Human Immunodeficiency Virus (HIV) gag Antigen-Specific T-Helper and Granule-Dependent CD8 T-Cell Activities in Exposed but Uninfected Heterosexual Partners of HIV Type 1-Infected Individuals in North India

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Repeated exposure to human immunodeficiency virus (HIV) does not always result in HIV infection, and several cohorts of HIV-exposed but uninfected (EU) individuals have been described. We studied T-helper and granule-dependent cytotoxic T-lymphocyte (CTL) activities in a group of 30 EU partners of HIV type 1 (HIV-1)-infected individuals. HIV-1-specific helper-T-cell activity was studied by measuring the levels of interleukin 2 (IL-2) produced by peripheral blood mononuclear cells (PBMCs) and the granule-dependent CTL activity by measuring the intracellular levels of perforin and granzyme B expression in CD8+ T cells after stimulation with gag p24 antigen. Elevated IL-2 production by PBMCs after p24 stimulation occurred in EU individuals. The levels of perforin and granzyme B expression in CD8+ T cells were also higher among EU individuals than among healthy controls. HIV-specific helper-T-cell and granule-dependent CTL activities inversely correlated with the time since the last unprotected sexual exposure in these individuals. In our cohort, activation of T-helper and granule-dependent CTL activities against HIV might be due to unprotected sexual contact. These results indicate that HIV-1-specific T-cell responses could play a role in protection against acquiring infection in this cohort of EU individuals.

There is considerable variation in susceptibility to human immunodeficiency virus (HIV) infection, with some individuals remaining seronegative despite repeated exposure to the virus (28, 30). These exposed uninfected (EU) individuals are a useful subset of individuals with whom mechanisms that protect against HIV infection can be studied. Several host factors, either alone or in combination, might be important in conferring protection. These include host genetic factors (30), noncytotoxic CD8+ T-cell responses (34), HIV-specific cytotoxic T-cell activity (3, 30, 33), neutralizing antibodies to HIV coreceptors (23), mucosal antibodies against HIV (9), and increased β-chemokine production (11, 38) with associated CD4+ T-cell resistance (11, 25).

Several reports have demonstrated the relevance of HIV-specific CD4+ T-helper cells in the induction and maintenance of the host immune response against HIV and, in particular, of HIV-specific cytotoxic T lymphocytes (CTLs) in EU individuals (13, 19, 29, 30, 38). PBMCs from EU individuals have been shown to proliferate and secrete interleukin 2 (IL-2) on exposure to HIV antigens (5). HIV type 1 (HIV-1)-specific CTLs have been described in several EU individuals, and the generation of HIV-1-specific CTLs is a key goal in the development of a potential vaccine (13, 19, 29, 33). This effect is mediated by two different mechanisms: direct lysis of virus-infected cells and secretion of antiviral soluble factors (2, 15). CTL-mediated lysis of infected targets is achieved by granule-dependent and granule-independent pathways. In the former, perforin, a lytic enzyme that mediates the death of target cells and that facilitates the penetration in these cells of apoptosis-inducing granzymes, is released by CTLs through exocytosis.

In this study, HIV-specific helper-T-cell activity and granule-dependent CTL activity and their association with the last unprotected sexual activity in a group of exposed but persistently seronegative heterosexual partners of HIV-infected individuals were analyzed. We studied the HIV gag p24-specific IL-2 responses in our cohort of EU individuals and also the granule-dependent CTL activities by measuring the gag p24-stimulated perforin- and granzyme B-expressing CD8+ T cells.

**MATERIALS AND METHODS**

**Study group and controls.** Thirty heterosexual couples with discordant HIV serological status were selected from the Immunodeficiency Clinic at the Post Graduate Institute of Medical Education and Research, Chandigarh, India. The study group included the seronegative partners of HIV-positive patients who had been exposed to HIV through unprotected heterosexual contact over various periods of time. All individuals had had regular unprotected sexual contact with their partner within 6 months prior to sampling. All these couples with discordant HIV serological status received active counseling on HIV prevention/safe sex methods at the time that their samples were drawn. HIV antibody-negative serostatus was confirmed by enzyme-linked immunosorbent assays (ELISAs; Span Diagnostics Ltd., Surat, India) for HIV-1 and HIV-2 antibodies. Additionally, the qualitative detection of HIV provirus DNA from genomic DNA was carried out by PCR with gag gene-specific primers. Thirteen HIV-infected treat-
TABLE 1. Summary of demographic characteristic of EU individuals

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value or description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of subjects</td>
<td>30</td>
</tr>
<tr>
<td>No. of males/no. of females</td>
<td>7/23</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>Mean ± SD 34.2 ± 6.6</td>
</tr>
<tr>
<td>Range</td>
<td>24–49</td>
</tr>
<tr>
<td>Mode of exposure</td>
<td>Heterosexual contact</td>
</tr>
<tr>
<td>Duration (mo) of unprotected sexual activity</td>
<td>Mean ± SD 71.6 ± 39.5</td>
</tr>
<tr>
<td>Range</td>
<td>6–160</td>
</tr>
<tr>
<td>Known duration (mo) of HIV positivity of partner</td>
<td>Mean ± SD 17 ± 9.9</td>
</tr>
<tr>
<td>Range</td>
<td>6–48</td>
</tr>
<tr>
<td>Time (days) since last unprotected sexual contact</td>
<td>Mean ± SD 42.1 ± 40.2</td>
</tr>
<tr>
<td>Range</td>
<td>4–180</td>
</tr>
<tr>
<td>CD4 count (no. of cells/µl) of partner</td>
<td>Mean ± SD 91.9 ± 124.5</td>
</tr>
<tr>
<td>Range</td>
<td>11–447</td>
</tr>
<tr>
<td>Median (range) frequency of unprotected sexual contact in preceding 6 mo</td>
<td>12 (1–24)</td>
</tr>
</tbody>
</table>

ment-naïve individuals and 25 sexually active healthy controls (HCs) whose partners were not HIV infected were also included in the study.

**PBMC culture and IL-2 estimation.** Whole blood (heparinized) was collected, and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation by using Histopaque-1.077 (Sigma) (21). The cells in the interface containing PBMCs were collected and washed twice in phosphate-buffered saline (PBS; pH 7.2) and resuspended in 1 ml RPMI 1640 (Gibco, Germany) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated fetal calf serum. The PBMC suspension (1 x 10⁶ cell/ml) was used for culture in a 96-well sterile tissue culture plate (Greiner, Germany). Three sets of cultures were performed for each sample: (i) PBMCs alone in RPMI 1640 without any stimulant, (ii) PBMCs in the presence of 4 µg/ml phytohemagglutinin (PHA; Sigma), and (iii) PBMCs in the presence of 4 µg/ml HIV gag p24 antigen (Sigma). The cultures were incubated at 37°C for 48 h in a CO₂ incubator with 5% CO₂. The culture supernatants were harvested after 48 h. Isotype controls and unstained permeabilized cells were also used in the experiments as controls.

**RESULTS**

**Characteristics of EU individuals.** The demographic characteristics of the EU individuals are shown in Table 1. The 30 EU individuals had a mean age of 34.2 ± 6.6 years (range, 24 to 49 years). The mode of exposure to HIV infection was through heterosexual contact in all individuals. The mean time since the spouse tested positive for HIV was 17 ± 9.9 months (range, 6 to 48 months), and the mean duration of their unprotected sexual activity was 71.6 ± 39.5 months (range, 6 to 160 months). The median frequency of their unprotected sexual contact in the preceding 6 months was 12, with a range of 1 to 24.

**Control groups.** Thirteen HIV-infected individuals (seven men) with early HIV disease who were not receiving antiretroviral therapy were also studied. Their mean age was 34.4 ± 8.2 years (range, 25 to 57 years). The mean absolute CD4 count of the HIV-infected controls was 545.7 ± 249.7 cells/µl (range, 351 to 1,046 cells/µl), and the mean absolute CD8 count was 1,382.9 ± 555.7 cells/µl (range, 628 to 2,000 cells/µl). All had acquired HIV infection through heterosexual contact. Twenty-five HCs (14 men) had a mean age of 29.9 ± 6.1 years (range, 24 to 53 years). All HCs tested negative for HIV-1 and HIV-2 antibodies.

**IL-2 estimation.** The median levels of IL-2 produced by the three groups are shown in Table 2. The levels of IL-2 production by the PBMCs after stimulation with the gag p24 antigen for 48 h were measured from the culture supernatants by ELISA. The median IL-2 levels were 72.5 pg/ml, 20 pg/ml, and 10 pg/ml for EU individuals, HIV-positive controls, and HCs, respectively. The IL-2 level of the EU individuals after p24 stimulation was significantly higher than those of the HIV-infected controls (P < 0.001) and the HCs (P < 0.0001). The HIV-infected controls produced low levels of IL-2 compared...
with the levels produced by the EU and HC individuals after PHA stimulation ($P < 0.0001$). Among the EU individuals, the time since their last unprotected sexual contact inversely correlated with the level of p24-specific IL-2 production ($r = -0.52; P = 0.002$) (Fig. 1). Those with recent exposure to the virus showed higher responses.

**CTL activities.** Intracellular granzyme B and perforin produced by CD8$^+$ T cells after stimulation with gag p24 antigen was detected by triple-color flow cytometry. For each analysis, 10,000 events were acquired and gated on CD8-PE-Cy5 expression and side-scatter properties (Fig. 2A). Intracellular perforin and granzyme B levels were further analyzed within these CD8$^+$ T-cell populations by using granzyme B-FITC and perforin-PE antibodies in EU individuals (Fig. 2B), HIV-infected individuals (Fig. 2C), and HC individuals (Fig. 2D). The EU individuals showed a higher percentage of CD8$^+$ T cells than the HCs did (Fig. 3A). The mean percentage of CD8$^+$ T cells was 31.5 ± 6.3 among the EU individuals, whereas among the HCs it was 25.7 ± 4.28 ($P = 0.0003$). Among the HIV-infected controls, the mean percentage of CD8$^+$ T cells was significantly higher (57.3 ± 5.21) than that among the EU individuals and the HCs ($P < 0.0001$). The mean percentages of perforin-expressing CD8$^+$ T cells were 30.3 ± 4.8, 41.52 ± 7.7, and 16.7 ± 4.1 in the EU individuals, HIV-positive controls, and HCs, respectively. The percentage of CD8$^+$ T cells expressing perforin was significantly higher in the EU individuals and HIV-positive controls than in the HCs ($P < 0.0001$) (Fig. 3B). The mean percentage of CD8$^+$ T cells expressing perforin was higher in the HIV-infected controls than in the
EU individuals ($P < 0.0001$). The mean percentages of CD8$^+$ T cells expressing granzyme B were also higher in the EU individuals and the HIV-positive controls. The mean percentages of CD8$^+$ T cells expressing granzyme B were 16.5 ± 3.9 in the EU individuals and 20.2 ± 4.2 in the HIV-infected controls, whereas in the HCs the mean percentage was 6.9 ± 3.7 ($P < 0.0001$). HIV-infected controls had significantly higher levels of granzyme B than EU individuals ($P = 0.008$) (Fig. 3C). The mean percentage of CD8$^+$ T cells expressing both granzyme B and perforin in EU individuals was 8.3 ± 2.2, and in HCs it was 3.5 ± 0.8 ($P < 0.0001$). Among the HIV-infected controls, the mean percentage of CD8$^+$ T cells expressing perforin and granzyme B was 16.9 ± 3.0. The percentage of granzyme B- and perforin-expressing CD8$^+$ T cell was significantly higher in the HIV-infected controls than in the EU individuals and the HCs ($P < 0.0001$) (Fig. 3D). In the EU individuals, the time since the last unprotected sexual contact correlated inversely with the percentages of CD8$^+$ T cells expressing perforin (Fig. 4A), granzyme B (Fig. 4B), and both perforin and granzyme B (Fig. 4C). There was a direct correlation between the p24-specific IL-2 response and the percentage of CD8$^+$ T cells expressing perforin (Fig. 5A), the percentage of CD8$^+$ T cells expressing granzyme B (Fig. 5C), and the percentage of CD8$^+$ T cells containing perforin and granzyme B (Fig. 5D).

**DISCUSSION**

Understanding of the immune responses that might protect individuals from HIV infection can contribute to the development of an effective vaccine. One group of individuals who may be able to provide potential insights into protective immunity consists of people with repeated exposures to HIV who remain seronegative. In this first report from India, we describe the T-helper and granule-mediated CD8$^+$ T-cell activities against HIV infection in a group of HIV-exposed but uninfected heterosexual partners of HIV-infected individuals. Our earlier analysis of chemokines and their receptor gene polymorphisms did not show any significant association (35). However, there was elevated spontaneous and HIV gag p24 antigen-induced production of beta chemokines (regulated on activation, normal T-cell-expressed and secreted protein [RANTES], macrophage inflammatory protein 1α [MIP-1α], and MIP-1β) but not alpha chemokine (stromal cell derived factor 1) by PBMCs in these individuals (36).

In this study, the increased levels of IL-2 production by
PBMCs in response to HIV gag p24 antigen in EU individuals suggest that a specific sensitization to HIV occurred in these individuals as a result of unprotected sexual activity. There was a higher IL-2 response to gag and PHA in EU individuals than in the HIV-infected controls. Early defects in T-helper-cell function in HIV infection are known to include a reduced ability to respond to recall antigen by IL-2 production (8, 12). Our results are in agreement with those presented in previous reports, in which higher frequencies of gag-specific IL-2-producing CD4+ T cells were identified in EU individuals (10, 16). Schenal et al. (31) reported that gag-specific IL-2-producing cells were predominant in EU individuals and that gag-specific gamma interferon-secreting lymphocytes were mostly observed in HIV-infected patients. Taken together, our results indicate that HIV exposure in EU individuals might favor the generation of IL-2 and that the helper-T-cell activity observed in EU individuals not only is enhanced but also may even be greater than that in HIV-infected patients. This in turn helps maintain specific CD8+ T cells, which may be an important factor in nontransmission in EU individuals.

The results for granule-dependent CTL activity showed that in response to gag p24 antigen, higher levels of intracellular expression of perforin, granzyme B, and both perforin and granzyme B occurred in the CD8+ T cells from the EU individuals than in those from the HCs. Earlier studies have reported elevated levels of perforin expression in HIV-infected individuals, suggesting that HIV-infected cell lysis by antigen-specific CD8+ T cells occurs mostly by this pathway (1, 17, 26, 32). Perforin- and granzyme B-mediated CTL activity could be an important mechanism operating against the virus in EU individuals. IL-2 induces the synthesis of perforin and granzymes and stimulates their release into the extracellular milieu (17).

The direct correlation between the IL-2 response and the levels of perforin and granzyme B expression on CD8+ T cells that was observed in EU individuals suggests that helper-T-cell responses play a direct role in activating the granule-dependent CD8 T-cell activity in these individuals. Moreover, in EU individuals, terminally differentiated CD8+ T cells are perforin enriched (31). We suggest that the enhanced granule-dependent CTL activity observed in our EU individuals could be a marker of immunologic memory to repeated viral exposure.

Previous studies have reported that HIV-specific T-helper and CTL responses disappear within months after the cessation of virus exposure in uninfected newborns of HIV seropositive women (7) and in health care workers reporting a single occupational exposure to HIV-infected body fluids (6). Jennes et al. (16) reported that gag-specific T-helper responses were associated with the frequency of exposure in a cohort of seronegative female sex workers (FSWs). Consistent with these findings, there was an inverse correlation between gag-specific IL-2 production and the time of the last unprotected sexual activity in our EU individuals. Similar to this observation, an inverse correlation was observed between the time since the
last unprotected sexual contact and the percentage of CD8+ T cells expressing perforin, granzyme B, and both perforin and granzyme B in these individuals. Granule-dependent CTL activity might have been activated during a previous greater-risk sexual contact and maintained through low-grade continuous exposure in these individuals. Two earlier reports by Kaul et al. have demonstrated a positive correlation between the occurrence of HIV-specific CTLs and the duration of prior sex work among EU FSWs in Kenya (18, 20). The follow-up of these group showed that some of these FSWs became infected, despite preexisting CTL responses. This finding was associated with a break in sex work or a reduction in the number of clients, which could have led to a waning of the CTL response (20). Significantly, the members of our cohort had monogamous relationships with their partners, unlike commercial sex workers.

The induction and maintenance of HIV-1-specific T-helper and CTL activities in our EU individuals can be explained by several possible mechanisms. Low viral loads in the partner could result in a lack of transmission, despite long-term exposure. The existence of an HIV-specific immune response could be due to self-limited HIV replication in these individuals. This low dose of HIV-1 exposure without productive infection might give rise to antigen-specific immune responses. This has been suggested by studies demonstrating the induction of CD8+ T-cell responses in the absence of de novo viral protein synthesis (4). Another possibility for these HIV-specific immune responses in EU individuals might be the result of an alternate pathway for exogenous antigen processing known as antigen cross presentation (24, 27), and as a result the T cells may be primed directly with defective nonreplicating particles or viral proteins. The role of antigen cross presentation in protection against HIV disease progression has been reported (22).

We conclude that in our cohort of EU individuals, activation of T-helper and granule-dependent CTL activities against HIV occurred as a result of unprotected exposure. These correlate inversely with the duration of the last unprotected exposure, and there is a direct correlation between T-helper and granule-dependent CTL activities.

ACKNOWLEDGMENT

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