Phenotypic Analysis of Human Immunodeficiency Virus (HIV) Type 1 Cell-Mediated Immune Responses after Treatment with an HIV-1 Immunogen

Ronald B. Moss,1 Mark R. Wallace,2 Wiesława K. Giermakowska,1 Erin Webb,1 Jay Savary,1 Carolyn Chamberlin-Brandt,2 Georgia Theofan,1 Roy Musil1, Steven P. Richieri,1 Fred C. Jensen,1 and Dennis J. Carlo1

It was hypothesized that immune recognition could be stimulated with combined immune-based and potent antiviral drug therapies. This study examined human immunodeficiency virus type 1 (HIV-1)-specific lymphocyte proliferation before and after treatment with an inactivated HIV-1 immunogen in 15 chronically infected HIV-1 seropositive subjects. Lymphocyte proliferation to the immunizing antigen (gp120-depleted HIV-1; P < .001), purified native p24 (P < .001), and recombinant p24 (P < .05) increased after treatment with the HIV-specific immune-based therapy. By HIV-1 antigen-specific flow cytometry, T helper CD4 lymphocytes, CD8 lymphocytes, and NK cells (all P < .001) were the predominant cell types proliferating in vitro after treatment. Additional phenotyping of proliferating cells revealed predominantly CD4 and CD8 memory (both P < .001) phenotypes. This study supports the concept that in vitro lymphocyte proliferation to HIV-1 antigens, augmented after treatment with an inactivated HIV-1 immunogen, involves primarily CD4 and CD8 cell memory immune responses.

Cell-mediated immune responses against human immunodeficiency virus (HIV) type 1 are progressively lost in HIV-1 infection [1–4]. A notable exception occurs in persons with non-progressive disease who display strong lymphocyte proliferative T helper (Th; CD4) immune responses against core (p24) HIV-1 antigens that are associated with control of viral replication [5, 6]. Studies of subjects with chronic HIV-1 infection treated acutely with highly active antiretroviral therapy (HAART) have not shown a complete reversal of anergy against the virus, despite nearly complete suppression of plasma HIV-1 RNA [7–10]. This may be due in part to the concomitant loss of antigenic stimulation that may be required to increase the frequency of HIV-1–specific memory cells. In contrast, the results of a recent study of subjects on HAART suggested that stimulation with an inactivated HIV-1 immunogen in a potent adjuvant can augment recognition of HIV-1 antigens [11]. Treatment with this immunogen also resulted in an increase in HIV-1 antigen-stimulated β-chemokine (MIP-1β) production from cultured peripheral blood mononuclear cells (PBMC). The production of β-chemokines has been associated predominantly but not exclusively with CD8 and NK cell lymphocytes [12–14]. The proliferative response to HIV-1 antigen, in contrast, has been associated primarily with CD4 Th cells. The relationship between CD4 Th cell and effector functions of CD8 cells and other cells in HIV-1 infection is poorly understood.

Immune responses to HIV-1 are progressively lost during the course of HIV-1 infection. One explanation for the lack of recognition of HIV-1 antigens may be that repeated antigen stimulation in chronic HIV-1 infection and hyperactivation of the immune system may result in an irreversible immune unresponsiveness. Alternatively, we hypothesized that anergy against HIV-1 could be reversed under the condition of specific immune stimulation and controlled viral replication [15]. Furthermore, the in vitro recognition of HIV-1, induced by an HIV-1–specific immune-based therapy, might involve a memory immune response of different effector cell types orchestrated by the Th cell.

Here we report the results of a study that assessed immune recognition after stimulation with combined immune-based therapy and potent antiviral drug therapy. This study examined HIV-1–specific lymphocyte proliferation before and after treat-
ment in 15 HIV-1-seropositive subjects with chronic HIV-1 infection.

Methods

Fifteen HIV-1 seropositive subjects were enrolled in an open-label research study as part of an expanded access program of Remune (HIV-1 immunogen; Immune Response). The baseline demographics for this cohort are listed in table 1. Subjects had a mean of 586 CD4 cells/mm³ at baseline and a mean of 953 copies/mL of plasma HIV-1 RNA by Amplicor assay (Hoffman La Roche, Nutley, NJ). Concomitant antiviral drug therapies are also listed in table 1. Subjects received 1 intramuscular injection of the HIV-1 immunogen at day 1 and every 12 weeks thereafter, which consisted of gp120-depleted inactivated HIV-1 (HZ321) at a dose of 10 U of p24 antigen in incomplete Freund’s adjuvant (IFA).

Both gp120-depleted HIV-1 (HZ321), immunizing antigen) and native p24 antigens were utilized for in vitro immune function assays. gp120-depleted HIV-1 (HZ321) immunogen is highly purified by ultrafiltration and ion exchange chromatography [16] from the filtered (0.45 μm) extracellular supernatant fluid of HZ321 HuT-78 cells [17]. HIV-1 antigen (HZ321) is clade A envelope and clade G gag [18]. The outer envelope protein (gp120) is depleted at the ultrafiltration stage of the purification process. Antigen preparations were inactivated through a sequential application of β-propiolactone (BPL) [19] and 60Co irradiation [20]. Native p24 (np24) was lysed from purified gp120-depleted inactivated HIV-1 (HZ321) with 2% Triton X-100 and then purified using Sepharose fast flow S resin (Pharmacia, Uppsala, Sweden). Chromatography was done at pH 5.0, and p24 was eluted by using a linear salt gradient. Purity of the final product was estimated as >99% by both SDS-PAGE and reverse-phase high-performance liquid chromatography. Recombinant p24 (rp24) was obtained from Protein Sciences (Meridian, CT). Candida antigen was obtained from Greer Laboratories (Lenoir, NC).

For the lymphocyte proliferation assays (LPAs), fresh PBMC from HIV-1-seropositive subjects were purified and cultured with medium alone, Candida antigen (5 μg/mL), or inactivated HIV antigens, including whole gp120-depleted HIV-1 (5 μg/mL), np24 (5 μg/mL), and rp24 (5 μg/mL). PBMC were seeded in a round-bottom 96-well plate (Becton Dickinson, San Jose, CA) at 2 × 10⁵ cells/well in complete RPMI (Hyclone Laboratories, Logan, UT) containing 10% heat-inactivated (56°C, 30 min) human AB serum (Gemini, Calabasas, CA), and 1% antibiotics (100 U/mL penicillin; 100 μg/mL streptomycin [Gibco, Gaithersburg, MD], and L-glutamine 1% [Hyclone]). All assays were done in triplicate. After 6 days of incubation, cells were labeled with 1 μCi of [3H]thymidine in complete RPMI (Hyclone Laboratories, Logan, UT) containing 10% heat-inactivated (56°C, 30 min) human AB serum (Gemini, Calabasas, CA), and 1% antibiotics (100 U/mL penicillin; 100 μg/mL streptomycin [Gibco, Gaithersburg, MD], and L-glutamine 1% [Hyclone]). All assays were done in triplicate. After 6 days of incubation, cells were labeled with 1 μCi of [3H]thymidine in complete RPMI for 16–18 h. On day 7, 20 μL of BPL (1:1600 final concentration) was added to each well to neutralize any virus produced during the incubation period. Cells were harvested after a 2-h incubation with BPL at 37°C, and the incorporated label was determined by scintillation counting in a beta counter. Geometric mean counts per minute (cpm) were calculated from the triplicate wells with and without antigen. Results were calculated as a lymphocyte stimulation index (LSI), which is the geometric mean cpm of cells incubated with antigen divided by the geometric mean cpm of cells incubated without antigen.

Table 1. Baseline human immunodeficiency virus (HIV) RNA, CD4 count, and antiviral drugs.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Baseline HIV RNA copies/mL</th>
<th>Baseline CD4 cells/mm³</th>
<th>Baseline antivirals</th>
<th>Months before baseline on antivirals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4625</td>
<td>332</td>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>448</td>
<td>605</td>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>&lt;400</td>
<td>1147</td>
<td>Lamivudine, stavudine, indinavir</td>
<td>18, 11, 11</td>
</tr>
<tr>
<td>4</td>
<td>&lt;400</td>
<td>496</td>
<td>Lamivudine, zidovudine, saquinavir</td>
<td>26, 46, 19</td>
</tr>
<tr>
<td>5</td>
<td>3478</td>
<td>695</td>
<td>Zidovudine, lamivudine, indinavir</td>
<td>34, 32, 24</td>
</tr>
<tr>
<td>6</td>
<td>&lt;400</td>
<td>545</td>
<td>Lamivudine, stavudine, nefavir</td>
<td>35, 38, 1</td>
</tr>
<tr>
<td>7</td>
<td>400</td>
<td>326</td>
<td>Lamivudine, zidovudine, indinavir</td>
<td>25, 70, 17</td>
</tr>
<tr>
<td>8</td>
<td>852</td>
<td>815</td>
<td>Lamivudine, stavudine, nefavir</td>
<td>3, 3, 3</td>
</tr>
<tr>
<td>9</td>
<td>&lt;400</td>
<td>689</td>
<td>Stavudine, didanosine, ritonavir, saquinavir</td>
<td>10, 7, 7, 7</td>
</tr>
<tr>
<td>10</td>
<td>&lt;400</td>
<td>507</td>
<td>Stavudine, didanosine, indinavir</td>
<td>14, 14, 20</td>
</tr>
<tr>
<td>11</td>
<td>529</td>
<td>114</td>
<td>Zidovudine, lamivudine, nefavir</td>
<td>8, 8, 8</td>
</tr>
<tr>
<td>12</td>
<td>&lt;400</td>
<td>569</td>
<td>Indinavir, lamivudine, zidovudine</td>
<td>23, 23, 86</td>
</tr>
<tr>
<td>13</td>
<td>&lt;400</td>
<td>319</td>
<td>Lamivudine, ritonavir, zalcitabine</td>
<td>32, 25, 36</td>
</tr>
<tr>
<td>14</td>
<td>&lt;400</td>
<td>643</td>
<td>Stavudine, didanosine, nefavir</td>
<td>12, 12, 12</td>
</tr>
<tr>
<td>15</td>
<td>758</td>
<td>586</td>
<td>Means</td>
<td>953, 586</td>
</tr>
</tbody>
</table>
Figure 2. gp120-depleted human immunodeficiency virus type 1 (HIV-1) antigen-stimulated MIP-1β production in peripheral blood mononuclear cells at baseline and 4 weeks after treatment with HIV-1 immunogen (*P = .002).

Figure 3. Distribution of blasting (proliferating *) lymphocytes by flow cytometry in response to gp120-depleted human immunodeficiency virus type 1 (HIV-1) antigen at baseline (base) and 4 weeks after treatment (Wk 4) with HIV-1 immunogen expressed as % of total (no. of blasting lymphocytes/total lymphocytes; A) and by absolute no. of blasting lymphocytes (/B). CD4, T helper cells; CD8, CD8 lymphocytes; NK, natural killer cells; φ, monocytes; B, B lymphocytes. *P ≤ .001.
Gating was done on the forward and side light scatter blot. The nonblasting lymphocyte population (R1) and the blasting lymphocytes (R2) were identified as follows: % total = (number of blasting positive lymphocytes in RII/total events) × 100 in the light scatter plot. We also analyzed the absolute number of positive blasting cells (R1I). Lymphocyte phenotyping was done by antibody labeling, and the same total of events was acquired for each analysis.

We used the Mann-Whitney U statistic to compare lymphocyte proliferation responses before and after treatment, the percentage of total positive cells proliferating without antigen compared with cells stimulated with antigen, the number of positive cells, and MIP-1β responses before and after treatment. A one-way analysis of variance followed by Dunnett’s multiple comparison test was used to compare the effect of CD4, CD8, and CD56 depletion on lymphocyte proliferative responses. The strength and direction of associations were determined by the Spearman rank-order correlation. All P values are two tailed.

Results

As shown in figure 1, lymphocyte proliferative response to the immunizing antigen were significantly increased at 4 weeks after one treatment (all data shown as ± SE; baseline mean LSI, 6.4 ± 2.1; mean LSI 4 weeks after treatment, 42.4 ± 12.0). Proliferative responses to np24 (P < .001) were baseline mean, 2.7 ± 0.8; mean LSI 4 weeks after treatment, 29.0 ± 10.7. rp24 also increased (P < .05; baseline mean LSI, 13.1 ± 8.0; mean LSI 4 weeks after treatment, 30.2 ± 11.8), compared with pretreatment levels. No baculovirus control was available, and the high baseline LSI for rp24 may have been due to the control baculovirus proteins. In contrast, lymphocyte proliferative response to Candida did not significantly change after treatment with HIV-1 immunogen (P > .05; baseline mean LSI ± 63.1 ± 14; mean LSI ± 4 weeks after treatment, 85.1 ± 18.1). Proliferative responses to np24 and rp24 appeared to be strongly correlated (r = .80, P < .001). Proliferative responses to np24 and Candida were less strongly associated (r = .40, P = .03).

Lymphocyte proliferative responses remained augmented 4 weeks after the second treatment at week 16 (n = 14) to HIV-1 antigen (P < .002; mean LSI, 45.6 ± 8.7), np24 (P < .001; mean LSI, 32.0 ± 6.7), and rp24 (P = .03; mean LSI, 29.6 ± 7.6). Lymphocyte proliferative responses remained elevated 4 weeks after the third treatment at week 28 (n = 11) to HIV-1 antigen (P = .002; mean LSI, 32.5 ± 9.0), np24 (P < .001; mean LSI, 28.6 ± 7.7), and rp24 (P = .16; mean LSI, 19.3 ± 6.1).

MIP-1β production in response to the immunizing antigen also increased after treatment (P = .002; baseline mean, 810.9 ± 290.5 pg/mL; mean 4 weeks after treatment, 5902 ± 1775 pg/mL), as shown in figure 2. MIP-1β also increased in response to np24 (P = .06; baseline mean, 621.8 ± 186.0 pg/mL; mean ± SE 4 weeks after treatment, 2923 ± 1084 pg/mL) and rp24 (P = .03; baseline mean, 1323.0 ± 458.8 pg/mL; mean 4 weeks after treatment, 5521.0 ± 1762.0 pg/mL).
The CD4 lymphocytes ($P < .001$), CD8 lymphocytes ($P < .001$), and NK cells ($P < .001$) were the predominant cell types proliferating in in vitro cultures with HIV-1 or p24 antigen 4 weeks after treatment (figure 3), as determined by flow cytometry. Figure 4 illustrates the determination of blasts of cultured cells by forward and side scatter. A representative example of CD4 and CD8 gated blasting cells in a subject before and after treatment is shown in figure 5A and 5B, respectively.

CD4 ($r = .83, P < .001$), CD8 ($r = .80, P < .001$), and NK ($r = .78, P < .001$) cells proliferating in response to HIV-1 antigen or np24 antigen (data not shown) by flow cytometry were strongly associated with the corresponding lymphocyte proliferation index multiplied by the thymidine incorporation assay as shown in figure 6. Additional phenotyping of antigen-stimulated proliferating cells revealed predominantly CD4 and CD8 memory (both $P < .001$) phenotypes after treatment (figure 7). Depletion of CD4 lymphocytes, compared with other cell types, most efficiently abrogated the HIV-1–specific lymphocyte proliferative response ($P < .05$, figure 8).

**Discussion**

In this study, we examined the recognition of HIV-1 antigens by in vitro measurement by standard LPA and by a flow cytometry method. By the standard thymidine incorporation LPA, we observed an augmentation of responses to the immunizing antigen, purified np24, and rp24. The specificity of the immune response to the HIV-1 immunogen was confirmed by the observation that, in contrast to HIV-1 antigen proliferative responses, responses to *Candida* antigen did not significantly change after treatment.

**Figure 5.** CD4- and CD8-positive cells (*), left and right columns, respectively, illustrate blasting cells from peripheral blood mononuclear cell culture before (A) and after (B) treatment with human immunodeficiency virus type 1 (HIV-1) immunogen in response to (i) no antigen (medium only), (ii) gp120-depleted HIV-1 antigen, and (iii) native p24 (np24) antigen.

**Figure 6.** Correlation between gp120-depleted human immunodeficiency virus (HIV) type 1 antigen-stimulated lymphocyte proliferation measured by thymidine incorporation assay vs. flow cytometry assay at time 0 and week 4. Solid lines, fitted linear regression; dotted lines, 95% confidence interval.
Observations of persons with nonprogressive HIV-1 disease, who also mount strong responses to the more genetically conserved parts of HIV-1 (e.g., p24 [gag]), suggest that such responses are associated with control of HIV infection [5, 6]. Such important observations need to be examined in natural history cohorts and therapeutic efficacy trials, to better define this relationship. Previously, we [21] and others observed lymphocyte proliferative responses across different HIV-1 clades after treatment with an immune-based therapy, which may in part be due to gag (p24) and other conserved sequences of the virus within the immunogen. In this study, immune responses to p24 (both native and recombinant forms) were stimulated after one treatment with immunogen, supporting the specificity of this approach. These responses were not further stimulated with additional injections but remained significantly elevated from pretreatment levels. Most importantly, in subjects on potent antiretroviral drug therapy, the magnitude of the recognition of HIV-1 antigens in some subjects after treatment with an inactivated HIV-1 immunogen is comparable to that in some subjects with nonprogressive disease [11]. In addition to the proliferative response, the production of a β-chemokine (MIP-1β) in response to HIV-1 antigens (HIV, np24, rp24) also increased after treatment with the HIV-1 immunogen in this study, as previously noted [22]. Of interest, high β-chemokine production by PBMC has been associated with asymptomatic disease and lower virus load in other studies [23, 24], but no association was determined in this cohort at baseline.

By use of an antigen-specific flow cytometric assay, we found that CD4, CD8, and NK cell phenotypes were the predominant cells proliferating in response to HIV-1 antigen. Furthermore, the number of these cell types measured by flow cytometry correlated strongly with lymphocyte proliferation as measured by the standard thymidine incorporation assay.

It is important to note that a complex cascade of cytokines and chemokines produced by a variety of cell types collaborates in the host response to HIV-1 [25, 26]. Previously, we observed increased HIV-1 antigen-stimulated interferon (IFN-γ) production [13] and decreased tumor necrosis factor–α serum levels [27] after treatment of chronically infected persons with this HIV-specific immune-based therapy. Of interest, IFN-γ can up-regulate class I antigen expression for CD8 cell activation and class II antigen expression to activate Th cells.

In this study and others [13, 22], we observed increased production of an HIV-1 antigen-stimulated β-chemokine (MIP-1β) after treatment with the HIV-1 immunogen. We and others have also reported that β-chemokines can be made principally, but not exclusively, by CD8 and NK cells [12–14]. Thus, the cell-mediated immune response to HIV-1 may involve a Th response with subsequent release of cytokines that may stimulate the proliferation and activation of antiviral effectors, including CD8 and NK cells. Strong Th responses to HIV-1 antigens have also been associated with cytolytic CD8 responses in some studies of clinical nonprogressors [28].

Essential in the recognition of HIV-1 induced by an immunogen is the adjuvant [29]. Although not examined in the current study, it is possible that IFA with an antigen containing the genetically conserved parts of the virus activates or recruits antigen-presenting cells that may include macrophages or dendritic cells. The role of antigen-presenting cells in the HIV-1–specific immune response after treatment with HIV-1–specific immune-based therapy is currently under study.

This study further suggests that HIV-1–specific immune recognition, which results in the proliferation of Th cells, can be associated with the activation and proliferation of CD8 and NK cells with the potential for cytolytic [24] and noncytolytic activity [12] against HIV-1. The proliferating CD8 and NK cells may be secondary yet important phenomena. This observation is supported by the finding that depletion of Th cells resulted in the strongest abrogation of the HIV-1–specific lymphocyte proliferative response, as shown here and by others [5].
study further suggests that CD8 and NK precursors may also be important required subsets in the LPA response to HIV-1 antigens.

Functional immunologic unresponsiveness or anergy might be due to a number of factors in HIV-1 infection. One possibility is that HIV antigen-reactive cells are lost (clonal deletion). Potent antiviral drug therapy may allow regeneration of naive cells that can be primed by HIV-1 immunogen treatment. Another possibility is that the clonal frequency of HIV antigen-reactive cells is below the detection limits of current assays but are stimulated with HIV-1 immunogen treatment. As demonstrated in this study, after treatment with an HIV-1 immunogen, cells proliferate and differentiate into predominantly CD4 and CD8 memory cells.

Taken together, the results of this study suggest that HIV-1 functional immune unresponsiveness can be reversed by an HIV-1-specific immune-based therapy in chronic HIV-1 infection. As potent antiviral drug therapy can suppress viral replication and augment the circulating pool of naive T cells [30, 31], these data provide further rationale for examining the combination of HIV-1-specific immune-based therapies with potent antiviral drug therapy in larger ongoing clinical trials.

Acknowledgments

We thank Michele Harden for manuscript preparation; Peter Salk, Joceilyn DiVeley, and Alan Landay for critical review of the manuscript; and Christopher Nardo for statistical comments.

References

17. Choi DJ, Dube S, Spicer TP, Slade HB, Jensen FC, Poiesz BJ. Sequence note: HIV type 1 isolate Z321, the strain used to make a therapeutic HIV type 1 immunogen, is intersubtype recombinant. AIDS Res Hum Retroviruses 1997;13:357–61.
29. Choi DJ, Dube S, Spicer TP, Slade HB, Jensen FC, Poiesz BJ. Sequence note: HIV type 1 isolate Z321, the strain used to make a therapeutic HIV type 1 immunogen, is intersubtype recombinant. AIDS Res Hum Retroviruses 1997;13:357–61.


