Human Immunodeficiency Virus Type 1-Specific Cytotoxic T Lymphocytes Release Gamma Interferon, Tumor Necrosis Factor Alpha (TNF- α), and TNF- β When They Encounter Their Target Antigens

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Human immunodeficiency virus type 1 (HIV-1) infection is associated with elevated levels of inflammatory cytokines in the serum and cerebrospinal fluid of infected persons, but the sources of these proteins as well as the specific stimuli which trigger their production and release have not been fully defined. In this study, we evaluated the ability of HIV-1-specific cytotoxic T-lymphocyte (CTL) clones derived from seropositive persons to release gamma interferon (IFN- γ), tumor necrosis factor alpha (TNF- α), and TNF- β upon contact with target cells presenting viral antigen. Peripheral blood- and cerebrospinal fluid-derived HIV-1-specific CD3+ CD4- CD8+ CTL clones as well as freshly isolated peripheral blood mononuclear cells from infected persons were tested in parallel for HIV-1-specific cytotoxicity and cytokine release. Target cells consisted of autologous and allogeneic B-lymphoblastoid cell lines sensitized with synthetic HIV-1 peptides containing the epitopes recognized by these CTL. Cytokine production was measured by specific enzyme-linked immunosorbent assay of culture supernatant fluid. HIV-1-specific CTL clones directed at envelope, Gag, reverse transcriptase, and Nef epitopes specifically released IFN- γ , TNF- α , and TNF- β upon contact with their relevant target epitopes but not following contact with irrelevant epitopes. These cytokines were released in an HLA class I-restricted fashion, and release was detectable as early as 4 to 6 h of incubation and remained elevated at 48 h. Fresh peripheral blood mononuclear cells from a seropositive person likewise released IFN- γ in an antigen-specific and HLA class I-restricted manner when incubated with target cells presenting a peptide containing a CTL epitope, paralleling the HIV-specific cytolytic activity of these cells. These studies indicate that in addition to mediating direct cytotoxicity, HIV-1-specific CTL may affect other immune responses by releasing IFN- γ , TNF- α , and TNF- β . Elevated levels of these cytokines which have been detected in serum and cerebrospinal fluid of infected persons may be due at least in part to the persistent HIV-1-specific CTL response.

Human immunodeficiency virus type 1 (HIV-1) infection is associated with progressive and ultimately profound immunosuppression, yet a number of markers of chronic immune activation are present in infected persons. Neopterin (4, 10, 11), β -2 microglobulin (5, 30, 50), soluble interleukin-2 (IL-2) receptor and CD8 (12), and quinolinic acid (17) may be elevated in serum and cerebrospinal fluid of HIV-1-infected individuals. Recent studies have also demonstrated elevated levels of gamma interferon (IFN- γ) in the peripheral blood (15, 44, 53) and cerebrospinal fluid (12) of HIV-1-seropositive persons, and elevated levels of IFN- γ staining have been observed in germinal centers of lymph nodes (7) as well as brain tissue (53) of such individuals. Likewise, elevated levels of tumor necrosis factor alpha (TNF- α) in serum (25, 27), cerebrospinal fluid (53), and brain tissue (53) have been detected in a number of studies.

One potential source of these cytokines is activated lymphocytes (51). HIV-1 infection is associated with a vigorous and persistent virus-specific cytotoxic T-lymphocyte (CTL) response. HIV-1-specific CTL have been detected in the peripheral blood (26, 40, 58, 60), lung (42), spleen and lymph nodes (14), and cerebrospinal fluid (18, 48) of infected

persons and are thought to contribute to inhibiting virus replication (reviewed in reference 56). This response is mediated predominantly by HLA class I-restricted CD8+ lymphocytes, and the specific CTL epitopes recognized by these cells have begun to be defined (reviewed in reference 39). Although cytokine release patterns by murine virus-specific CTL have been extensively characterized (23, 24, 35, 41, 51), relatively little is known of the cytokine release patterns of human class I-restricted, virus-specific CTL. In this study, we examined cytokine release by HIV-1-specific CTL clones which had been established by limiting dilution cloning from infected persons, to determine whether the virus-specific CTL response may contribute to the elevated levels of inflammatory cytokines observed in these individuals.

(This study was presented in part at the VIII International Conference on AIDS, Amsterdam, The Netherlands, 19–24 July 1992 [18a].)

MATERIALS AND METHODS

Subjects. HIV-1-specific CTL clones were established from peripheral blood and cerebrospinal fluid from four seropositive subjects as previously described (18, 59). Fresh peripheral blood mononuclear cells (PBMC) for use as effector cells were obtained from one of these subjects (subject 010-035i), an asymptomatic seropositive subject

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with a CD4 count of 92/mm³ who was receiving zidovudine at the time of study. All subjects gave informed consent, and the study was approved by the Massachusetts General Hospital Human Studies Committee.

Cell lines. B-lymphoblastoid cell lines (B-LCL) were established by transformation of PBMC with Epstein-Barr virus as previously described (57). B-LCL were maintained in RPMI 1640 medium supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μ g/ml), and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; 10 mM) and containing 20% (vol/vol) heat-inactivated fetal calf serum (medium R-20). Allogeneic B-LCL were also obtained from the American Society for Histocompatibility and Immunogenetics B-Cell Line Repository.

HLA typing. HLA typing was performed by the Massachusetts General Hospital Tissue Typing Laboratory, using standard serological techniques.

Synthetic HIV-1 peptides. Synthetic HIV-1 peptides (p17/2, p17/3, reverse transcriptase [RT]/49, Env/127, Nef/180, Nef/181, p24/122, and p24/124) were synthesized by Multiple Peptide Systems, San Diego, Calif., as previously described (20, 21). The 10-amino-acid (aa) peptide Env/128N and the 9-aa peptide Env/128O were synthesized by Cambridge Research Biochemicals, Wilmington, Del. (20). Peptides were synthesized according to the HIV-1 PV 22 (p17, RT, Env, and Nef peptides) or the BH10 (p24 peptides) sequences (37). The sequences of these peptides in the single-letter amino acid code are as follows: p17/2, KIRLRPGGKKKYKLKHIVWASRELE; p17/3, VWASRELERFAVNPGLLTSEGCRQ; RT/49, AIFQSSMTKILEPFRKQNPDIYIQ; Env/127, AIEAQHLLQLTVWGKQLQA RILA; Env/128N, VERYLKDQQL; Env/128O, ERYLK DQQL; Nef/180, DLSHFLKEKGGLGLHSQRRQDIL; Nef/181, SQRRQDILDWYHTQGYFPDWQNY; p24/122, KRWILGLNKKIVRMYSPTSILD; and p24/124, LDIRQGPKEPFRDYVDRFYKTL.

Preparation of target cells. B-LCL were sensitized with synthetic peptides by incubating 1×10^6 to 5×10^6 cells with 10 to 80 μ g of synthetic HIV-1 peptide per ml for 1 h in the presence or absence of 3.0 to 3.7 MBq of Na²⁵¹CrO₄ and were then washed and used as target cells in the chromium release and cytokine release assays. Similarly, the HIV-1-specific CD3⁺ CD4[−] CD8⁺ CTL clone 63D35 (59) was also incubated with synthetic peptide, employing the same method for sensitization, for use as target cells in selected experiments.

Preparation of effector cells. Cerebrospinal fluid-derived mononuclear cells and PBMC, obtained by Ficoll-Hypaque density gradient centrifugation, were seeded at limiting dilution in 96-well plates, in the presence of 100 U of recombinant IL-2 (rIL-2; Hoffmann-La Roche) per ml and irradiated fresh PBMC (2×10^5 cells per well) from HIV-1-seronegative donors (18, 21, 59). The CD3-specific monoclonal antibody 12F6 was added at 0.1 μ g/ml as a polyclonal stimulus for T-lymphocyte proliferation. Cells were at no time exposed to exogenous viral antigen *in vitro*, in order to ensure that any virus-specific CTL responses would be reflective of *in vivo*-determined specificities. After 2 to 4 weeks, wells demonstrating cell growth were restimulated with irradiated feeder cells and the CD3-specific monoclonal antibody. Cells were then screened for HIV-1-specific cytotoxic activity in chromium release assays, using recombinant HIV-1-vaccinia viruses to express viral antigen in autologous B-LCL (59). HIV-1-specific CTL clones were subsequently characterized with regard to surface phenotype, HLA restriction pattern, and epitope specificity (18, 59).

Clones were maintained in RPMI 1640 medium containing 10% (vol/vol) heat-inactivated fetal calf serum, L-glutamine, HEPES, and antibiotics (medium R-10) supplemented with 100 U of rIL-2 per ml and were periodically restimulated with 12F6 (59). Clones were tested for HIV-1-specific cytotoxicity and for production of cytokines 1 to 3 weeks following restimulation. Freshly isolated nonstimulated PBMC were also used directly as effector cells in selected ⁵¹Cr release and cytokine release assays.

Cytotoxicity assay. CTL clones were incubated in 96-well U-bottom microtiter plates with 10^4 ⁵¹Cr-labeled target cells in a 200- μ l final volume. Clones were plated at effector-to-target (E/T) ratios ranging from 10:1 to 1:1 and incubated for 4 h at 37°C in a 5% CO₂ chamber. Freshly isolated PBMC were plated at E/T ratios ranging from 100:1 to 10:1, using 10^4 ⁵¹Cr-labeled target cells, and incubated for 6 hours. Supernatants were harvested and counted on an LKB Clinii Gamma gamma counter (Pharmacia, Turku, Finland). Percent specific lysis was determined from the following formula: $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})]$. Maximum release was determined by lysis of targets in 2.5% Triton X-100. Spontaneous release was less than 30% of maximum release unless otherwise indicated.

Cytokine release assay. CTL clones and freshly prepared PBMC were incubated at indicated E/T ratios with 10^5 peptide-sensitized target cells per well in 96-well U-bottom microtiter plates in a total volume of 200 μ l of R-10 medium for 4 to 48 h at 37°C in a 5% CO₂ chamber. No rIL-2 or anti-CD3 monoclonal antibody was added to the assay medium. Since Epstein-Barr virus-transformed B-LCL constitutively produce TNF- β (19), for most assays these target cells were irradiated with 100 Gy before being used in TNF- β release assays. IFN- γ and TNF- α release assays were performed with irradiated and nonirradiated target cells without any qualitative difference in the release of these cytokines. Preliminary experiments indicated that specific cytokine release was greater with use of 10^5 target cells per well and 10^5 effector cells per well than with use of 10^4 target cells per well and 10^5 effector cells per well (data not shown). Subsequent assays of cytokine release were therefore performed with 10^5 target cells per well. If not stated otherwise, clones were tested for cytokine release at an E/T ratio of 1:1. PBMC were tested at an E/T ratio of 10:1. Supernatant fluid was harvested and stored at -70°C until testing for cytokine concentrations by using specific enzyme-linked immunosorbent assays (ELISAs) for IFN- γ , TNF- α (Genzyme Corp., Cambridge, Mass.; Biosource International, Camarillo, Calif.), and TNF- β (Biosource International) (1) according to the manufacturers' instructions. Chromium release assays were always performed simultaneously as a control for virus-specific lysis.

RESULTS

HIV-1-specific CTL clones derived from cerebrospinal fluid and peripheral blood release IFN- γ upon contact with the relevant viral epitope. The ability of HIV-1-specific CTL to produce IFN- γ was investigated by using clones derived from three infected individuals. Clone 1-101 was derived from the cerebrospinal fluid of a subject with AIDS dementia; it recognizes an epitope in the p17^{gag} protein (aa 18 to 31), and recognition is restricted by the HLA class I molecule A3 (18). Clone 2-71B was derived from the peripheral blood of a second subject with AIDS dementia. This clone recognizes an epitope in a conserved region of the trans-

membrane envelope glycoprotein gp41 (aa 584 to 592), and recognition by this clone is restricted by HLA-B14. Clone 63D35 was established from the peripheral blood of an asymptomatic subject; it recognizes an epitope in the RT protein (aa 325 to 349) and is restricted by HLA-A11 (59). All CTL clones were CD3⁺ CD4[−] CD8⁺ (18, 59; data not shown). Autologous B-LCL were sensitized with peptides containing the relevant CTL epitopes, and these cells were then incubated with the respective CTL clone at an E/T ratio of 1:1. As controls, autologous target cells were sensitized with irrelevant peptides. Supernatant fluid was harvested at intervals over a 48-h period and tested for IFN- γ by ELISA. As shown in Fig. 1, all three clones produced IFN- γ specifically in response to target cells sensitized with peptide containing the relevant CTL epitope. Significant levels of IFN- γ were detectable as early as 4 h and were still detectable at 48 h for each of the clones tested. No IFN- γ was produced when these CTL clones encountered target cells sensitized with the irrelevant peptide, indicating that the response was specific for the sensitizing epitope alone. Likewise, incubation of target cells with peptide, without the addition of the cloned effector cells, did not stimulate the release of IFN- γ .

HIV-1-specific CTL derived from cerebrospinal fluid and peripheral blood release TNF- α and TNF- β upon contact with the relevant viral epitope. Studies similar to those outlined above were undertaken to determine whether these CTL also produce TNF upon recognition of their relevant target antigens. As shown in Fig. 2, the HIV-1-specific CTL were induced to produce TNF- α and - β upon stimulation with the relevant autologous peptide-sensitized target cell. The cerebrospinal fluid-derived clone 1-101 as well as the PBMC-derived clone 63D35 produced significant levels of TNF- α at 6 h after incubation with the relevant target, and levels were higher at 24 to 48 h (Fig. 2A and B). There was no increase of TNF- α production beyond the background levels when these clones were incubated with target cells incubated with irrelevant peptides, and target cells in the absence of added effector cells produced only background levels of this cytokine. Similar results were obtained for TNF- β production by these cells (Fig. 2C and D). Higher background levels of TNF- β release in Fig. 2C likely indicate constitutive production of this cytokine by B-LCL. Negligible background TNF- β production was observed in the assay shown in Fig. 2D, in which B-LCL were irradiated prior to use as target cells to suppress protein production.

To further clarify the contribution of constitutive TNF- β production by B-LCL, we performed an additional series of experiments in which irradiated and nonirradiated B-LCL were compared as target cells in the TNF- β release assays. The viability of the B-LCL directly after irradiation was comparable to that of cells before irradiation (data not shown), and irradiation of the target cells did not alter the induction of cytokine release by CTL in our assays but did lower the background TNF- β production by these B-LCL (Table 1).

IFN- γ , TNF- α , and TNF- β production by HIV-1-specific CTL is HLA class I restricted. Previous studies of human virus-specific CTL have suggested that the release of IFN- γ occurs in an HLA-unrestricted fashion when these cells encounter virus-infected target cells (31, 32). To determine whether HLA molecules served to restrict cytokine release by these HIV-1-specific CTL, HLA-matched and -mismatched B-LCL were incubated with either the relevant or an irrelevant HIV-1 peptide and used as targets in cytokine release assays. For these experiments, two additional CD3⁺

FIG. 1. HIV-1-specific CTL release IFN- γ when they encounter their target antigen. Target cells consisted of autologous B-LCL incubated with the relevant or an irrelevant HIV-1 peptide. Clones were incubated with target cells for 4 to 48 h. Supernatants were harvested, and IFN- γ concentrations were determined by ELISA. Target-specific IFN- γ release was detectable as early as 4 h of incubation and was still elevated after 24 to 48 h. T, target cells (autologous B-LCL); E, effector cells; lower limit of detection, 100 pg/ml. (A) The cerebrospinal fluid-derived CTL clone 1-101 released IFN- γ upon contact with the Gag p17/2 peptide (aa 18 to 42); (B) CTL clone 2-71B released IFN- γ upon contact with peptide Env/128N (aa 583 to 592); (C) clone 63D35 released IFN- γ upon contact with the RT/49 peptide (aa 325 to 349).

CD4[−] CD8⁺ HIV-1-specific CTL clones were also examined. Clone 2-50C was derived from the cerebrospinal fluid of subject 2, demonstrating the same epitope specificity and HLA restriction as did clone 2-71B, derived from the peripheral blood of the same individual. Clone 51C-51 was derived from the cerebrospinal fluid of an HIV-1-infected individual with meningitis and recognizes an epitope in the Nef protein (aa 103 to 127). Cytokine release was shown to be HLA class I restricted for all clones tested, paralleling the restriction observed for cytolytic activity of these cells. Cytokines were

FIG. 2. HIV-1-specific CTL release TNF- α and TNF- β when they encounter their relevant target. (A) TNF- α release by CTL clone 1-101. TNF- α was detectable only when target cells (T) were sensitized with the cognate peptide p17/2, and levels increased over 28 h. E, effector cells. (B) TNF- α release by clone 63D35. Large amounts of TNF- α are detected at 6 h when target cells are incubated with the relevant peptide RT/49 and increase during the 48-h period. (C) TNF- β release by clone 1-101. TNF- β was detectable at 6 h with use of target cells sensitized with peptide p17/2 and was even more elevated after 24 to 48 h. (D) TNF- β released by clone 63D35. This clone released significant amounts of TNF- β at 24 and 48 h of incubation in the presence of target cells sensitized with peptide RT/49.

produced when HLA-matched target cells were sensitized with the relevant peptide but not with an irrelevant peptide. For example, cytolytic activity and IFN- γ , TNF- α , and TNF- β release of CTL clone 63D35 were greatest when target cells expressed the class I allele HLA-A11 (Table 2). Likewise, cytotoxicity and IFN- γ and TNF- α release of the CTL clones 2-50C and 51C-51 were demonstrable when the target cells shared the class I molecules HLA-B14 (2-50C; Tables 3 and 4) and HLA-B13 (51C-51; Table 5), respectively. IFN- γ release by clone 1-101 was dependent upon

presentation of the cognate peptide by HLA-A3 (Table 6), and IFN- γ release by clone 2-71B was observed only when the target cells shared the class I molecule HLA-B14 (Table 7). Because clones 1-101 and 2-71B could be maintained in culture for a limited time only, the role of HLA antigens in restricting TNF- α and - β release by these clones could not be determined. Likewise, the role of HLA antigens in restricting TNF- β release by clone 2-50C was not determined because of limited availability of this clone, although preliminary data suggested epitope-specific TNF- β release by this clone (data not shown). No significant TNF- β release was detected with use of clone 51C-51 incubated either with

TABLE 1. Evidence that irradiation of target B-LCL reduces the amount of TNF- β constitutively produced by B-LCL but does not inhibit TNF- β release by the CTL

Target cells ^a	HIV-1 peptide	Effector cells ^b	Specific lysis (%) ^c	TNF- β (pg/ml)	
				Nonirradiated target cells ^d	Irradiated target cells ^{d,e}
+	RT/49	—		290	85
+	RT/49	+	82	1,300	1,000
+	Env/127	—		320	85
+	Env/127	+	0	230	85
—		+		54	54

^a Autologous B-LCL were sensitized with the synthetic HIV-1 peptide RT/49 (aa 325 to 349) or Env/127 (aa 558 to 582).

^b CTL clone 63D35, recognizing peptide RT/49 presented by the HLA-A11 molecule.

^c E/T ratio, 3:1; incubation time, 4 h.

^d E/T ratio, 1:1; incubation time, 24 h.

^e Target cells were irradiated with 100 Gy in a gamma irradiator.

TABLE 2. HLA-A11 restriction of HIV-1-specific IFN- γ , TNF- α , and TNF- β release by the CTL clone 63D35

Target cells ^a (shared HLA class I allele)	HIV-1 peptide	Specific lysis (%) ^b	Concn (pg/ml) ^c		
			IFN- γ	TNF- α	TNF- β
Autologous ^d	RT/49	86	2,700	2,200	200
	Env/128O	0	<100	20	24
A11	RT/49	63	3,200	2,600	265
	Env/128O	0	<100	37	48
None	RT/49	3	1,250	860	130
	Env/128O	0	<100	40	62

^a Target cells were sensitized with synthetic HIV-1 peptide RT/49 (aa 325 to 349) or Env/128O (aa 584 to 592), washed, and irradiated with 100 Gy.

^b E/T ratio, 3:1; incubation time, 4 h.

^c E/T ratio, 1:1; incubation time, 24 h.

^d Complete HLA type: A1, A11; B8, Bw62; Cw4; DR3; DRw52; DQw2.

TABLE 3. HLA-B14 restriction of TNF- α release of the cerebrospinal fluid-derived CTL clone 2-50C

Target cells (shared HLA class I allele)	HIV-1 peptide	Specific lysis (%) ^a	TNF- α (pg/ml) ^b
Autologous ^c	Env/127	2	170
	Env/128O	42	900
B14	Env/127	1	110
	Env/128O	48	940
None	Env/127	5	125
	Env/128O	0	110

^a Clone 2-50C was incubated with target cells at an E/T ratio of 3:1 for 4 h.^b E/T ratio, 1:1; incubation time, 24 h.^c Complete HLA type: A24, A28; B7, B14; Cw7, Cw8; DR2, DR12; DRw52; DQw1, DQw3.

the autologous or with an HLA-matched target cell line, suggesting either that the release was below detection limits of this assay system or that there are different cytokine release patterns for some clones.

HIV-1-specific CTL serve as target and effector cells in cytotoxicity and cytokine release assays. A minor amount of IFN- γ , TNF- α , and TNF- β release by clone 63D35 was also observed when the peptide containing the relevant CTL epitope was presented by HLA-mismatched B-LCL (Table 2). Because even nanomolar amounts of peptide have been shown to be sufficient to sensitize target cells for lysis by the CTL clone 63D35 (22a), it was hypothesized that small amounts of peptide still present in the supernatant after preparation of the targets might be able to sensitize the clone itself for self-lysis. This hypothesis was supported by the observation that the CTL clone 63D35 could serve both as a target and as an effector in the cytotoxicity and cytokine release assays (Tables 8 and 9). Incubation of 63D35 effector cells with peptide-sensitized 63D35 target cells presenting the relevant HIV-1 peptide resulted in lysis of the ⁵¹Cr-labeled 63D35 cells and in specific cytokine release. Smaller amounts of cytokines were also released when 63D35 target cells were incubated with the relevant but not with the irrelevant peptide without addition of 63D35 effector cells, further supporting the hypothesis that peptide can sensitize these cells for self-lysis and further confirming the ability of this clone to release IFN- γ , TNF- α , and TNF- β . Measured spontaneous release values in the parallel CTL assays using 63D35 as target cells were higher in the presence of cognate peptide (46 and 32%; Tables 8 and 9) than irrelevant peptide

TABLE 4. HLA-B14 restriction of IFN- γ release of the cerebrospinal fluid-derived CTL clone 2-50C

Target cells (shared HLA class I allele)	HIV-1 peptide	Specific lysis (%) ^a	IFN- γ (pg/ml) ^b
Autologous ^c	Env/127	0	<100
	Env/128O	17	430
B14	Env/127	0	<100
	Env/128O	19	225
None	Env/127	0	<100
	Env/128O	1	<100

^a Clone 2-50C was incubated with target cells at an E/T ratio of 10:1 for 4 h.^b E/T ratio, 1:1; incubation time, 24 h.^c Complete HLA type: A24, A28; B7, B14; Cw7, Cw8; DR2, DR12; DRw52; DQw1, DQw3.TABLE 5. HLA-B13 restriction of HIV-1-specific IFN- γ and TNF- α release by the CTL clone 51C-51

Target cells ^a (shared HLA class I allele)	HIV-1 peptide	Specific lysis (%) ^b	Concn (pg/ml) ^c	
			IFN- γ	TNF- α
Autologous ^d	Nef/180	0	<100	26
	Nef/181	79	1,100	365
B13	Nef/180	7	<100	41
	Nef/181	81	2,520	410
None	Nef/180	4 ^e	<100	<25
	Nef/181	1 ^f	<100	<25

^a Target cells were sensitized with peptide Nef/180 (aa 86 to 110) or Nef/181 (aa 103 to 127) and irradiated with 100 Gy.^b E/T ratio, 3:1; incubation time, 4 h.^c E/T ratio, 1:1; incubation time, 24 h.^d Complete HLA type: A30, A32; B13, B44; Cw5, Cw6; DR11; DRw52; DQw3.^e Spontaneous release, 39%.^f Spontaneous release, 37%.

(32 and 15%; Tables 8 and 9), presumably as a result of self-lysis in the presence of cognate peptide.

Circulating HIV-1-specific CTL produce IFN- γ in response to recognition of their target viral epitope. Unstimulated PBMC obtained from subject 010-035i were previously observed to exert a particularly vigorous HIV-1-specific CTL response directed at an epitope contained in peptide p24/122 of Gag, affording the opportunity to study antigen-specific cytokine release of these PBMC in vitro. This response has been previously demonstrated to be mediated by CD3+ CD4+ CD8+ lymphocytes and to be restricted by the HLA-Bw62 allele (21). Freshly prepared PBMC were incubated with target cells presenting either the relevant or an irrelevant peptide for 6 to 48 h at an E/T ratio of 10:1, and cytokine release was determined in the supernatant. PBMC from this patient released significant amounts of IFN- γ when incubated with the relevant target cells at 24 to 48 h, paralleling the cytolytic activity (Fig. 3). These results demonstrate that IFN- γ release is not merely a phenomenon observed in IL-2-dependent cell lines in long-term culture. Attempts to detect TNF- α in freshly isolated PBMC have been unsuccessful thus far, possibly in part because high levels of TNF- α are spontaneously produced by PBMC in this experimental system (data not shown). TNF- β production by the PBMC was not detected above background levels with this method (data not shown), possibly because of limitations in the sensitivity of the assay.

TABLE 6. HLA-A3 restriction of IFN- γ release of the cerebrospinal fluid-derived CTL clone 1-101

Target cells (shared HLA class I allele)	HIV-1 Gag p17 peptide	Specific lysis (%) ^a	IFN- γ (pg/ml) ^b
Autologous ^c	p17/2	46	220
	p17/3	0	<100
A3	p17/2	48	380
	p17/3	0	<100
None	p17/2	1	<100
	p17/3	0	<100

^a Clone 1-101 was incubated with target cells sensitized with peptide p17/2 (aa 18 to 42) or p17/3 (aa 35 to 59) at an E/T ratio of 10:1 for 4 h.^b E/T ratio, 1:1; incubation time, 6 h.^c Complete HLA type: A3, A23; B7, B35; Cw4, Cw7; DR2, DR6; DRw52; DQw1.

TABLE 7. HLA class I restriction of IFN- γ release of the blood-derived CTL clone 2-71B

Target cells (shared HLA class I allele)	HIV-1 peptide	Specific lysis (%) ^a	IFN- γ (pg/ml) ^b
Autologous ^c	Env/127	6	185
	Env/128N ^d	82	5,700
A28, B14	Env/127	1	185
	Env/128N	78	5,400
A24	Env/127	4	185
	Env/128N	1	195

^a E/T ratio, 10:1; incubation time, 4 h.^b E/T ratio, 1:1; incubation time, 24 h. Effector cells alone released 200 pg of IFN- γ per ml.^c Complete HLA type: A24, A28; B7, B14; Cw7, Cw8; DR2, DR12; DRw52; DQw1, DQw3.^d aa 583 to 592.

DISCUSSION

This study demonstrates that HIV-1-specific CTL derived from the blood and cerebrospinal fluid of infected individuals release IFN- γ , TNF- α , and TNF- β in an epitope-specific and HLA class I-restricted fashion, paralleling the HIV-1-specific cytotoxic response of these cells. Although previous studies of murine and human major histocompatibility complex (MHC) class I-restricted T lymphocytes have demonstrated that these cells are capable of producing a variety of cytokines (2, 8, 24, 32, 34, 35, 41, 45, 51), the present study provides the first evidence in humans that MHC class I-restricted IFN- γ , TNF- α , and TNF- β release can occur in specific response to CTL epitope recognition. The observed release of IFN- γ by CD4⁺ CD8⁺ CTL in an HLA class I-restricted fashion is in accordance with previous studies using murine MHC class I-restricted CTL (24, 34, 35) but is in contrast to the HLA-nonrestricted IFN- γ release by virus-specific CD8⁺ CTL derived from humans reported by other investigators (31, 32). Differences in methodology, including the use of virus-infected target cells and bioassays rather than specific ELISA to measure cytokine release, may account for lack of HLA-restricted responses observed by these authors. In the present study, target cells were incubated with synthetic viral peptides (18, 20, 21, 40, 52, 59), thereby excluding the potentially confounding variable of virus infection of the target cells and demonstrating the requirement of CTL epitope presentation in the proper HLA

TABLE 8. Evidence that CTL clone 63D35 serves as target and as effector for specific lysis and IFN- γ and TNF- α release

Target cells ^a	HIV-1 peptide	Effector cells ^b	Specific lysis (%) ^c	Concn (pg/ml) ^d	
				IFN- γ	TNF- α
+	RT/49	—		160	145
+	RT/49	+	22 ^e	1,200	580
+	Env/128O	—		<100	34
+	Env/128O	+	0 ^f	<100	52
—		+		<100	36

^a 63D35 CTL (10⁵ per well) were sensitized with HIV-1 peptides as indicated.^b CTL clone 63D35.^c E/T ratio, 10:1; incubation time, 4 h; spontaneous release.^d E/T ratio, 1:1; incubation time, 24 h.^e Spontaneous release, 46%.^f Spontaneous release, 32%.TABLE 9. Evidence that CTL clone 63D35 serves as target and as effector for specific lysis and TNF- β release

Target cells ^a	HIV-1 peptide	Effector cells ^b	Specific lysis (%) ^c	TNF- β (pg/ml) ^d
+	RT/49	—		55
+	RT/49	+	62 ^e	770
+	Env/128O	—		<16
+	Env/128O	+	0	<16
—		+		<16

^a 63D35 CTL (10⁵ per well) were sensitized with HIV-1 peptides as indicated.^b CTL clone 63D35.^c E/T ratio, 10:1; incubation time, 4 h.^d E/T ratio, 1:1; incubation time, 24 h.^e Spontaneous release, 32%.

context for the induction of the cytokine release by these CTL.

Although B-LCL target cells were used for the majority of the assays reported here, a number of lines of evidence demonstrate that the B cells are not the source of these cytokines observed in our studies. Irradiation of the B-LCL abrogated the background levels of cytokine production but did not affect the total levels of cytokine production. In addition, the demonstration that cytokine production occurred at similar levels when T lymphocytes were used as target cells provides additional evidence that these cytokines were not released by B-LCL. Although 63D35 released inflammatory cytokines to a lesser degree by incubation with an HLA-mismatched target cell line, this non-HLA-restricted cytokine release may be due to T cell-T cell presentation. Supportive evidence for this interpretation lies in the demonstration that this CTL clone is able to present the relevant epitope for lysis and cytokine release by the very same clone and the observation that an irrelevant peptide did not stimulate effector cells. Experiments in Tables 8 and 9 demonstrate conclusively the release of TNF- α , TNF- β , and IFN- γ by clone 63D35 in assays in which this clone alone was used as both effector and target cell. By using the cognate peptide to sensitize clone 63D35 as target cells, both cytolysis and cytokine release by 63D35 effector cells were observed. CTL-mediated lysis of CTL targets has been reported recently with use of both human and murine CTL

FIG. 3. Release of IFN- γ by fresh PBMC in response to recognition of their target HIV-1 epitope p24/122 (aa 263 to 284). Target-specific IFN- γ release was detectable at 24 h of incubation and was still elevated after 48 h. Methods are as described in the legend to Fig. 1. Specific lysis of autologous target cells (T) presenting the p24/122 peptide after 6 h of incubation was 53%; specific lysis of target cells presenting peptide p24/124 was 0%. E, effector cells.

(36, 55). In the study presented here, we demonstrate that CTL-CTL presentation of the cognate epitope leads also to cytokine release paralleling lysis of radioactively labeled CTL.

These studies suggest that elevated levels of inflammatory cytokines observed in HIV-1 infection are derived at least in part from HIV-1-specific CTL. IFN- γ , TNF- α , and TNF- β release were observed with use of CD4⁺ CD8⁺ CTL derived from the peripheral blood and from the cerebrospinal fluid of four different HIV-1-infected individuals and with use of CTL specific for a variety of HIV-1 proteins and restricted by a variety of HLA class I molecules. The hypothesis that HIV-1-specific CTL contribute to the elevated cytokine levels in vivo is further supported by experiments using freshly isolated unstimulated PBMC from an HIV-1-infected individual. When PBMC were cocultured with autologous target cells sensitized with an HIV-1 Gag peptide containing a CTL epitope recognized by these PBMC, significant levels of IFN- γ were released in an antigen-specific manner paralleling the HIV-1-specific cytolytic activity of these cells (Fig. 3). Although levels of cytokine release may vary somewhat, it appears that IFN- γ , TNF- α , and TNF- β release is a common feature of HIV-1-specific CD3⁺ CD4⁺ CD8⁺ CTL. This cytokine release pattern has also been described for murine Th1 helper and cytotoxic T lymphocytes (51). Whether other cytokines besides IFN- γ , TNF- α , and TNF- β are released by CD4⁺ CD8⁺ HIV-1-specific CTL and whether these patterns differ from those observed in human virus-specific CD4⁺ positive CTL (28, 29) remain to be investigated.

The results reported here may have important implications for understanding HIV-1 disease pathogenesis. Release of IFN- γ may contribute to the host defense against HIV-1 by local direct antiviral effects (16). In addition, IFN- γ induces MHC class I and class II molecule expression on a variety of cells, including endothelial cells, astrocytes, and cells of the monocyte/macrophage lineage (22, 49, 54), which may facilitate recognition of these cells by CTL if they are infected with HIV-1. Alternatively, IFN- γ may induce HIV-1 expression as recently observed in persistently infected promonocytic cells in vitro (3). TNF release may also have an adverse effect on controlling virus replication, since both TNF- α and TNF- β have been shown to stimulate HIV-1 replication in infected T-cell lines (33).

The cytokines released by HIV-1-specific CTL may also contribute directly to the pathogenesis of HIV-1-related neurologic disorders such as AIDS dementia complex. A vigorous HIV-1-specific CTL response has been observed in the cerebrospinal fluid of infected persons, and the present study indicates that target cell recognition by these CTL results in release of IFN- γ , TNF- α , and TNF- β . Elevated levels of IFN- γ and TNF- α have been reported in the cerebrospinal fluid of HIV-1-infected individuals with AIDS compared with levels in healthy individuals (12, 53). There are several different mechanisms by which cytokine production by HIV-1-specific CTL may potentially contribute to the observed neuropathologic changes. IFN- γ induces metabolic changes in macrophages and microglial cells such as altered metabolic pathways for kynurenine (17) and tryptophan (61), which could lead to elevated production of neopterin and quinolinic acid. IFN- γ also stimulates macrophages and microglial cells to release TNF- α (9). TNF- α and TNF- β stimulate astrocytes to proliferate (46) and may contribute to the reactive gliosis, which is one of the characteristic histopathologic alterations in encephalopathic brains of HIV-1-infected individuals (6, 13, 38, 43). In

addition, TNF- α has been implicated in demyelination in the central nervous system (47). The release of these cytokines by intrathecal HIV-1-specific CTL provides a potential physiologic link between the virus-specific immune response and some of the neuropathologic sequelae of HIV-1 infection.

In summary, the results presented here demonstrate that HIV-1-specific CTL not only lyse target cells presenting HIV-1 peptides but also release inflammatory cytokines in response to recognition of these targets. Release of inflammatory cytokines by HIV-1-specific CTL may contribute to the state of immune activation observed in HIV-1-infected individuals and may play an important role in the pathogenesis of HIV-1 infection.

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