to nonspecific or HIV-1 antigen-specific stimulation as described above. Inconsistent and lower CTLm responses were mediated by nonspecifically stimulated, frozen-thawed PBMC compared with freshly donated PBMC in both assays (data not shown). In contrast, the anti-HIV-1 CTLm reactivity mediated by frozen-thawed PBMC after HIV-1 antigen-specific stimulation assessed by both limiting-dilution analysis (Fig. 2) and the bulk lysis assay (Fig. 3) was comparable to that of the freshly donated, matched PBMC from the same person tested prior to freezing. Subjects B and C had high CTLm responses with either fresh or frozen-thawed PBMC, whereas subject D, with the lowest $CD4^+$ T-cell numbers, had the lowest anti-HIV-1 CTLm responses by both assays with both the fresh and frozen-thawed preparations. We confirmed that frozen-thawed PBMC from two other subjects (E and F; $CD4^+$ T-cell counts, 784 and 446 per mm^3, respectively) exhibited vigorous anti-HIV-1 CTLm responses by either assay, e.g., anti-Vac and anti-GPE CTLm precursor frequencies were $192/10^6$ cells and $1{,}541/10^6$ cells, respectively, for subject E and $35/10^6$ cells and $566/10^6$ cells, respectively, for subject F. Likewise, the anti-Vac and anti-GPE percent lysis in the bulk CTLm assay was 10 and 42%, respectively, for subject E and 3 and 39%, respectively, for subject F.

The variation in CTLm activity stimulated by GPE in either fresh and frozen-thawed PBMC (Fig. 2 and 3) was not accounted for by intra-assay fluctuations. That is, samples of frozen-thawed PBMC from three HIV-1-infected subjects (C, E, and F) were split in half and stimulated concurrently with VV-GPE-infected, autologous B-LCL to assess the validity of the CTLm assay results. The variation in CTLm activity averaged <0.1 $\log_{10}$ between the replicate, split specimens for the precursor frequency assay and less than 1% for the bulk lysis assay (data not shown).

We next examined the reproducibility of the CTLm assays with replicate samples of PBMC from two HIV-1-infected subjects (C and F) that were tested at different times after freezing. The results show that both the precursor frequency and bulk lysis responses stimulated by VV-GPE-infected, autologous B-LCL were reproducible when assessed several months apart (Table 4). As expected, there was some interassay variation, but the levels of CTLm activity remained relatively stable against the various targets, i.e., a mean variance of

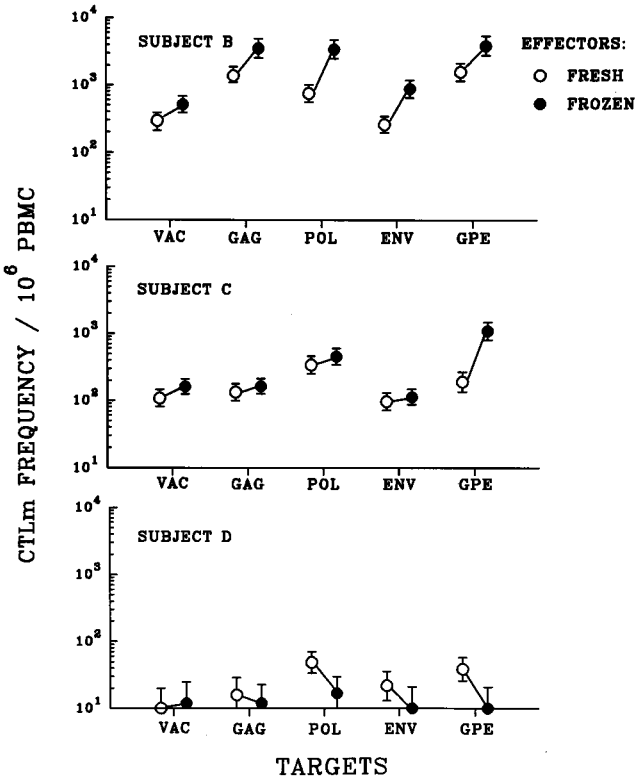

FIG. 2. Anti-HIV-1 CTLm precursor frequencies and confidence intervals induced by HIV-1 antigen-specific stimulation of freshly donated and matched, frozen-thawed PBMC from the same three HIV-1-seropositive subjects ($CD4^+$ T-cell numbers per mm^3: 684 for B, 261 for C, and 16 for D).

0.3 $\log_{10}$ for the precursor frequency assay and <2% for the bulk lysis assay. This interassay variation therefore was responsible for at least a portion of the variation in CTLm responses between fresh and frozen-thawed preparations (Fig. 2 and 3).

Determination that the CTLm effector cells were $CD8^+$. We (17) and others (10) have previously shown that HIV-1-specific lytic activity stimulated by anti-CD3 MAb is mediated by $CD8^+$ T cells. In the present study, $CD8^+$ T-cell-enriched populations that were stimulated with GPE had greater precursor frequencies (representative results shown in Fig. 4) and percent lysis at an effector-target cell ratio of 10:1 (representative results shown in Fig. 5) than did $CD8^-$ cell populations against Gag, Pol, Env, and the GPE triplex. Thus, the GPE-stimulated CTL activity was mediated by $CD8^+$ cells.

MHC-restricted CTLm cytolysis. MHC class I-restricted, anti-HIV-1 CTLm activity by antigen-stimulated PBMC from HIV-1-infected individuals was detected by both precursor frequency analysis (Fig. 6) and the bulk lysis assay (Fig. 7). The effector cells were tested against allogeneic targets that were mismatched at all MHC class I loci except the public allele Bw4. CTLm reactivity was very low or absent against the HIV-1 antigen-expressing, MHC class I-mismatched target cells but was high against the autologous targets.

Viability and phenotype of the stimulated cell cultures. Study subjects with either high or low levels of anti-HIV-1 CTLm activity had similar increases in cell number during the in vitro culture period. As in our previous study (17), freshly donated or frozen-thawed PBMC stimulated with anti-CD3 MAb had increases in cell numbers of approximately 56- to 1,400-fold after the 14 days of culture (data not shown). In contrast, there was a 38- to 800-fold increase in cell numbers by 14 days in PBMC cultures stimulated with HIV-1 antigen ($P < 0.0001$, Scheffe's multiple comparison test), with the greater relative increases occurring in wells seeded at the lower concentrations. The cells from the anti-CD3 MAb-stimulated or antigen-stimulated cultures of either freshly donated or frozen-thawed samples had comparable viability of 89 to 99%, with the lower viability being observed in wells seeded with low concentrations of PBMC. They also had similar increases in numbers of $CD8^+$ cells, from 44% (± 6%) initially to 79% ± 5%, by 14 days of culture. However, there was greater outgrowth of activated $CD8^+$ $HLA\text{-}DR^+$ cells in the antigen-stimulated cultures, i.e., an increase from 11% ± 2% prior to culture to 68% ± 9% in the HIV-1 antigen-stimulated cultures and 52% ± 9% in the anti-CD3 MAb-stimulated cultures ($P < 0.05$, paired t test).

DISCUSSION

We have shown that psoralen- and UV light-inactivated, autologous B-LCL infected with a VV vector encoding the three major structural proteins of HIV-1 stimulated greater HIV-1-specific CTLm responses in PBMC from HIV-1-infected subjects than did anti-CD3 MAb. The higher degree of stimulation by the antigen-specific method was demonstrable in bulk lytic assays at a low effector-to-target ratio of 10:1, confirming the results of Lubaki et al. (12) and van Baalen et al. (22). Of more importance, however, is that antigen-specific stimulation induced greater anti-HIV-1 CTLm precursor frequencies in our limiting-dilution assay. Anti-HIV-1 cytotoxic

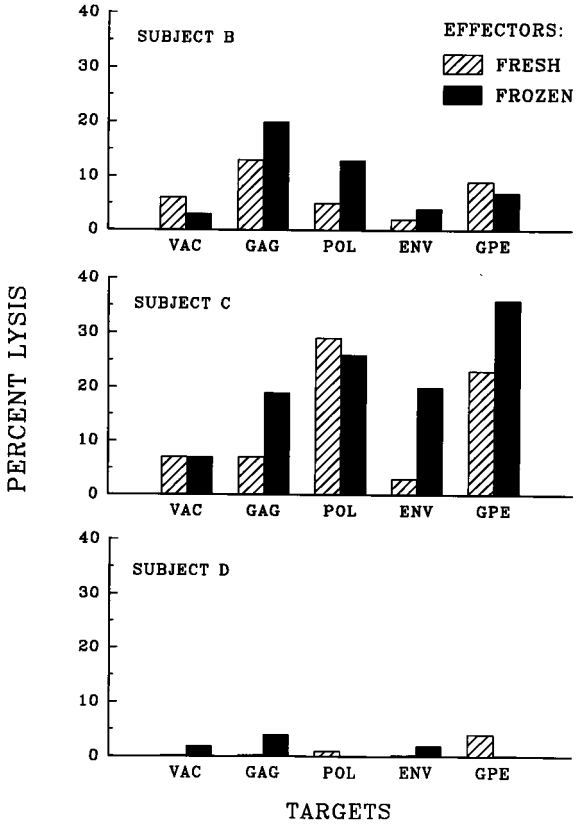

FIG. 3. Bulk lysis mediated by freshly donated and matched, frozen-thawed PBMC from the same samples used for the data in Fig. 2, stimulated by HIV-1 antigen-specific methods (10:1 effector-to-target cell ratio).

TABLE 4. Reproducibility of anti-HIV-1 CTLm responses by frozen-thawed PBMC stimulated by VV-GPE-infected B-LCL

Subject ($CD4^+$ T cells/mm^3)	Time from freezing of PBMC to CTLm assay (mo)	No. of precursors/10^6 PBMC (95% confidence interval)					% Bulk lysis[a]				
		Vac	Gag	Pol	Env	GPE	Vac	Gag	Pol	Env	GPE
C (310)	1.5	162 (125–210)	164 (127–213)	456 (344–606)	114 (87–149)	1,094 (807–1,482)	7	19	26	20	36
	5	79 (59–106)	77 (57–103)	884 (655–1,194)	75 (56–101)	1,022 (755–1,383)	11	20	34	22	24
F (446)	9.5	35 (24–51)	897 (664–1,212)	262 (198–347)	94 (69–128)	566 (423–756)	3	41	56	36	39
	16	204 (157–264)	1,329 (978–1,805)	737 (547–989)	269 (207–351)	614 (458–822)	26	57	30	38	35

[a] Effector-to-target cell ratio, 10:1.

responses detected by either the bulk lysis or precursor frequency assay were mediated by $CD8^+$ T lymphocytes and were MHC class I restricted.

The superior induction of anti-HIV-1 CTLm responses by antigen-specific than by nonspecific stimulation could be related to the interplay of naive, memory, and activated effector $CD8^+$ T-cell subpopulations (20). We hypothesize that the autologous B-LCL present HIV-1 antigen in the context of MHC class I molecules directly to HIV-1-specific memory $CD8^+$ T cells that continuously evolve from activated effector T cells during chronic HIV-1 infection. The B-LCL could also be activating naive T cells with HIV-1 specificity, albeit at a much lower efficiency than other types of antigen-presenting cells (4). This stimulation would result in an expansion of $CD8^+$ CTLm into effector cells in vitro with specificity for Gag, Pol, and Env immunodominant T-cell epitopes. The inclusion of IL-2 in the cultures augments this process and serves to overcome the loss of IL-2 production by $CD4^+$ Th1 lymphocytes during HIV-1 infection (3). Pertinent to this hypothesis is that there were significantly greater levels of activated $CD8^+$ T cells and HIV-1-specific lytic reactivity in the HIV-1 antigen-stimulated cultures, even though there was less outgrowth of cells, than in the anti-CD3 MAb-stimulated cultures.

Although VV-GPE-infected B-LCL also stimulated Vac-specific CTLm in this system, the levels of HIV-1-specific lytic reactivity were consistently higher than the anti-Vac responses. This may obviate the need for the very laborious method of cold-target inhibition, wherein nonradioactive VV-Vac-infected cells are added to the assay mixtures to decrease the background lysis by VV-Vac-specific CTL (12).

By contrast, anti-CD3 MAb stimulates a polyclonal CTL response in memory and activated effector T cells of various antigenic specificities, whereas naive T cells react poorly (4). Moreover, anti-CD3 MAb can induce apoptosis of activated T cells (18), which are known to account for a portion of circulating anti-HIV-1 CTL effectors during HIV-1 infection (7). These factors could result in the lower levels of HIV-1-specific CTLm responses detectable in PBMC cultures stimulated with anti-CD3 MAb than in the antigen-stimulated cultures. Thus, although elevated anti-HIV-1 CTLm precursor frequencies stimulated by anti-CD3 MAb are associated with control of primary HIV-1 infection (1, 11) and low viral load in HIV-1-infected, long-term nonprogressors (17), our data suggest that antigen-specific stimulation is a more sensitive method for assessing HIV-1-specific CTLm responses. This is supported by the association of higher anti-HIV-1 CTLm precursor frequencies stimulated by HIV-1 antigen and not those stimulated by anti-CD3 MAb with higher $CD4^+$ T-cell numbers in the subjects tested in this study.

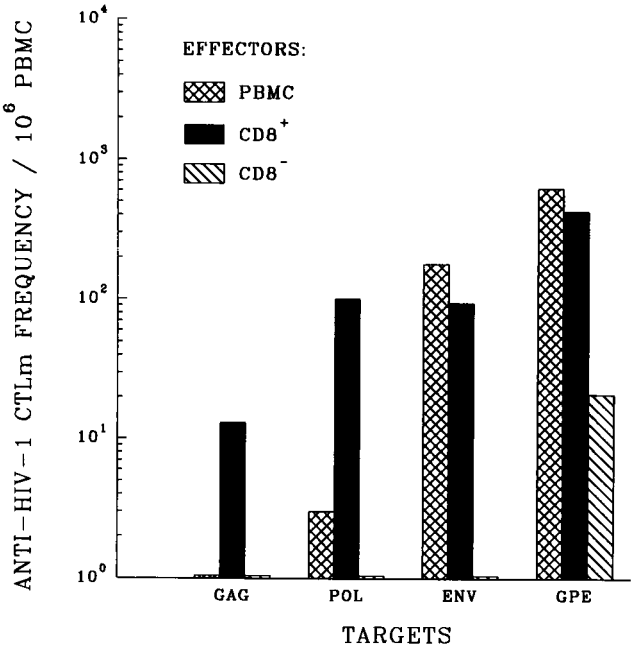

FIG. 4. Anti-HIV-1 CTLm precursor frequencies induced by HIV-1 antigen-specific stimulation in $CD8^+$ and $CD8^-$ T-cell subpopulations obtained from freshly donated blood of study subject A (409 $CD4^+$ T cells per mm^3).

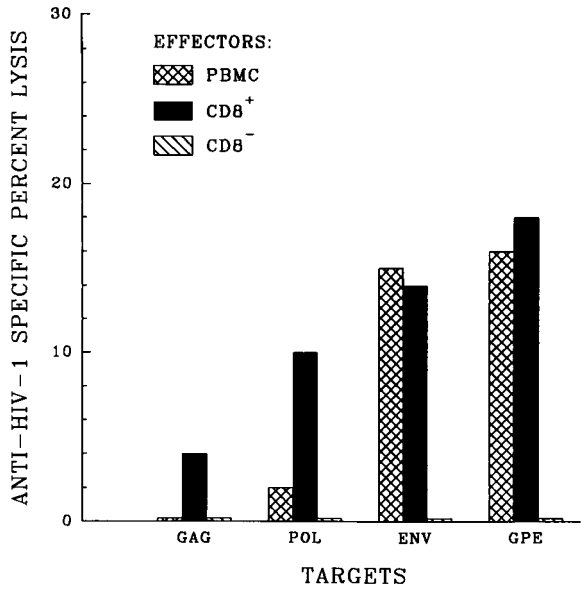

FIG. 5. Bulk lysis mediated by HIV-1 antigen-stimulated $CD8^+$ and $CD8^-$ T-cell subpopulations derived from the same PBMC used for the data shown in Fig. 4 (10:1 effector-to-target cell ratio).

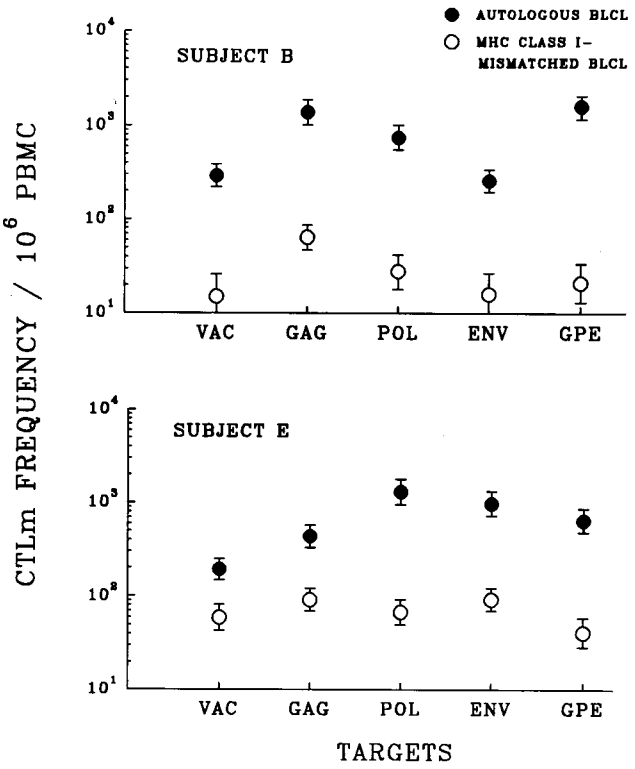

FIG. 6. Precursor frequencies and confidence intervals of anti-HIV-1, MHC class I-restricted lysis mediated by HIV-1 antigen-stimulated, freshly donated PBMC from study subject B (MHC class I type A1, A2; B44, Bw57, Bw4; allogeneic PBMC donor MHC class I type A3, A24; B27, B63, Bw4) and frozen-thawed PBMC from subject E (MHC class I type A3, B7, B44, Bw4; allogeneic PBMC donor MHC class I type A1, A2; B51, B57, Bw4). The $CD4^+$ T-cell counts were 684 per mm^3 for B and 784 per mm^3 for E.

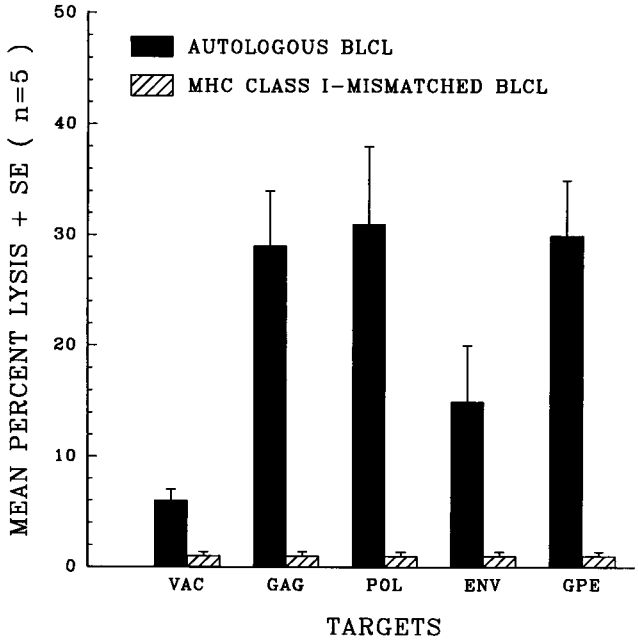

FIG. 7. MHC class I-restricted bulk lysis specific for HIV-1 mediated by PBMC from five study subjects (A, B, E, F, and G) that were stimulated with VV-GPE-infected, autologous B-LCL.

An important determination of this study is that HIV-1-specific CTLm responses can be efficiently activated by HIV-1 antigenic stimulation of frozen-thawed PBMC to levels similar to those induced in freshly donated PBMC cultures. This CTL response can be assessed by either precursor frequency or bulk lysis assays. Although others have recently used a similar method for stimulation of anti-HIV-1 CTLm precursors from frozen-thawed cells (9), no direct comparisons with freshly donated PBMC were made. We show further that anti-HIV-1 CTLm activity is less evident in anti-CD3 MAb-stimulated cultures of frozen-thawed PBMC. To our knowledge, the antigen stimulation method is the most sensitive and consistent assay for analysis of HIV-1-specific CTLm responses in frozen-thawed PBMC. Moreover, the highly quantitative nature of the precursor frequency assay (19) makes it the method of choice, even though it is more labor intensive and costly than the bulk lysis assay. This is supported by the lack of correlation between the two assays shown by our results. Finally, the CTLm assays were shown to be reproducible with PBMC that were stored frozen for up to 16 months. Preliminary studies indicate that anti-HIV-1 CTLm can be reproducibly detected by the antigen-specific stimulation method in PBMC that have been stored for 6.5 years prior to testing (15). This suggests that the assay is applicable to PBMC stored for many years at −135°C.

The significance of our findings is that nonconcurrent prospective studies can now be done with a quantitative assay for CTLm specific for Gag, Pol, and Env with longitudinal specimens of frozen PBMC obtained in natural history, vaccine, and other studies of HIV-1 infection. Such an approach may allow more precise delineation of the role of anti-HIV-1 CTLm in the host response to HIV-1 infection.

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