Human Immunodeficiency Virus (HIV) *nef*-specific Cytotoxic T Lymphocytes in Noninfected Heterosexual Contact of HIV-infected Patients

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Abstract

We report on the detection of HIV-specific cytotoxic T lymphocytes (CTL) among 23 regular partners of HIV-infected individuals. 15 of the 46 individuals enrolled in the study were positive for HLA-A2.1 typing. Among the 23 contacts studied, 7 were seropositive and 16 were seronegative on repeated tests. None of the 16 seronegative contacts were positive for p24 antigenemia nor were they positive by the lymphocytes coculture assay, although, in two instances HIV-1 DNA could be detected by PCR (in one case using a *gag* SK 38/39 primer, and in the other using a primer for the *pol* P3/P4 primer). These two individuals remained seronegative for 18 and 36 mo, respectively. HIV-specific cytotoxicity was performed in the 15 HLA-A2.1 subjects (7 indexes, 2 seropositive contacts, and 6 seronegative contacts) and in 4 HLA-matched HIV negative donors. CTL specific for *env, gag,* or *nef* proteins could not be detected in unstimulated bulk cultures of peripheral blood lymphocytes in any of the six seronegative contacts. However, using a limiting dilution assay we found an usually high frequency of HIV *nef*-specific CTL in six seronegative contacts studied. The frequency of CTL precursors (CTLp) for HIV *env* and *gag* was very similar to that observed in seronegative HLA-matched healthy donors. Because no presence of HIV could be demonstrated in these individuals, these findings argue against the possibility of a silent HIV infection and suggest that a CTL response against *nef* may be involved in a rapid and effective clearance of the virus after sexual exposure. (*J. Clin. Invest.* 1994. 93:1293–1297.) Key words: human immunodeficiency virus • cytotoxic T lymphocytes • HIV-noninfected individuals • vaccination • prophylaxis

Introduction

The possibility that HIV infection could occur in the absence of demonstrable serum antibodies to HIV has generated concern both in the scientific community and among the general public. By using an ELISA for the detection of serum p24 antigen, lymphocyte coculture for HIV isolation, and PCR for the identification of HIV DNA in PBL, it is generally possible to detect viremia in patients during the 6 mo preceding seroconversion (1–5). In some instances HIV has been detected by PCR or by lymphocyte coculture, respectively, at 12–42 and 11–18 mo before seroconversion (3, 5). However, Imagawa et al. (6) were unable to reisolate HIV in 26 of the 27 culture-positive, seronegative men who participated in their initial study. Based on these findings the authors advanced the hypothesis of incomplete rather than latent, persistent infection (6). In other studies conducted in high risk seronegative individuals, the possibility of silent viral infection in the absence of seroconversion remains very controversial (7–18). It has been appreciated that a specific cytotoxic T lymphocyte (CTL)[1] response to HIV is of much greater magnitude than that observed in almost any other viral infection. This is thought to be one of the main factors contributing to the long symptom-free periods observed in AIDS patients (19). Mucosal immunity is probably an effective barrier against viral infection. However, during an invasive exposure to HIV a rapid and effective CTL response could participate in clearing the organism from the first HIV-infected cells. Unusually high frequencies of HIV-specific CTL precursor cells have been demonstrated in some healthy seronegative donors (20). It is conceivable that a rapid recruitment and expansion of these cells after HIV exposure could play an important role in protection against HIV infection. In recent studies, cell-mediated immune responses to HIV have been detected in seronegative homosexual men exposed to HIV (21). Moreover, strong HIV-specific CTL activity was observed in uninfected infants born from HIV-infected mothers (22, 23).

In this study we sought the presence of CTL specific for three HIV proteins among seronegative partners of HIV-infected individuals. We found high frequency of CTL precursor (CTLp) against the nef antigen in six seronegative contacts. These data are interpreted as evidence that CTL could have played a protective role in killing HIV-infected cells immediately after infection via sexual exposure and that they preexisted in the immune system as memory CTL.

Methods

Subjects. 23 HIV-infected patients and their 23 regular heterosexual partners were enrolled in this study. One member of each couple, whether male or female, was known to be HIV seropositive (index).

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1. *Abbreviations used in this paper:* CTLp, cytotoxic T lymphocyte precursor; LDA, limiting dilution analysis.

The heterosexual partner (contact) excluded any other possible exposure to HIV. The couples enrolled in this study maintained a stable, unprotected sexual relationship (condoms were never used) for at least 2 yr at the onset of the study.

15 index cases were male and 8 were female. Nine of these patients presented with symptomatic HIV infection and were classified in group IV according to the Centers for Disease Control (CDC) classification. Two had AIDS. The mean CD4 cell count was 319×10^6/liter (range, 5–1,080), while 16 of the index cases had a CD4 cell count < 400 $\times 10^6$/liter (Table I). All of the 23 contact cases were asymptomatic, except for the presence of persistent generalized lymphadenopathy in three of the seven seropositive contacts. 2 yr after the end of the study the HIV^- contacts remain negative and in good condition while most of the HIV^+ individuals have died.

Virologic studies. Detection of serum HIV antibody was performed using both ELISA and Western blot analysis. Serum p24 antigen detection was performed by immunoassay (Abbott Laboratories, North Chicago, IL).

A standard cocultivation procedure was used for HIV isolation. PBMC were isolated from heparinized blood of each individual by Ficoll-Hypaque density gradient centrifugation. 3×10^6 cells were used for cocultivation with 9×10^6 PHA-stimulated normal PBMC from healthy donors. The culture supernatants were tested directly for HIV-1 p24 antigen twice a week for a period of 6 wk (24).

The PCR assay detected any HIV-1 DNA present in uncultured PBMC. Two primer pairs were used: SK38/39 for a *gag* sequence (25) and P3/P4 for a *pol* sequence (26), along with their corresponding oligonucleotide probes. 1 μg of DNA was subjected to 40 cycles of amplification, and the amplified fragments were analyzed by Southern blot. Each sample was assayed twice with each primer pair and was considered positive only when repeatedly reactive. Amplification with the β-globin primers PCO_3/PCO_4 was used as a standard control (27).

HIV-specific cytotoxicity. Murine cell clones stably expressing HIV-1 (BRU) gene products were produced by transfection of cloned *env, gag,* and *nef* genes into P815 mastocytoma cells. Double transfectants expressing HIV-1 gene products and human HLA-A2.1 were produced as described previously (28–30). PBL were isolated by Ficoll-Hypaque gradient centrifugation and stored in liquid nitrogen after freezing in FCS containing 10% DMSO. PHA blasts were generated by incubation of 3×10^6 PBL in 15 ml RPMI 1640 supplemented with 20% FCS, 1 mM sodium pyruvate, and PHA HA15 (1:100). After 2 d, human IL-2 (10 U) was added to the medium and the cells were incu-

Table I. Main HIV Disease Markers in 23 Index Cases and in 7 Seropositive Contacts

Cases	Sex	Transmission	CDC classification*	CD4 cell count	p24 antigen§
				×10⁶/liter	*pg/ml*
1	F	Heterosexual	II	294	0
2	M	IV drug user	III	166	0
3	M	Bisexual	II	238	0
4	M	IV drug user	IV	391	0
5	M	IV drug user	II	204	0
6	M	Transfusion	II	434	0
7	F	IV drug user	IV	115	160
8	M	Transfusion	III	400	0
9	M	Heterosexual	IV	5	0
10	F	IV drug user	IV	72	80
11	M	Bisexual	II	851	0
12	F	IV drug user	II	400	0
13	M	IV drug user	II	305	0
14	M	Heterosexual	II	340	0
15	F	Transfusion	IV	16	0
16	F	Heterosexual	III	1080	0
17	M	Bisexual	III	660	0
18	M	IV drug user	IV	80	0
19	F	Heterosexual	III	420	390
20	M	IV drug user	IV	84	250
21	F	Heterosexual	IV	180	0
22	M	IV drug user	IV	252	5200
23	M	Bisexual	III	360	380
102	F	Heterosexual	II	340	0
103	F	Heterosexual	II	663	0
109	F	Heterosexual	II	280	0
110	M	Heterosexual	IV	108	0
120	F	Heterosexual	II	456	0
122	F	Heterosexual	II	416	0
123	F	Heterosexual	III	391	0

* Centers for Disease Control classification: II, asymptomatic; III, persistent generalized lymphadenopathy; IV, symptomatic disease. ‡ Flow cytometry. § ELISA (Abbott, North Chicago, IL).

Table II. Virological Study on 23 Heterosexual Couples

	Positive PCR*			
Lymphocyte coculture‡	P3/PR (pol)	SK38/39 (gag)	P3/PR and SK38/39	PCO3/PCO4 (globin)
All seropositive patients (n = 30)	25/29	27/29	23/29	29/29
Sero + and coculture + (n = 24)	21/24	23/24	20/24	24/24
Sero + and coculture − (n = 6)	4/5	4/5	3/5	5/5
All seronegative contacts (n = 16)	1/16	1/16	0/16	16/16
Sero − and coculture − (n = 16)	1/16	1/16	0/16	16/16

* HIV-1 DNA detection using PCR was performed with three primer pairs: SK38/39 for a gag sequence, P3/PR for a pol sequence, and PCO3/PCO4 for a globin-positive control. ‡ Standard cocultivation procedure: 3×10^6 PBMC of the subject are cocultivated with 9×10^6 PHA-stimulated PBMC from healthy donors.

bated in upright tissue culture flasks at 37°C for 10–12 d. The supernatants changed every 4 d.

Lymphocyte suspensions were tested against P815 transfectants for cytotoxic activity in a standard 18-h ^{51}Cr release assay. Percent specific lysis (PSL) of 10^4 ^{51}Cr-labeled target cells in 200 ml was determined for various effector/target ratios.

HIV-specific CTLp cells were quantified using limiting dilution analysis (LDA) (31). Microcultures were initiated under LDA conditions with 10^2–10^4 PBL in 24 replicate wells. Each microculture received 2.5×10^4 x-irradiated autologous PHA blasts as feeder cells in 200 ml RPMI 1640 supplemented with 20% FCS, five U/ml human IL-2, and PHA (1:1,000 dilution; Difco Laboratories Inc., Detroit, MI) (25). On days 8 and 13, each well was split, and on day 13, they were assayed for cytotoxicity on ^{51}Cr-labeled target cells. Supernatants were collected and counted for radioactivity after a 12-h incubation at 37°C. Individual microcultures were considered positive when ^{51}Cr release values exceeded the mean of control wells by 3 SD. CTLp cell frequencies were estimated by Poisson distribution analysis (31). Minimal estimates were verified by using the statistical method of χ^2 minimization designed by Taswell (32). Frequencies were normalized to the number of CTLp cells per 10^4 PBL plated.

Results

Virology studies. In 23 index cases serum HIV antibodies were detected by both ELISA and Western blot analysis. 7 contact cases were seropositive and 16 seronegative by both techniques. Among the 30 seropositive individuals, there were 23 indexes and 7 contacts.

Serum p24 antigenemia was detectable in six instances (20%). HIV isolation using a standard cocultivation procedure was positive in 24 of 30 cases (80%). HIV-1 DNA was detected by the PCR assay in 29 of 30 cases. Both *pol* and *gag* sequences were found in 23 patients, *pol* alone in 2 and *gag* alone in 4 cases, respectively. In the 16 seronegative contacts, serum p24 antigenemia and HIV isolation were negative. In one contact case there was amplification of the P3/P4 (*pol*) primer and in another case there was amplification of the SK38/39 (*gag*) primer but never of both primers together (Table II). These two subjects were tested for the presence of antibodies to HIV 18 and 26 mo later, respectively, and remained seronegative.

HIV-specific cytotoxicity. The analysis of HIV-specific cytotoxicity from PBL was performed in 19 HLA-A2 subjects, using the P815-A2, P815-A2-env, P815-A2-gag, and P815-A2-nef transfectants as target cells.

Among the seropositive subjects, HIV-specific cytotoxicity against *env* or *gag* was detected in three indexes using unstimulated CTL from PBMC (data not shown). By limiting dilution the relative frequencies of HIV-specific CTLp against *env, gag,* or *nef* proteins were either undetectable or lower than in HLA-matched HIV-negative donors (Table III). The HIV-specific CTLp frequencies were $> 5 \times 10^4$ in five of nine cases. Interestingly, all five patients had a CD4 cell count $> 400 \times 10^6$/liter, while the eight patients with undetectable HIV-specific CTLp had a CD4 cell count $< 400 \times 10^6$/liter. The two patients with the highest CTLp frequencies for *env, gag,* and *nef* antigens had the highest CD4 cell counts. These two patients are still alive after 42 and 37 mo, with a CD4 cell count of 354 and 294 $\times 10^6$/liter.

Among the seronegative individuals, HIV-specific cytotoxicity could not be detected in bulk cultures against *env, gag,* or *nef* proteins in any of the five HLA-A2 seronegative contacts nor in the four HLA-A2 healthy donors. In contrast, by LDA we found high frequencies of HIV *env*-specific CTLp in all 10 individuals (Table III). The relative frequencies of CTLp ranged from 5 to 25×10^{-4} in the six contacts, and were somewhat comparable to those in the four healthy donors (5.5–20 $\times 10^{-4}$) studied.

HIV *gag*-specific CTLp were detected with relative frequencies $> 5 \times 10^{-4}$ in two of six contacts as well as two of four donors. HIV *nef*-specific CTLp could be detected in all six contacts, with relative frequencies varying from 6 to 25×10^{-4}, values higher than in the four healthy donors (0–3×10^{-4}) ($P < 0.02$; Mann-Whitney U test).

Discussion

In this paper we describe six seronegative individuals in stable unprotected sexual relationships with an HIV-infected partner that remained protected from viral infection and demonstrated to have high frequency of CTLp for HIV *nef* protein. Failure to develop HIV infection 2 yr or longer after beginning a relationship with an HIV-infected individual while having a high number of CTLp for HIV *nef* protein is unprecedented and deserves discussion concerning: (*a*) the origin of the CTL response against *nef* in these individuals, and (*b*) a cause–effect relationship between high frequency of CTLp and protection from HIV infection.

Table III. Relative Frequencies of HIV-specific CTL Precursor Cells Using LDA

	Target cells*			
	P815-A2	P815-A2-env	P815-A2-gag	P815-A2-nef
HIV^+ indexes				
8	<1	1	7.5	1
12	<1	1	5	3
13	<1	3	3	4
14	2	2	2	2
16	1	13	17	13
17	2	10	40	8
18	<1	<1	<1	<1
HIV^+ contacts				
103	<1	6	17	14
110	<1	2.5	<1	2
HIV^- contacts				
101	2	7.5	2	16.5
104	1	5	20	12
105	3	25	3.5	25
108	2	12.5	3	7
116	1	10	25	25
117	3	12.5	3	6
HIV^- donors				
A	2	20	5	2
B	<1	5.5	3	<1
C	4	12.5	6.5	3
D	2	8.5	2.5	2

* P815 mouse mastocytoma cells were doubly transfected with HIV-1. Data are CTLp cells/10^4 PBL plated.

It could be argued that these individuals had been exposed to HIV in a way sufficient to elicit an CTL response. Interestingly, in these individuals only the response to *nef* was markedly higher than in HIV-negative HLA-matched controls. This suggests that contact with the virus expanded selectively the repertoire for the *nef* protein. The reasons why a response to *nef* was favored can only be speculated.

It has been proposed that *env* and *gag* proteins cross-react with self-antigens (33, 34). Thus, molecular mimicry at the T cell level could account for the high frequency of CTLp for *env* or *gag* proteins. Similarly, one could consider that HIV *nef* protein restimulated CTLp originally directed against a cross-reactive self molecule (35) or a peptide antigen borne on ubiquitous bacteria or others viruses. However, the fact that HIV-negative HLA-matched control had low frequency of CTLp against the *nef* protein argues against this hypothesis.

A second possibility would be that HIV induced a very early cytotoxic response effective in ridding the host of virus-infected cells at this early stage.

Within this view one cannot discount a synergistic effect by NK cells that would contribute to limiting the number of HIV-infected cells. Interestingly, we found a high level of NK activity in most seronegative contacts (data not shown).

Whatever the mechanisms might be, a CTL response against *nef* is a fundamental advantage to the host because of the low variability of the *nef* gene in different isolates (36). Further, little evolution of *nef* was noted from the asymptomatic to the disease stage; thus, it has been shown that in a patient over a 4-yr period in vivo, there was no obvious selection for, or outgrowth of, any particular *nef* or U3/R sequence (37), so that *nef*-specific CTL could still be effective against the emergence of escape HIV mutants. In contrast, it has been reported that *gag*-specific CTL became ineffective against escape mutants for the *gag* genes (38, 39).

An intriging aspect of our study is the detection of CTLp for the *nef* antigen in the absence of seroconversion. Although HIV infection is expected to induce seroconversion, it is possible that the individuals studied herein were exposed to quantities of HIV sufficient to prime T cell immunity-inducing antibody response.

It has been reported (21) that low-dose (40–80 μg) immunization of seronegative volunteers with recombinant glycoprotein (rgp) 160 induces a T cell (Th1) response with little if any antibody production. In contrast, immunization with a high dose of rgp 160 induces both cell-mediated immunity and an antibody production (Th2 response).

From the foregoing, it appears as if the six seronegative contacts with high CTLp for the *nef* antigen were "locked in" a Th1 response by contact with the virus that allowed them to mount an adequate immune response for protection. As a corollary, our results may also suggest that a high frequency of CTLp for the *nef* antigen in the absence of seroconversion could be a sign of favorable prognosis, although one can not exclude that CTL against other regulatory gene proteins may have a similar role as that describe herein for *nef*.

In conclusion, we demonstrated a high frequency of CTLp against *nef* in seronegative individuals exposed to HIV infection by sexual contact. These finding have general implications for understanding the mechanisms of immune response to HIV infection, for new approaches to diagnosis and prognosis of infected individuals, and for designing strategies of vaccination and prophilaxis against HIV.

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