ENV-specific Cytotoxic T Lymphocyte Responses in HIV Seronegative Health Care Workers Occupationally Exposed to HIV-contaminated Body Fluids

Ligia A. Pinto,* James Sullivan,‖ Jay A. Berzofsky,‡ Mario Clerici,¶ Harold A. Kessler,§‖ Alan L. Landay,§‖ and Gene M. Shearer*

**Experimental Immunology and ‡Metabolism Branches; National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892; §Departments of Immunology/Microbiology and ‖Medicine, Rush Presbyterian St. Luke's Medical Center, Chicago, Illinois 60612; and ¶Cattedra Immunologia, Universita degli Studi di Milano, Milano, Italy*

Abstract

Identification of the components of protective immunity are crucial for the development of effective prophylactic and therapeutic vaccine strategies. Analysis of HIV-specific responses in exposed but uninfected individuals might thus provide a unique resource to elucidate the components and correlates of protective immunity to HIV. In the present study we analyzed HIV-specific cytotoxic and helper T lymphocyte responses in health care workers (HCW) exposed to body fluids from HIV-positive individuals. HCW exposed to blood from HIV-negative individuals as well as healthy donors served as controls. Cytotoxic T lymphocyte (CTL) responses to HIV envelope (*env*) peptides were detected in 7/20 (35%) HCW exposed to HIV-positive blood and in none of the 20 health care workers exposed to uninfected blood or the seven healthy blood donors studied. HIV-specific CTL responses were detected only after in vitro stimulation, and were MHC class I restricted. No MHC class I restriction elements were uniformly identified among the different responders. 21/28 (75%) HCW exposed to contaminated blood responded to *env* as measured by IL-2 production to the peptides, in contrast to only 9/38 (24%) HCW exposed to HIV seronegative blood and 3/35 (9%) healthy blood donors. All the HIV exposed individuals were seronegative on repeated ELISA tests, and no evidence of infection was obtained by PCR analysis. These findings indicate that a single exposure to HIV can induce CTL immunity to HIV antigens, in the absence of other evidence of infection. (*J. Clin. Invest.* 1995. 96:867–876.) Key words: HIV-specific CTL • class I-restricted CTL • T helper responses • HIV-exposed health care workers

Introduction

A better understanding of the reason that some individuals remain uninfected despite exposure to HIV is critically important for an understanding of the mechanisms of protective immunity and therefore for the development of more effective prophylactic and therapeutical vaccine strategies. T lymphocyte immunity may play an important role in the host's response to HIV infection. T cell immunity appears to be transient and declines simultaneously with other immune functions in the HIV-infected host as disease progresses (1–3). Several lines of evidence suggest that cytotoxic T lymphocyte (CTL)[1] activities might be involved in delaying the onset of disease in HIV-infected patients (4–6). Large numbers of circulating CTL and CTL precursor cells are present in asymptomatic HIV-infected (HIV^+) individuals. Circulating CTL numbers decrease gradually with time, such that HIV-specific CTL become undetectable before major clinical deterioration (7, 8). A balance between T cell responses and HIV titers appears to occur during latent infection and AIDS. Evidence for CD8+ cytotoxic T lymphocyte precursors specific for cells expressing HIV-1 *gag, pol,* and *env* with clearance of viremia in acute infection suggests that cellular immunity is involved in the initial control of virus replication in primary HIV infection and imply a role for CTL in protective immunity to HIV in vivo (9, 10). Further evidence for a role of CTL activity in the delay of disease progression has been provided in studies on animal models of SIV and HIV infection (11, 12).

Several lines of evidence suggest that exposure to HIV does not necessarily result in seroconversion or infection defined by standard criteria. Nevertheless, several laboratories have recently reported that exposure to HIV, in the absence of seroconversion, can induce HIV-specific cell mediated immune responses that have been suggested to contribute to virus clearance (13–23). Accumulating evidence suggests that a rapid and effective CTL response during an invasive exposure to HIV might be involved in clearing the organism of the first infected cells. Unusually high frequencies of HIV-specific CTL precursors have been demonstrated also in uninfected donors, presumably related to priming by cross-reactive antigens such as homologous bacterial and viral proteins, HLA antigens and other self proteins as well as endogenous retroviral proteins (4). It is plausible that rapid recruitment and expansion of these cells after limited HIV exposure could account for the protection against HIV infection. In this context, strong HIV specific CTL activity has been recently reported in uninfected infants born from HIV-infected mothers (17, 19, 20). Furthermore, high frequency of CTL precursors against *nef* antigen was recently demonstrated in uninfected individuals sexually exposed to HIV (23).

We recently reported T helper cell reactivity to HIV envelope peptides in six out of eight health care workers (HCW)

Address correspondence to Gene M. Shearer, Ph.D., National Cancer Institute, National Institutes of Health, Experimental Immunology Branch, Bldg. 10, Room 4B17, Bethesda, MD 20892. Phone: 301 402-3246, FAX: (301) 496-0887.

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1. *Abbreviations used in this paper:* CTL, cytotoxic T lymphocyte; HCW, health care workers.

exposed to body fluids from HIV $^+$ patients (22), as well as in PCR negative homosexual men who engage in high-risk sexual behavior (16). However, T helper cell responses could be caused by exposure to defective or dead virus, whereas CD8$^+$ CTL would be strongly suggestive that virus actually infected some cells to be presented with class I MHC molecules. HCW accidentally exposed to HIV represent a unique population for whom the time and type of exposure are specifically recorded. Furthermore, in contrast to other cohorts of HIV-exposed individuals, exposure of HCW should be lower and involve a single event. Although the risk of transmission of HIV in this population is estimated to be low ($< 0.3\%$) (24), it has been demonstrated that HIV can be readily cultured from the infected residual blood aspirated from needles (H. A. Kessler, unpublished observations), suggesting that most high risk percutaneous exposures of HCW can result in live HIV inoculation. To address whether a single documented high risk exposure to HIV results in activation of CTL immunity, we analyzed CTL activity specific for synthetic peptides corresponding to the envelope (*env*) of HIV in a well characterized group of HCW occupationally exposed to body fluids from HIV $^+$ patients, in parallel with the evaluation of HIV-specific T helper cell reactivity.

Methods

Subjects

Health care workers who received accidental parenteral exposures to body fluids from HIV-infected or uninfected patients reported immediately to the Employee Health Service, Rush Presbyterian-St. Luke's Medical Center, where they were interviewed, completed questionnaires concerning their accidents. At that time they were informed of this study and asked whether they wanted to participate. Those who were interested were given a copy of the protocol and consent form. 28 HCW reporting occupational exposures to HIV-infected blood or body fluids were prospectively enrolled from 9/90 to 5/94. Thirty-eight HCW reporting blood or body fluid exposures from HIV-1 seronegative source patients, as well as 33 healthy blood donors were enrolled as controls (Table I). The participants were recruited under protocols reviewed and approved by the Institutional Review Boards of both Rush-Presbyterian-St. Luke's Medical Center and the National Cancer Institute (NCI). Whole blood was obtained at various time intervals (range 0–99 wk) following the occupational exposure. Information about HCW post-exposure management and source patient clinical status was obtained by reviewing HCW employee health records, source patient medical records and the Infectious Disease clinical charts of HCW who elected to take AZT after their injuries, as well as those of the respective source patients.

Synthetic peptides

The peptides used in this study were synthesized as previously described (25–27). The peptides, based on the sequence of gp160 HIV-1 IIIB are: *env* T1 (KQIINMWQEVGKAMYA, aa residues 428–443; gp120); *env* T2 (HEDIISLWDQSLK, aa residues 112–124; gp120); *env* Th4.1 (DRVIEVVQGAYRAIR, aa residues 834–848; gp160) and *env* P18 (P18 IIIB; RIQRGPGRAFVTIGK; aa residues 315–329; gp160). An *env* peptide based on the sequence of gp160 HIV-1 MN was also used (P18 MN, RIHIGPGRAFYTTKN; homologous to P18 IIIB; gp160). Peptides were dissolved in RPMI 1640 and stored at −80°C.

Preparation of mononuclear cells

Blood was collected into heparinized vacuum tubes (Becton Dickinson Vacutainer Systems, Rutherford, NJ) in Chicago, coded and shipped overnight at ambient temperature to the NCI in Bethesda, where the samples were tested. The peripheral blood mononuclear cells (PBMC) were separated by centrifugation at 1,800 *g* for 20 min on Ficoll-Hypaque gradients. The PBMCs were washed twice in PBS (GIBCO) and resuspended in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and Hepes buffer (GIBCO). In most cases, the samples were coded and tested in a blinded manner such that the investigators in Bethesda did not know which samples came from unexposed individuals or from HCW exposed to HIV $^+$ or HIV $^-$ body fluids. In most cases, repeat bleeds of the same health care worker carried different code numbers, and the investigators in Bethesda were unable to distinguish between the experimental and control groups.

Table I. Health Care Worker Demographic Characteristics

	HIV exposed (n = 28)	Control HIV− exposed (n = 38)
Sex		
Male	19	36
Female	9	2
Race		
White	17	28
Black	5	8
Hispanic	2	1
Asian	4	1
Age (mean)	34.7	33.5
Job category		
Nurses	9	30
Physicians	11	2
Laboratory workers	3	3
Other	5	3
Prior exposures		
HIV− and unknown source	11/21	19/29
HIV+ source	1/21	3/29
Injury type		
Mucous membrane splash	2	0
Needle puncture	22	29
Scratch/laceration	5	8
Wound contamination	0	1
AZT administration	10	0
HIV PCR Negative	28	ND
HIV Ab (ELISA) Negative	28	38

ND, not done.

In vitro assay for T helper function

The production of IL-2 was tested by stimulation of 3×10^5 PBMC/well (in triplicate) in 96-well microtiter plates (Costar, Cambridge, MA) in 0.2 ml of RPMI + 5% human serum (28). Synthetic peptides corresponding to the *env* of HIV-1 (25–27) were used as previously described at 2.5 μM (28, 29). Influenza A/Bangkok RX73 [H3N2] (FLU) prepared and used at an optimal stimulatory concentration as a positive control for each individual's CD4-mediated response to a recall antigen (28, 29). The cultures also contained 2 μg/ml of the anti-Tac anti-IL-2 receptor monoclonal antibody (generously provided by Dr. John Hakimi, Hoffman-La Roche, Nutley, NJ) to block IL-2 consumption. Culture supernatants were harvested after 7 d. The total IL-2 produced throughout the culture period was determined by testing each supernatant for ability to stimulate proliferation of an IL-2–dependent mouse continuous T lymphocyte line (CTLL). Four successive two-fold dilutions in triplicate were used to test the supernatants for ability to stimulate the proliferation of 8×10^3 CTLL/well in 96-well microtiter plates (28). After 24 h, the CTLL cultures were pulsed with 1 μCi of [^{3}H] thymidine, and harvested 18 h later. A sample was scored as positive if there was a positive IL-2 response to two or more of the 5

HIV peptides tested. An IL-2 response was considered positive to a given peptide when the proliferation (in cpm) of CTLL cells in the presence of the supernatants of cultures with the peptides exceeded fivefold or more the proliferation of cells cultured in the presence of unstimulated cultures.

Results were expressed as the mean counts per minute (CPM) for triplicate wells at the highest supernatant dilution tested, as well as stimulation index (S.I.) calculated as:

$$\frac{\text{cpm of cultures in the presence of peptide-stimulated supernatants}}{\text{cpm of cultures in the presence of unstimulated supernatants}}$$

Cytotoxic T lymphocyte assays

Preparation of effector CTL. CTL assays were performed according to the method previously reported (30). 3×10^6 freshly isolated PBMC were incubated for 7 d with the HIV *env* synthetic peptides (2.5 μM) at 37°C in a humidified 5% CO_2 incubator in RPMI 1640 supplemented with 5% human serum. The cells were then washed and resuspended in RPMI 1640-10% FCS and used in the CTL assays at a concentration of 3×10^6 cells/ml.

Preparation of target cells. Target cells were EBV-transformed B lymphoblastoid cell lines. Autologous B lymphoblastoid cell lines were generated by incubating PBMCs with the supernatant of B95.8 cells, a cell line that chronically produces Epstein Barr virus, and an anti-CD3 monoclonal antibody (OKT3; Ortho Biotech, Raritan, NJ), provided by the Division of Cancer Treatment, National Cancer Institute. Target cells were labeled with Chromium-51 (150 μCi $Na_2{}^{51}CrO_4$; Amersham Corp, Arlington Heights, IL) and pulsed overnight with either no peptide or 5 μM of the peptides. After three washes with PBS, the targets were resuspended at 5×10^4 cells/ml in RPMI 1640 containing 10% FCS and were dispensed into the wells of a 96-well round bottom microtiter plate at 5×10^3 cells/well. 100 μl of effector cells at concentrations of 3×10^5, 15×10^4, and 7.5×10^4 was added in triplicate to the target cells. Spontaneous release was determined in targets cultured in media alone. Maximal release was determined from 100 μl of each target incubated with 5% Triton X-100. After 6 h of incubation, supernatants were harvested and counted in a gamma counter (Micromedic Systems Inc., Horsham, PA). Percent specific lysis was determined as 100% × (test cpm-spontaneous cpm)/(maximum cpm-spontaneous cpm). A CTL response was considered positive when the difference between the percent lysis of peptide pulsed targets and percent lysis of RPMI-pulsed targets was > 15%. In cases of high percent lysis against RPMI-pulsed targets, responses were considered positive when the percent specific lysis against peptide-pulsed targets was three fold above the RPMI-pulsed targets. Antibody-blocking experiments were performed by incubating target cells with an anti-class I antibody W6/32 (anti-HLA-A,-B,-C) at a fixed E:T.

Generation of HIV-specific $CD8^+$ cell lines

$CD8^+$ cells were isolated using Dynabeads (Dynal Inc., Lake Success, NY) according manufacturer's instructions. 1×10^6 cells were cultured in the presence of irradiated peptide-pulsed autologous PBMC (1.5×10^6 cells/well) in 48-well plates in media supplemented with 10% human serum. Interleukin-2 (10 U/ml; Boehringer Mannheim, Indianapolis, IN) was added 3 d after culture. The cells were restimulated weekly with irradiated autologous peptide-pulsed PBMC and maintained in IL-2 (10 U/ml) containing media, that was changed at 3-d intervals. CTL assays were performed as described above after four rounds of stimulation.

HIV-1 provirus detection by PCR

PCR was performed according to the manufacturer's instructions (Roche Molecular Systems). Briefly, an ethylenediaminotetracetic acid-anticoagulated blood sample (0.5 ml) was processed with specimen wash reagent to lyse the blood red blood cells. The cell pellet was extracted with proteinase K and non-ionic detergents and amplified with SK431/462 primers (31). To prevent carry over in this system uracil-*N*-glycosylase was utilized in the amplification mixture. Amplified product was detected by enzyme immunoassay using the SK102 probe.

Haplotype determination

HLA antigens were determined by the tissue typing laboratory at Maryland Medical Laboratories (Baltimore, MD), using standard serologic assays.

HIV antibody

HIV antibody was measured with a commercial solid phase enzyme immunoassay (ELISA; Abbot Diagnostic, Abbot Park, IL).

Results

HIV specific cytotoxic T lymphocyte responses. Env-specific CTL responses were evaluated in 20 HIV-seronegative, PCR negative HCW with exposure to HIV contaminated blood/body fluids, as well as in 20 health care workers with exposure to seronegative blood and seven healthy blood donors, presumably unexposed to HIV. To induce expansion of HIV-specific primed T cells, PBMC were stimulated in vitro with each of the five individual peptides or a pool of peptides for 7 d. After this stimulation, bulk cultures of PBMC were assayed for cytotoxic activity against HIV *env* peptide pulsed-EBV transformed autologous B lymphoblastoid cell lines in a 6-h-^{51}Cr-release assay. Seven of 20 individuals with known HIV exposure exhibited cytolytic activity against the peptide-pulsed targets at least once during the study (Fig. 1). HIV-specific lysis was observed only in peptide-stimulated cultures and was not detected in fresh blood tested for CTL activity. Donor 1 (*A*) responded to a combination of T1 and T2 peptides as well as to P18 IIIB. Donor 2 (*B*) responded to a pool of T1 and T2 peptides. Donors 3 (*C*) and 4 (*D*) responded to a pool of P18 MN/P18IIIB peptides or to P18 MN, respectively. Donor 5 (*E*) and 7 (*G*) showed specific cytolytic activity against a pool of T1, T2, and Th4.1 peptides. Significant T1-specific CTL activity was detected in the T1-stimulated cultures from donor 6 (*F*). Although peptide-specific CTL responses were detected only after in vitro stimulation with the peptides, the reported activity does not appear to represent a primary in vitro response to the peptides, since no *env*-specific lysis was detected in PBMC from any of the individuals exposed to HIV-negative blood or healthy donors. A representative negative CTL assay on *env*-stimulated cultures of an individual exposed to an HIV uninfected sample is shown in Fig. 1 *H*.

To investigate the HLA restriction of these responses, CTL activity of stimulated cultures were assayed against peptide-pulsed HLA class I-mismatched targets in some individual cases (for example, donor 2) (Fig. 2 *A*.) No HIV-specific cytolytic activity of *env*-stimulated cultures was observed against heterologous EBV-transformed mismatched targets pulsed with the stimulatory peptide. In addition, there was complete inhibition of *env*-specific lysis of autologous peptide-pulsed targets in the presence of the anti-class I monoclonal antibody (W6/32) (donor 4) (Fig. 2 *B*). The W6/32 reagent did not exhibit any inhibitory effects on antigen-specific MHC class II restricted responses (data not shown).

No clear relationship emerged from the comparison between the *env*-specific CTL responses and the HLA haplotype of the CTL responders (data not shown). Nevertheless, based on a previous study (30), it is noteworthy that three of the CTL

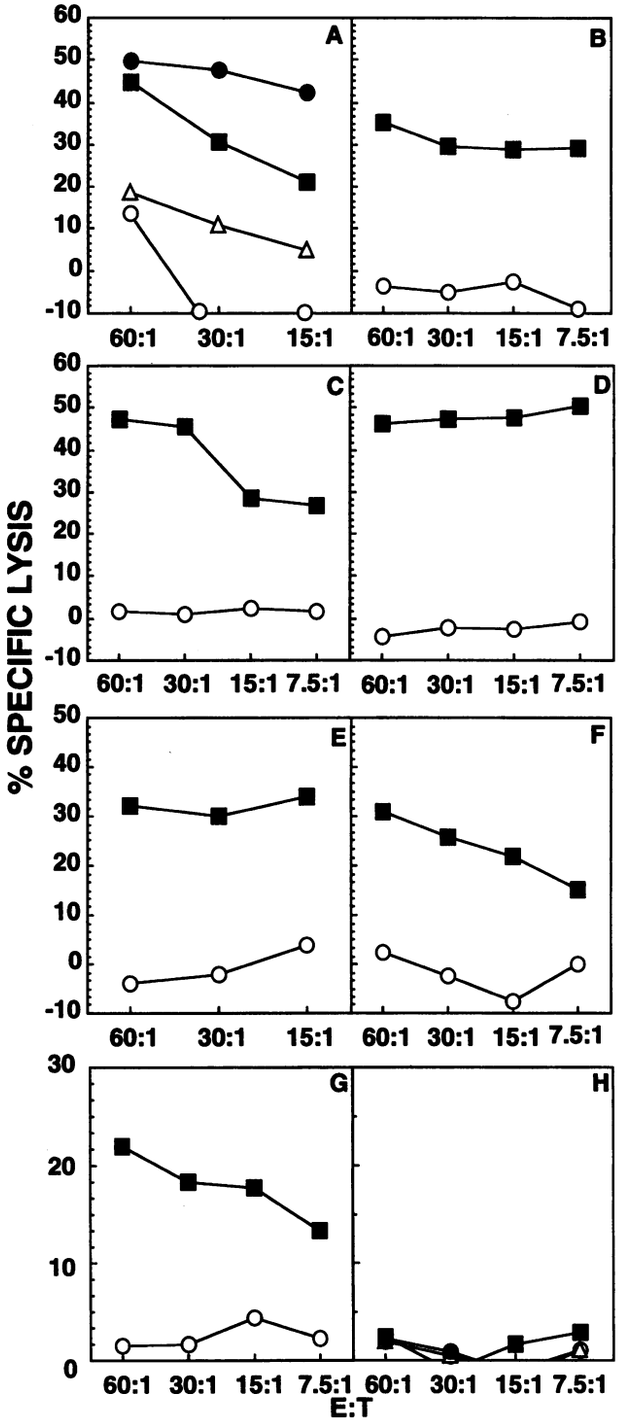

Figure 1. CTL activity of cultures stimulated with *env* peptides against autologous EBV-transformed targets pulsed with the stimulating peptide (■,●) or with RPMI (○,△). Donor 1–7 (*A–G*) had been exposed to body fluids from HIV-infected patients. The donor shown in *H* is representative of donors exposed to body fluids from a seronegative patient. (*A*) Donor 1: CTL response of cultures stimulated with P18 IIIB (■,○) or with a pool of T1/T2 peptides (●,△). (*B*) Donor 2: CTL response of cultures stimulated with a pool of T1/T2 peptides. (*C*) Donor 3: CTL response of cultures stimulated with a pool of P18 MN/IIIB peptides. (*D*) Donor 4: CTL response of cultures stimulated with the P18 MN peptide. (*E*) Donor 5: CTL responses of cultures stimulated with a pool of T1/T2/Th4.1 peptides. (*F*) Donor 6: CTL responses of cultures stimulated with the T1 peptide. (*G*) Donor 7: CTL responses of cultures stimulated with a pool of T1/T2/Th4.1 peptides. (*H*) CTL responses from cultures stimulated with T1/T2/Th4.1 or P18 MN/IIIB pools of peptides. Donor 1: HLA-A2,33, B27,60, C-,DR2, 4, w53, DQ 1,3. Donor 2: HLA-A 2,28, B18, B50, Cw6, w7, DR7, 11, w52,w53 DQw2,7. Donor 3: HLA-A28, 33, B4, B57, Cw6,7. Donor 4: HLA-A1, A-, B7,62, Cw3,w7, DR1,4, w53 DQ5,8. Donor 5: HLA-A1, 11, B44, 55, Cw3, DR1,6,w52, DQ1. Donor 6: HLA-A10,24, B18,-, Cw5, DR11, 15, w52, DQw6, w7. Donor 7: HLA-A2,33; B7,38; C-; DR11,15,w52,DQ1,3.

responders shared HLA-A2 and all three recognized the T1/T2 pool of peptides.

The lack of cytolytic activity of peptide-stimulated cultures on RPMI-pulsed targets, in addition to the fact that CTL responses to *env* peptides were observed upon stimulation with only certain peptides and not others, suggest that these responses are peptide-specific (Fig. 3). Thus, PBMC from the donor in Fig. 3 *A* responded to P18 MN but not any of the other four peptides used individually as stimulators, despite the fact that the effectors were assayed on autologous targets pulsed with a pool of all five peptides. The PBMC from the donor in panel B were stimulated and assayed on targets pulsed with the same peptides used for stimulation. This experiment demonstrates that this donor responded to P18 MN and/or P18 IIIB but not to the pool of T1/T2/Th4.1 peptides.

Temporal analysis of CTL responses. The analysis of CTL responses performed at different time points after exposure to HIV indicated variability with time among the different individuals tested (Table II). In one individual (donor 6), CTL responses against *env* peptides were observed 1 wk after exposure to HIV and no cytolytic activity from 7-d peptide-stimulated cultures were observed 69 d after exposure. In donor 1, *env*-specific responses were detected 56 d after exposure, but were absent 149 d after exposure. The CTL response studied at a single time point in donor 4 demonstrated P18 MN-specific CTL 81 d after exposure. T1, T2, and MN specific lysis was observed 94 d after exposure in donor 3. These responses were absent 220 d after exposure. In donor 2, CTL responses to T1T2 peptide pool were seen 143 d after exposure to HIV, but not at previous time points, with disappearance of *env*-specific responses 227 d after exposure. Similarly, responses to T1/T2/Th4.1 peptide pool observed in donor 5 after 197 d of exposure were absent 237 d after exposure to HIV. Thus, CTL responses were seen as early as 7 d after exposure and as late as 197 d after exposure. However, the CTL memory was lost from the peripheral circulation in each case within 237 d, and often < 40 d after a previous positive response. It is not clear whether this loss is actual loss of memory cells or compartmentalization out of the circulation. It is also important to note that the blood samples were tested in a blinded fashion, and there was no statistically significant differences (Student's *t* test) between the number of times that HCW exposed to HIV-positive and HIV-negative body fluids were tested (mean = 2.45±1.32 SD vs. 1.95±1.05 SD, range between 1 and 5). Thus, the substantially higher frequency of positives in the former group was not simply an artifact of the number of times they were tested.

Relationship between CTL and T helper responses. IL-2

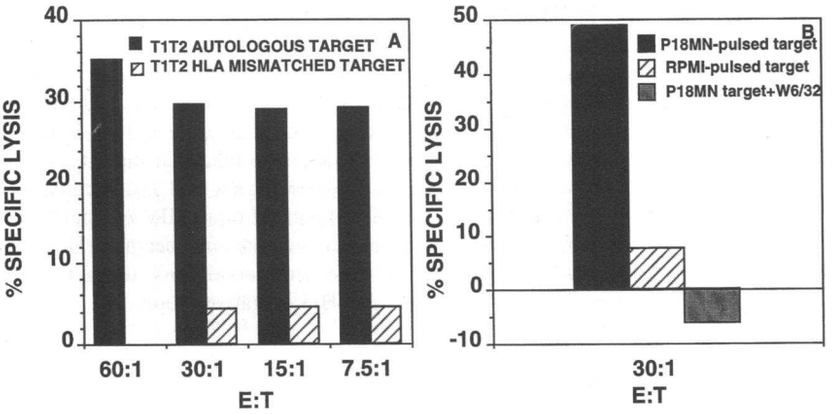

Figure 2. (*A*) CTL activity of a T1/T2 peptide pool-stimulated culture assayed against autologous (■) (Donor 2: HLA-A2,28; B18,50; Cw6,w7; DR7,11,w52,w53, DQw2,7) and HLA-mismatched EBV targets (▨); (Donor 5: HLA-A1,11; B44,55; Cw3,w5,DR1,6,w52; DQ1) peptide-pulsed targets. (*B*) CTL activity of a P18 MN-stimulated culture assayed against autologous (Donor 4) media-pulsed targets (▨) and peptide-pulsed targets in the presence (▩) or absence (■) of anti-class I W6/32 mAb (25 μg/ml). The W6/32 monoclonal antibody did not inhibit T cell proliferation to tetanus toxoid (data not shown).

production in response to the *env* peptides was tested simultaneously with the CTL assays. All of the CTL responders (7/20, 35%) were T helper reactive to the HIV *env* peptides at least once during the course of the study (Table III). A comparison between CTL responses and T helper responses at several times after exposure to HIV indicates that CTL responses can be stimulated in vitro in the absence of a detectable positive *env*-stimulated IL-2 response. These findings are compared in summary in Table II, and in detail in Fig. 4. In two of the cases (*A* and *E*) CTL responses appear to parallel the T helper (IL-2) responses. There was concordance between the two assays at both time points for the donor shown in Fig. 4 *E*, and at the first time and third time points for the donor shown in Fig. 4 *F*. For one time point in three of the donors (*C, D,* and *F*) and for two time points for one donor (*B*) at least 30% *env*-specific lysis was observed when the IL-2 stimulation index was less than three. Conversely, only one donor exhibited strong IL-2 responses to env when CTL activity was < 10% (*C*). These results demonstrate that CTL activity to HIV peptides can occur in the absence of T helper activity. Nevertheless, in seven of the CTL responders the HIV peptides recognized as T cytolytic epitopes were also recognized as T helper epitopes, in some cases at nonconcordant time points, which may reflect differences in the kinetics of CTL and T helper activity. It is also possible that some of the IL-2 was generated by virus-specific $CD8^+$ class I restricted T cells (32).

Env-specific T helper responses evaluated in a total of 28 HIV seronegative, PCR negative HCW with HIV exposures, indicated that 21 of them (75%) showed responses to two or more of the five peptides and in certain individuals these responses were observed more than 23 wk after exposure. Surprisingly, 24% (9/38) of HCW with HIV negative exposures exhibited responses to the peptides in contrast to 9% (3/33) in healthy blood donors (Table IV), and < 3% in > 200 previous low risk seronegative controls (M. Clerici et al., unpublished observations) and the difference between the groups were statistically significant. It cannot be ruled out that some of the exposures to negative fluids were actually to fluids from HIV-infected individuals who had not yet seroconverted. In contrast to the IL-2 (T helper) data, no *env*-specific CTL activity was detected in either of the two control groups.

To further investigate the nature of the observed CTL re-

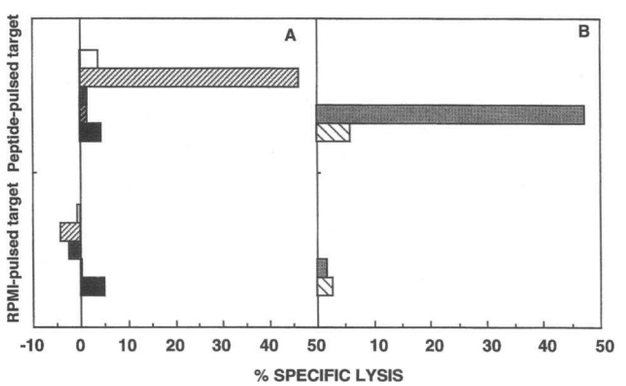

Figure 3. Specificity of peptide-induced CTL responses. (*A*) CTL activity of T1 (■), T2 (■), Th4.1 (■), P18MN (▧), or P18 IIIB (□)-stimulated cultures against targets pulsed with a pool of T1,T2,Th4.1,P18MN and P18IIIB at an effector:target ratio of 60:1. (*B*) CTL activity of T1/T2/Th4.1 (▧) or P18MN/IIIB (■)-stimulated cultures against T1/T2/Th4.1 or P18 MN/IIIB-stimulated targets respectively, at an effector:target ratio of 60:1.

Table II. Longitudinal Analysis of env-specific CTL Responses

Donor	Time (d)	CTL response to	Th response to
1	36	None	T2, TH4.1, P18 MN
	56	T1/T2 pool, P18 IIIB	T1, T2
	115	P18IIIB	None
	149	None	T1
2	51	None	P18 IIIB, TH4.1
	79	None	T1
	119	None	T1, T2, TH4.1
	143	T1/T2 pool	None
	227	None	None
3	75	P18 MN/P18 IIIB pool	TH4.1
	94	T1/T2 pool, P18 MN	P18 MN
	220	None	T2
5	197	T1/T2/TH4.1	T2, TH4.1
	237	None	None
6	7	T1T2TH4P18MNIIIB pool	T1, T2, TH4.1
	34	T1	P18 MN
	69	None	TH4.1

Table III. Frequency of HIV-env Peptide-specific Cytotoxic (CTL) and T Helper Lymphocyte Responses Detected in the PBMC of HCW Exposed to HIV-contaminated Body Fluids

	HIV^+ fluid exposed	HIV^- fluid exposed	Unexposed
TH^+CTL^+	7/20 (35%)	0/20 (0%)	0/7 (0%)
TH^+CTL^-	10/20 (50%)	5/20 (25%)	0/7 (0%)
TH^-CTL^+	0/20 (0%)	0/20 (0%)	0/7 (0%)
TH^-CTL^-	3/20 (15%)	15/20 (75%)	7/7 (100%)

sponses we generated *env*-specific T cell lines from CD8+ cells isolated from HIV exposed individuals. Specific lysis by these T cell lines could be induced after several rounds of stimulation using *env*-pulsed irradiated autologous PBMC as antigen presenting cells. T1 and P18 MN-specific CTL recognition of autologous targets was observed after four rounds of stimulation with autologous T1 and P18 MN-pulsed PBMC, from an HIV exposed HCW (donor 6) 425 d after exposure (Fig. 5). In addition, the CTL lysed autologous P18 MN-pulsed targets but not P18MN-pulsed allogeneic HLA class I mismatched targets, indicating that the cytotoxicity observed in this system is MHC restricted (*C*). It is also important to note that these CTL responses were T1 or P18 MN-specific (*A* and *B*), because no significant lysis was observed against other HIV *env* peptides. In contrast, we were unable to generate *env*-specific CTL lines by parallel culture, under the same conditions, using PBMC from two donors exposed to HIV-negative blood.

Discussion

In the search for a correlate of protective immunity against progression to AIDS, researchers have begun to investigate two populations of HIV^+ individuals: long term survivors (33), patients who have survived several years, despite a diagnosis

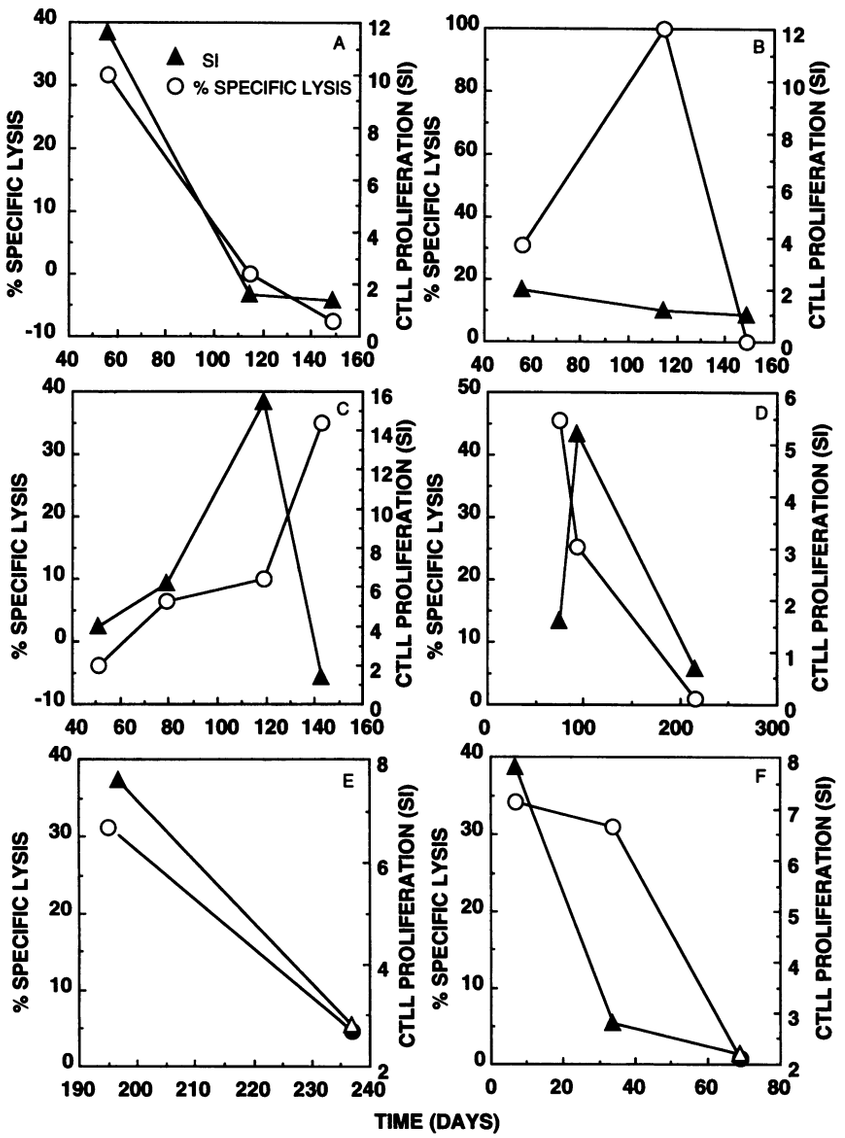

Figure 4. Relationship between *env*-specific CTL activity (○) and T helper responses (▽) in CTL responders at different time points after exposure to HIV. (*A*) Donor 1: CTL activity of a T1/T2-stimulated culture against T1/T2 pulsed-autologous EBV targets and T1/T2-induced IL-2 response. (*B*) Donor 1: CTL activity of a P18 IIIB-stimulated culture against P18 IIIB-pulsed EBV targets and P18 IIIB-induced IL-2 response. (*C*) Donor 2: CTL activity of a T1/T2-stimulated culture against T1/T2-pulsed targets and T1/T2-induced IL-2 response. (*D*) Donor 3: CTL activity of a P18 MN/IIIB peptide pool-stimulated culture against P18 MN/IIIB-pulsed EBV targets and P18 MN/IIIB-pulsed IL-2 response. (*E*) Donor 5: CTL activity of a T1-stimulated culture against T1-pulsed targets and T1-induced IL-2 response.

Table IV. Env-specific T Helper and CTL Reactivity in Exposed HCW and Unexposed Donors

Type of exposure	T helper$^+$	CTL$^+$
HIV$^+$ exposure	21/28 (75%)*	7/20 (35%)*
HIV$^-$ exposure	9/37 (24%)‡	0/20 (0%)
Unexposed	3/33 (9%)	0/7 (0%)

* Statistically significant differences between the individuals exposed to HIV$^+$ and HIV$^-$ body fluids or unexposed blood donors, $P = 0.0001$ (Chi-square test). ‡ Statistically significant differences between the HCW exposed to HIV$^-$ body fluids and unexposed blood donors, $P = 0.0005$ (Chi-square test).

of AIDS and a low CD4 count; and long-term nonprogressors (33–36), patients who appear to be healthy and have not exhibited a decline in CD4$^+$ T cell numbers during several years of follow-up. Another potentially useful population to study are individuals who have no evidence of infection, despite multiple exposures to HIV. In contrast to the objective of the studies of survivors and non-progressors which is to investigate mechanisms of survival after infection, this latter population can be used in the search for a correlate of protective immunity against HIV infection. At least 10 publications have reported detecting T cell responses in seronegative individuals including gay men (15, 16), discordant sexual couples (13, 18, 23), newborns of HIV-infected mothers (17, 19–21) and accidentally exposed HCW (22).

The present study extends the preliminary HCW report (22), which tested T helper cell responses in eight exposed HCW and nine controls, to now include 28 HIV-exposed seronegative HCW and 38 controls, and importantly also tests for *env*-specific CTL, as well for T helper responses. CTL activity is important to test because it suggests live virus infection of cells to get class I MHC presentation, whereas T helper cell responses could be due to exposure to dead virus. Thus, we demonstrate HIV *env*-specific CTL responses in 35% of HCW exposed to HIV contaminated body fluids and without evidence of infection by ELISA and PCR. The lack of recognition of peptide-pulsed HLA-mismatched EBV-transformed targets along with the demonstration of the blocking effect of an anti-MHC class I monoclonal antibody (W6/32) indicate that these responses were MHC class I restricted. MHC class I restricted CTL have been demonstrated to have potent anti-viral activity both in vitro and in vivo (1, 3, 37, 38). The alternative possibility that HIV peptides have stimulated cross-reactive CTL that were primed in vivo to an unrelated antigen seems unlikely because HIV-specific CTL responses were consistently not detected in the control groups stimulated similarly. The detection of MHC class I restricted CTL indicates introduction of the HIV antigens into the endogenous antigen presentation pathway. Induction of virus-specific CTL usually requires in vivo priming with infectious viruses. Inactivated virus, viral proteins and peptides are in most cases ineffective for in vivo induction of class I restricted CTL, although such antigen preparations can induce antibody responses to viral proteins (39, 40). Class I restricted T cells generally recognize antigens that are synthesized within an antigen-presenting cell (APC) or that otherwise enter the cytosol of the APC (41–44). It is conceivable therefore that a low level of infection occurred but was contained by cell mediated immune mechanisms, and was undetectable by PCR performed in the peripheral blood. The possibility of occurrence of HIV sequences in lymphoid tissues could not be addressed in this study. Although the mechanisms of in vivo induction of CTLs are incompletely understood, alternative explanations for the observed cell mediated immune responses to HIV include primary in vivo stimulation of CD8+ cells with defective viral particles that might gain access to the cytosol, or might bind to MHC class I molecules of cells with alternate pathways of antigen presentation by MHC class I involving endolysosomal processing of antigens (45, 46). Recent reports have demonstrated that peripheral blood dendritic cells are able to prime naive CD8+ cells to soluble antigens, including HIV peptides, leading to the generation of potent antigen-specific CTL (47, 48). In addition, a recent report indicates that intravenous infusion of HIV peptide-pulsed murine dendritic cells can induce HIV-specific CD8+ cells in vivo (49). However, the

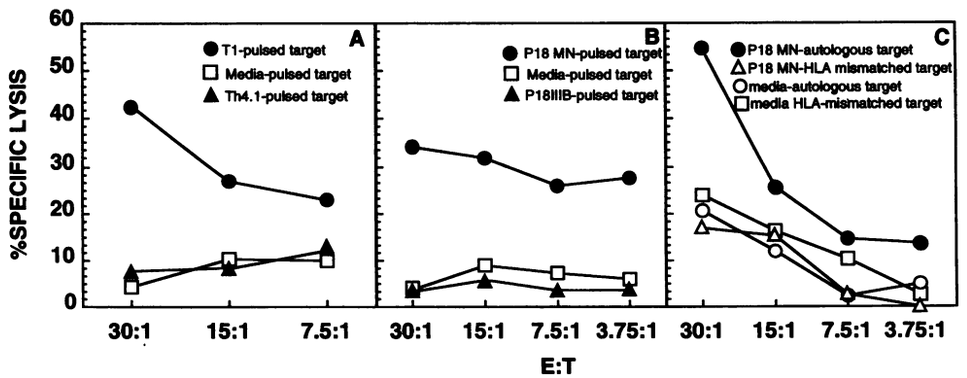

Figure 5. T1 and P18 MN-specific CTL activity of CD8+ cell lines generated from CD8+ cells isolated from the PBMC of Donor 6, 425 d after HIV exposure. The CTL were generated during four weekly rounds of stimulation with T1 or P18 MN. Lysis of autologous EBV targets pulsed with: (*A*) T1 (●), Th4.1 (▲) or media (□); or (*B*) P18 MN (●), P18 IIIB (▲) or media (□). (*C*) compares lysis of autologous EBV targets pulsed with P18 MN (●) or media (○) with that of HLA-mismatched EBV-transformed targets pulsed with P18 MN (△) or media (□).

possibility that this mechanism explains our findings seems unlikely based on the fact that dendritic cells represent 1 to 2% of circulating peripheral blood mononuclear cells and the volume of blood transferred was very small.

Two aspects of our data also suggest that we restimulated a recall response, rather than priming a CTL response from a previously naive population in vitro. First, specific CTL activity was observed only in *env*-stimulated cultures from HIV exposed individuals (7/20) and not in those exposed to HIV negative samples (0/20) or healthy blood donors (0/7). Second, a single round of peptide stimulation was sufficient to detect cytolytic activity from bulk cultures, suggesting the presence of HIV-specific CTL memory precursors in circulation in these individuals. In contrast, the in vitro primary response described required multiple stimulations (47, 48). Furthermore, we were unable to demonstrate CTL activity from fresh PBMC. This contrasts with the observation that HIV-specific CTL against several HIV antigens can be detected in fresh PBMC from many HIV-infected individuals (7, 8, 50, 51).

Several lines of evidence suggest that CTL are an important component of the immune response to HIV infection. HIV-specific CTL precursors are present at high frequency very early during infection, often detected before seroconversion (6). During the subsequent prolonged asymptomatic phase of infection, HIV specific $CD8^+$ MHC class I restricted CTL activity can be detected directly from peripheral blood in the absence of in vitro stimulation (7, 8, 50, 51). Progression to AIDS is accompanied by an increase in virus replication and a loss of $CD8^+$ HIV-specific CTL activity (6, 52). The association of the CTL response with the initial acute loss of viremia (9) and the subsequent loss of that control with progression to AIDS suggests that the CTL response contributes to the control of HIV replication during the asymptomatic phase of infection. Furthermore, studies on long-term non-progressors demonstrated that a vigorous and broadly reactive CTL response can be detected in ser-positive persons with normal CD4 counts who have been infected for up to 15 yr (37, 53). The presence of MHC class I restricted CTL responses has also been detected in multiply sexually or perinatally exposed, HIV seronegative individuals who remain virus-free by PCR (17, 19, 20, 23). It can be argued that these responses may have conferred resistance to HIV infection in the absence of antibody in the HIV exposed individuals without evidence for infection based on antibody production and PCR.

The variability of the kinetics of CTL responses might reflect differences in the CTL precursors in circulation between different individuals at different time points. Factors present at the moment of exposure, such as host's genetic susceptibility, inoculum type (defective or avirulent virus), inoculum size and pathogenicity of viral strain, as well as the host's ability to elicit a protective immune response could determine the outcome of the encounter with HIV. This possibility is supported by recent animal experiments, in which vaginal innoculation with less virulent simian immunodeficiency virus (SIV) appeared to result in transient detection of virus without persistent infection and development of low but detectable immune responses (54). Further support for this possibility comes from a study demonstrating resistance to SIV challenge in macaques previously exposed intravenously to sub infectious doses of SIV (55). The host's prior immunological background might also influence the immune system's ability to handle unrelated heterologous viral infections, as recently reported in murine viral infections (56, 57).

CTL responses were observed in the absence of simultaneous T helper reactivity to the *env* peptides, as measured by IL-2 production in response to the peptides. The apparent dichotomy between CTL and T helper reactivity is also in agreement with studies in which CTL responses could be induced in the absence of $CD4^+$ T cell help (58), and with the observation of intact $CD8^+$ CTL function in CD4-deficient knockout mice (59). Furthermore, recent studies on IL-2 negative mice suggested that this factor is not essential for either NK cell and or CTL activation during viral infection of mice (60, 61). It is very possible that T helper responses were present, with specificity for epitopes different from those that we tested.

Our finding of coincident *env*-specific T helper and CTL responses to the same peptides raises the possibility that we selected responses from individuals whose T cells responded to particular peptides for both helper and effector function, since the cultures were not supplemented with exogenous IL-2. Alternatively, some IL-2 could have been produced by $CD8^+$ T cells, as was reported for murine T cell responses from mice infected with vaccinia virus (32).

T helper responses were detected in a much higher proportion of HIV exposed HCW than CTL responses (75% versus 35%), which is consistent with promiscuous T cell recognition of the peptides in the contest of several MHC class II alleles (62). T helper but not CTL responses were observed in 24% of the control group exposed to seronegative blood. This high "background" could be accounted for by the putative cross-reactivity between HIV *env* and other antigens to which these individuals were previously exposed, particularly the alloantigens to which the control groups of HCW were exposed. This possibility is consistent with the reported homologies between HIV proteins and self antigens (63–68). However, since the fraction positive is significantly higher than in other control groups, it is possible that some of the fluids were infected even though the donor was seronegative (i.e., had not yet seroconverted).

The ability to generate HIV *env*-specific $CD8^+$ cell lines more than one year after HIV exposure favors the possibility that low dose exposure to HIV might generate HIV-specific long-term memory CTL precursors that could protect against subsequent exposure to HIV, as has been reported in animal models of other viral infections (69–71).

The demonstration of CTL responses along with the observation of T helper responses to HIV peptides in HIV exposed but uninfected health care workers indicate that in vivo priming of T helper and cytotoxic T lymphocytes can occur after single documented accidental occupational exposure to HIV, and raises the possibility that cell mediated immune mechanisms will be involved in the containment of HIV infection.

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