In Vitro Effect of Interleukin-12 on Antigen-Specific Lymphocyte Proliferative Responses from Persons Infected with Human Immunodeficiency Virus Type 1

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The relationship between CD4 lymphocyte count and the in vitro effect of interleukin (IL)-12 on lymphocyte proliferative responses to Candida, tetanus toxoid, and streptokinase antigens was studied in peripheral blood mononuclear cells (PBMC) from 30 human immunodeficiency virus (HIV)-infected persons and 10 seronegative controls. IL-12 significantly increased proliferative responses to microbial recall antigens of PBMC from HIV-infected persons with >200 CD4 lymphocytes/mm³ but had little effect on PBMC from patients with more advanced disease. The greatest increase was seen in patients with 200–500 CD4 cells/mm³. Results of limiting dilution analysis suggested that the increase in antigen-specific lymphocyte proliferation in the presence of IL-12 was due to an increase in the number of responding cells rather than an increase in the extent of proliferation of a fixed number of responder cells.

Infection with human immunodeficiency virus type 1 (HIV-1) is associated with a wide variety of immunologic abnormalities, including defects in NK and cytotoxic T cell activity [1], B cell differentiation [2, 3], monocyte accessory cell function [3], and T helper (Th) cell function [4, 5]. Th cell function, which is mediated by CD4 T lymphocytes, includes the ability to proliferate and elaborate cytokines in response to specific antigens. In vitro studies document the progressive loss of Th cell responses to recall antigens in peripheral blood mononuclear cells (PBMC) from HIV-infected subjects. Defective antigen-specific Th responses can be observed early in HIV infection [5], although responses to T cell mitogens and alloantigens remain intact until the later stages of disease [6–8].

Interleukin (IL)-12 is a cytokine that was originally described as NK cell stimulatory factor [9]. This cytokine may play an important role in resistance to infection by augmenting NK cell and cytotoxic T lymphocyte activity [10, 11], increasing production of interferon (IFN)-γ and other cytokines [3, 12], and stimulating Th1 cell responses [13]. IL-12 also stimulates proliferation of activated T lymphocytes and NK cells [14, 15]. Production of IL-12 in response to antigen stimulation is deficient in macrophages from HIV-infected persons [16].

Previous studies have shown that IL-12 can restore in vitro Th responses to antigens derived from the HIV-1 envelope and to microbial recall antigens when IL-12 is added to PBMC from HIV-infected persons [17–19]. Addition of IL-12 enhances production of IL-2 and IFN-γ by antigen- or phytohemagglutinin (PHA)-stimulated PBMC from HIV-infected subjects [19]. In the present study, we explored the relationship between CD4 lymphocyte count and proliferative responses to microbial recall antigens of PBMC from HIV-1-infected persons and uninfected controls in the presence or absence of recombinant human (rh) IL-12.

Methods

Study population and sample preparation. Blood samples were obtained from HIV-1-infected patients and from healthy HIV-seronegative laboratory workers. Patients with HIV-1 infection were stratified by CD4 cell count into 3 groups: group I, <200; group II, 200–500; and group III, >500/mm³. PBMC were separated from heparinized whole blood by ficoll-hypaque density gradient centrifugation and resuspended in R-10 medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin, 10 mM HEPES, and 10% [vol/vol] heat-inactivated human AB serum [Gemini Bio-Products, Calabasas, CA]).

Lymphocyte proliferation assays. Round-bottom 96-well microtiter plates were prepared by coating wells with 0.1 mL of the following antigens: 20 μg/mL Candida (Greer Laboratories, Lenoir, CA), 0.5 U/mL tetanus toxoid (Connaught Laboratory, Swiftwater, PA), and 20 μg/mL streptokinase (Boehringer, Mannheim, Germany). Antigen preparations were tested for nonspecific mitogenicity using neonatal PBMC obtained from cord blood. PBMC were seeded at 10⁵ cells/well into quadruplicate wells containing antigen, 5 μg/mL PHA (Sigma, St. Louis), or medium alone in the presence or absence of 5 U/mL rhIL-12 (provided by S. Wolf, Genetics Institute, Cambridge, MA) in a final volume of 200 μL.
Cultures were incubated for 6 days at 37°C, then pulsed with [methyl-3H]thymidine ([3H]Tdr, 1 μCi/well; DuPont NEN, Wilmington, DE). After 6 h, cells were harvested with a Filtermate 196 Harvester (Packard Instruments, Meriden, CT), the DNA collected onto Unifilter 96-well plates (Packard Instruments), and the extent of [3H]Tdr incorporation determined by liquid scintillation counting on a Microplate scintillation counter (Packard Instruments). The stimulation index (SI) was calculated by dividing the counts per minute (cpm) incorporated by cells in antigen- or mitogen-treated wells by the mean cpm incorporated in wells that contained medium alone. Since IL-12 had no effect on proliferation of PBMC in the absence of antigen or PHA, all SIs were calculated with reference to the control condition (no antigen, no IL-12).

Limiting dilution cultures. For limiting dilution assays, PBMC were seeded into 24 replicate wells of 96-well microtiter plates at four concentrations ranging from $3.7 \times 10^3$ to $10^5$ cells/well in 200 μL of R-10 medium in the presence or absence of antigen, rHL-12, or both. On day 6 of culture, cells were pulsed with [3H]Tdr and harvested as described. Wells stimulated with antigen were considered negative if the cpm were < 3 SD above the mean cpm for control unstimulated wells. The fraction of negative wells was plotted on a logarithmic scale against the number of cells per well, and the data were fitted to a line by minimum χ² analysis. The responder cell frequency was estimated as the number of cells per well calculated to give 37% negative wells [20].

Statistical analyses. A mixed model approach [21] was used to explore the association of in vitro lymphocyte responses and CD4 cell count with the effect of IL-12 on antigen-specific proliferation in vitro. For this analysis, the response in each replicate antigen-containing well was treated individually. For each subject, the data from these replicate wells were highly correlated, and therefore the error terms were not independent. Standard linear models were therefore inappropriate for analyses of these data. The mixed model generalizes the standard linear model as follows: $y = X\beta + Zv + e$, where X is the independent variable, y is the dependent variable, B is the parameter to be estimated, v is an unknown parameter vector of random effects with known model matrix Z, and e is an unknown random error vector whose elements are not required to be independent. For this analysis, Z was chosen to be an indicator specific to each patient. The mixed models were fit using SAS/STAT software (SAS Institute, Carey, NC). The dependent variable was the natural logarithm (ln) of the proliferation response to the recall antigens of PBMC divided by the mean of the control (no antigen) response. This measure is comparable to ln (SI). The independent variables were a classification of CD4 cell count ($<200$, $200–500$, or $>500$/mm$^3$) or HIV-seronegative control, combined with an indicator of in vitro treatment (with or without IL-12). Because responses from an individual subject were correlated, a subject indicator variable was utilized to model the random effects. Estimates of the SI for each group were obtained by taking the antilogarithm (e$^\beta$) of the predicted values. All P values were two-tailed.

Results

Study population. Samples were obtained from 30 HIV-infected subjects (10/stratum) and 10 seronegative controls. The median CD4 cell count was 61/mm$^3$ (range, 28–199) for patients in group I, 315/mm$^3$ (range, 218–464) for patients in group II, and 688/mm$^3$ (range, 513–1120) for patients in group III. Six patients in group I and 6 in group II had a history of oral candidiasis, but candidiasis was present in only 2 subjects from group I at the time of lymphocyte proliferation assay. Likewise, only 2 subjects (both in group I) had active AIDS-defining opportunistic infections (cryptosporidiosis and histoplasmosis). Use of antiretroviral therapy was reported by 8 subjects in group I and 4 subjects in group II. None of the patients in group III were receiving antiretroviral therapy. Additional subjects with similar characteristics were recruited for the precursor frequency and HIV outgrowth experiments.

Proliferative responses to recall antigens and T cell mitogen. In vitro proliferative responses of individual subjects to each microbial recall antigen and to the T cell mitogen PHA in the presence and absence of IL-12 are shown in figure 1. Although there was heterogeneity among patients in each group, antigen-specific responses appeared to increase with increasing CD4 cell count and in response to IL-12.

The association of lymphocyte responsiveness with CD4 cell count and the effect of IL-12 on antigen-specific lymphocyte proliferation were explored using a mixed model approach as described in Methods. The modeled proliferative responses of patients (groups I–III) and HIV-uninfected controls are given in table 1. In the absence of IL-12, proliferative responses of PBMC from HIV-infected patients to Candida, tetanus toxoid, and streptokinase recall antigens were significantly reduced compared with those in seronegative controls ($P < .003$, $P < .001$, $P = .006$, respectively; table 1).

PBMC from patients with <200 CD4 cells/mm$^3$ (group I) had significantly lower proliferative responses than did patients with 200–500 CD4 cells/mm$^3$ for Candida ($P < .001$) and tetanus toxoid antigens ($P < .001$; table 1). Streptokinase-specific proliferative responses of patients in group I were also lower than those in patients with higher CD4 cell counts, but the difference did not achieve statistical significance ($P = .118$; table 1). Candida- and streptokinase-specific proliferative responses were similar for patients in groups II and III ($P = .261$ and .130, respectively), but patients in group II had significantly lower proliferative responses to tetanus toxoid compared with group III patients ($P < .001$). Proliferation in response to PHA did not differ between groups (table 1).

Effect of IL-12 on antigen-specific lymphocyte proliferation. Addition of IL-12 resulted in significantly increased lymphocyte proliferative responses to tetanus toxoid, Candida, and streptokinase antigens for PBMC from HIV-infected subjects (table 1). There was no significant effect of IL-12 on proliferation of PBMC in the absence of antigen or PHA mitogen (data not shown). The IL-12-dependent increase in antigen-specific lymphocyte proliferation was related to CD4 cell count. IL-12 did not augment in vitro proliferative responses to tetanus toxoid ($P = .562$) or to streptokinase ($P = .164$) in PBMC from patients with <200 CD4 cells/mm$^3$ (group I); the effect of IL-12 on proliferative responses to Candida antigen in this group,
Figure 1. Effect of recombinant human interleukin-12 (rhIL-12) on in vitro lymphocyte proliferation to microbial recall antigens. Lymphocyte proliferation to Candida (A), tetanus toxoid (B), and streptokinase (C) antigens and to phytohemagglutinin (D) was determined by [3H]thymidine incorporation in the presence and absence of rhIL-12 as described in Methods. Stimulation index (SI) was calculated by dividing cpm incorporated by cells in antigen- or mitogen-treated wells by mean cpm incorporated into wells containing medium alone. Results for HIV-infected patients are displayed as natural logarithm (Ln) of individual SI values vs. CD4 cell count; results of HIV-uninfected controls are not ordered by CD4 cell count. Ln median SI in presence (solid line) or absence (dashed line) of rhIL-12 is indicated for patients with CD4 cell counts <200, 200–500, and >500/mm³ and for HIV-uninfected controls.
Table 1. Modeled antigen-specific proliferative responses of peripheral blood mononuclear cells from HIV-infected persons and uninfected controls in response to microbial recall antigens in the presence or absence of interleukin (IL)-12.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>HIV-infected</th>
<th>Group I (P)*</th>
<th>Group II (P)t</th>
<th>Group III (P)§</th>
<th>Uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida</td>
<td>Without IL-12</td>
<td>1.2 (.037)</td>
<td>17.5 (.261)</td>
<td>9.5 (.003)</td>
<td>20.3</td>
</tr>
<tr>
<td></td>
<td>With IL-12</td>
<td>1.5 (.001)</td>
<td>30.0 (.174)</td>
<td>14.2 (.001)</td>
<td>38.9</td>
</tr>
<tr>
<td>Tetanus toxoid</td>
<td>Without IL-12</td>
<td>1.2 (.001)</td>
<td>5.3 (.001)</td>
<td>23.1 (.001)</td>
<td>51.9</td>
</tr>
<tr>
<td></td>
<td>With IL-12</td>
<td>1.1 (.001)</td>
<td>13.1 (.020)</td>
<td>32.1 (.001)</td>
<td>94.6</td>
</tr>
<tr>
<td>Streptokinase</td>
<td>Without IL-12</td>
<td>1.0 (.118)</td>
<td>2.6 (.130)</td>
<td>6.3 (.006)</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>With IL-12</td>
<td>1.3 (.002)</td>
<td>7.9 (.541)</td>
<td>11.4 (.005)</td>
<td>17.5</td>
</tr>
<tr>
<td>PHA</td>
<td>Without IL-12</td>
<td>104.6 (.189)</td>
<td>202.4 (.422)</td>
<td>135.6 (.872)</td>
<td>151.4</td>
</tr>
<tr>
<td></td>
<td>With IL-12</td>
<td>194.4 (.652)</td>
<td>242.3 (.349)</td>
<td>151.4 (.436)</td>
<td>198.3</td>
</tr>
</tbody>
</table>

NOTE. Data are exponentiated values of estimated group stimulation index for each antigen under indicated conditions. Group I = <200, group II = 200–500, and group III = >500 CD4 cells/mm³. All statistical analyses are by mixed model as described in Methods. PHA, phytohemagglutinin.
* Group I vs. II.
† Group II vs. III.
§ HIV-infected (overall) vs. uninfected.

Although modest, was statistically significant (P = .037; table 1). By contrast, IL-12 induced significant increases in lymphocyte proliferative responses to all three microbial recall antigens among patients with higher CD4 cell counts (groups II and III: table 1). In vitro proliferative responses of PBMC from HIV-seronegative controls to Candida, tetanus toxoid, and streptokinase were also significantly enhanced by IL-12 (table 1).

Effect of IL-12 on HIV-1 outgrowth in vitro. In a previous report, IL-12 was found to enhance HIV-1 replication in prestimulated PBMC [22]. Stimulation of HIV-1 replication by IL-12 in the lymphocyte proliferation assays could lead to spurious results due to lytic infection of CD4 cells. To exclude this possibility, culture supernatants from lymphocyte proliferation assays performed on cells from additional subjects with CD4 cell counts of 49–298/mm³ (median, 204) were tested for HIV-1 p24 antigen production (Coulter Diagnostics, Hialeah, FL) at the time cells were harvested for analysis of [³H]TdR incorporation (day 6). No evidence for HIV-1 replication was detected when PBMC from these subjects were incubated with Candida, tetanus toxoid, or streptokinase antigens in the presence or absence of IL-12 (5 U/mL).

Limiting dilution analysis of lymphocyte proliferative responses. We investigated whether the observed effect of IL-12 on antigen-specific lymphocyte proliferation was due to an increase in the extent of proliferation by a fixed number of responding cells or to an increase in the number of cells proliferating in response to antigen. To this end, proliferative responses to Candida and tetanus toxoid antigens in the presence and absence of IL-12 were determined after limiting dilution of PBMC from 4 HIV-infected subjects and 1 seronegative control. The increased SI for Candida antigen in response to IL-12 was accompanied in each case by an increase in the frequency of responding cells (table 2). In the case of tetanus toxoid, subjects with the greatest increase in SI in response to IL-12 also had the greatest increase in responder cell frequency.

Discussion

Cytokines that stimulate cellular immune responses may have two potential roles in the treatment of HIV-1 infection: stimulation of HIV-specific immunity or general enhancement of cell-mediated immune responses (or both). Whereas the former may aid in controlling HIV-1 replication per se, the latter may still be of benefit in combating opportunistic infections in the late stages HIV-1 disease. It is this latter aspect toward which our investigation was directed.

Addition of IL-12 resulted in a significant increase in lymphocyte proliferative responses of PBMC from HIV-infected subjects to microbial recall antigens in vitro. Our results also demonstrated that in vitro lymphocyte proliferation to microbial antigens and the response to IL-12 were correlated with CD4 lymphocyte count. Although previous reports have dem-
Table 2. Limiting dilution analysis of responder cell frequency in HIV-infected persons and uninfected controls.

<table>
<thead>
<tr>
<th>Subject</th>
<th>HIV status</th>
<th>Without IL-12</th>
<th>With IL-12</th>
<th>Without IL-12</th>
<th>With IL-12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stimulation index*</td>
<td>No. of responder cells/10^6 PBMC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida antigen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>008</td>
<td>-</td>
<td>7.5</td>
<td>14.3</td>
<td>26</td>
<td>32</td>
</tr>
<tr>
<td>037</td>
<td>+</td>
<td>3.9</td>
<td>4.7</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>190</td>
<td>+</td>
<td>14.4</td>
<td>21.8</td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td>236</td>
<td>+</td>
<td>32.0</td>
<td>54.4</td>
<td>44</td>
<td>90</td>
</tr>
<tr>
<td>395</td>
<td>+</td>
<td>26.4</td>
<td>43.7</td>
<td>97</td>
<td>144</td>
</tr>
<tr>
<td>Tetanus toxoid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>008</td>
<td>-</td>
<td>5.6</td>
<td>7.1</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>037</td>
<td>+</td>
<td>13.7</td>
<td>15.3</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>190</td>
<td>+</td>
<td>1.3</td>
<td>4.3</td>
<td>7</td>
<td>16</td>
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<tr>
<td>236</td>
<td>+</td>
<td>5.6</td>
<td>7.4</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>395</td>
<td>+</td>
<td>1.9</td>
<td>4.7</td>
<td>5</td>
<td>25</td>
</tr>
</tbody>
</table>

NOTE. IL, interleukin; PBMC, peripheral blood mononuclear cells. Responder cell frequency was determined by limiting dilution in the presence or absence of IL-12 as described in Methods.
* Determined in the presence and absence of IL-12 and calculated as mean cpm incorporated by cells in antigen-treated wells divided by mean cpm incorporated into wells that contained medium alone.

It is possible that some of the heterogeneity in response to microbial recall antigens is explained by different patterns of antigen exposure in vivo—subjects in group I may have had more recent exposure to Candida species than to tetanus toxoid or to group A streptococcus. This possibility is supported by the observation that 6 patients in this group had a history of oral candidiasis, although none had a history of invasive candidal infection.

In another report, IL-12 enhanced activated lymphocyte proliferation and IL-2 production in response to HIV-1 envelope antigens, influenza virus, and alloantigens in HIV-infected patients [17]. In that study, proliferative responses to recall antigens were not enhanced by IL-12 in the small number of HIV-seronegative controls examined. By contrast, we did note significant increases in antigen-specific proliferative responses of PBMC from uninfected controls in the presence of IL-12. These differences might be explained by the larger number of
seronegative controls or the greater variety of microbial antigens tested in our study compared with earlier studies. A possible explanation for the blunted response to antigen and IL-12 in PBMC from patients with <200 CD4 cells/mm$^3$ is induction of HIV replication in the lymphocyte cultures. In one report, investigators noted significant enhancement of HIV-1 replication in an acute infection assay with prestimulated PBMC but not with freshly isolated PBMC from HIV-1-seronegative donors [22]. Activation of HIV-1 was associated with selective loss of CD4 cells in the stimulated cultures. Such loss could account for the lack of significant stimulation of antigen-specific lymphocyte proliferation by IL-12 in the present study in group I patients (<200 CD4 cells/mm$^3$). However, we did not find evidence of HIV-1 replication in PBMC from HIV-infected subjects when these cells were stimulated in vitro for 6 days with microbial recall antigens in the presence or absence of IL-12. This finding is in agreement with results of another study that likewise showed no evidence for enhancement of HIV-1 replication by IL-12 [23].

The increase in lymphocyte proliferation we observed in response to IL-12 could be due to an increase in the number of antigen-responsive lymphocytes in the assay or to an increase in the proliferative capacity of a fixed number of antigen-responsive cells. Results of limiting dilution analysis suggested that an increase in Candida- or tetanus toxoid-specific proliferation in the presence of IL-12 was due to an increase in the number of responding cells.

This finding could be explained by an additive or synergistic interaction between exogenous IL-12 and endogenous antigen-stimulated production of IL-2, which could lead to recruitment of a greater number of lymphocytes [17]. Preliminary data suggest that IL-12 enhances antigen-specific proliferative responses under conditions in which IL-2 was made limiting by the addition of low concentrations of anti-IL-2R monoclonal antibody (MAb) but has no effect when the action of IL-2 was completely blocked by higher MAb concentrations (unpublished observations).

It is also possible that IL-12 acts indirectly to increase the number of responding cells by stimulating production of IFN-γ. Interleukin-12 increases production of interferon (IFN)-γ [3, 12] by human neonatal CD4 cells and enhances proliferative responses and production of IFN-γ by PBMC from HIV-infected persons in response to PHA [24]. The apparent increase in responder cell frequency in the presence of IL-12 might also reflect the reported ability of IL-12 to prevent apoptosis of lymphocytes activated by signaling through the T cell receptor complex or CD4 cross-linking by gp120-antibody complexes [25].

In conclusion, IL-12 significantly increased in vitro proliferative responses to microbial recall antigens of PBMC from HIV-infected subjects with CD4 lymphocyte counts of >200/mm$^3$ but had little effect on PBMC from patients with more advanced disease. Clinical trials are needed to determine whether similar effects of IL-12 will be observed in vivo.

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References


