Effect of Granulocyte Colony-Stimulating Factor (G-CSF) in Human Immunodeficiency Virus–Infected Patients: Increase in Numbers of Naive CD4 Cells and CD34 Cells Makes G-CSF a Candidate for Use in Gene Therapy or to Support Antiretroviral Therapy

Susanne D. Nielsen, Pia Afzelius, Sanne Dam-Larsen, Claus Nielsen, Jens O. Nielsen, Lars Mathiesen, and John-Erik S. Hansen

The potential of granulocyte colony-stimulating factor (G-CSF) to mobilize CD4 cells and/or CD34 cells for use in gene therapy or to support antiretroviral therapy was examined. Ten human immunodeficiency virus–infected patients were treated with G-CSF (300 µg/day) for 5 days. Numbers of CD4 and CD34 cells were measured. To examine the numbers of naive and memory type CD4 cells, CD4 cell coexpression of CD45RA and CD45RO was measured. Functionality of mobilized CD4 cells was examined by use of the proliferation assay and interleukin-2 ELISA. The number of CD34 cells increased from 1.50 to 20.01/µL (P < .002). The CD4 cell count increased from 236 to 452/µL (P < .002). The CD45RA/CD45RO ratio increased from 0.50 to 0.57 (P < .03). Mobilized CD4 cells were functionally intact. In conclusion, G-CSF induced increases in numbers of CD34 cells and CD4 cells in HIV-infected patients. Furthermore, the fraction of naive CD4 cells increased. These findings have implications for the design of immunotherapy or gene therapy protocols.
Fluorescence of 5000 cells (lymphocyte subsets) was measured in tubes containing EDTA used to obtain a full blood cell count and for flow cytometry. On days -6, -3, 0, 4, and 8, additional blood samples were drawn into tubes containing heparin or EDTA to obtain peripheral blood mononuclear cells (PBMC) by means of density gradient centrifugation.

Flow cytometry. Flow cytometric analyses were done as described [4]. The fluorescence of 5000 cells (lymphocyte subsets) or 50,000 cells (CD34 and isotype controls) was measured. To determine the absolute numbers of CD34 cells and lymphocytes in peripheral blood, the percentage of the cells expressing CD34 was multiplied by the white blood cell count, while the percentage of cells expressing CD3 and CD8, and CD69 in a lymphocyte gate was multiplied by the lymphocyte count. The following combinations of monoclonal antibodies were used to determine phenotypes: isotype control; CD3 (Leu-4, SK7), CD4 (Leu-3a, SK3), CD8 (Leu-2a, SK1), CD4 (Leu-3a, SK3), CD45RA (Leu-18, L48), CD45RO (Leu-45RO, UCHL-1), CD4 (Leu-3a, SK3), CD25 (2A3), CD69 (Leu-23, L78); CD34 (anti-HPCA-2). All monoclonal antibodies were purchased from Becton Dickinson (San Jose, CA).

Proliferation assay. On days -6, -3, 0, 4, and 8, PBMC were used for proliferation assay, done essentially as described [5]. Cell cultures were prepared in microtitre plates (Nunc, Roskilde, Denmark) containing 5 × 10⁴ PBMC/well. Culture medium was RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and a mixture of penicillin, streptomycin, and gentamicin. To stimulate cultures, 5 µg/mL phytosphagglutinin (PHA; Sigma, St. Louis) was added. In each case, control experiments without stimulation were done. Results are reported as proliferation response to PHA minus response to plain culture medium. All results are medians of values from quadruplicate cultures.

Interleukin-2 (IL-2) ELISA. PBMC were used for IL-2 ELISA. Briefly, cell cultures were prepared in microtitre plates (Nunc) containing 10⁵ PBMC/well. To the cultures, 4 µg/mL PHA (Sigma) was added. In each case, control experiments without stimulation were done. After 3 days in a cell incubator, the culture supernatants were harvested. Predica IL-2 kit (Genzyme, Cambridge, MA) was used to quantitate IL-2 production. The assay was done according to the instructions of the manufacturer. Results are reported as IL-2 production in response to PHA stimulation minus response to plain culture medium. All results are medians of values from duplicate cultures.

Measurements of virus load. To determine virus load, quantification of viral RNA in plasma was done with a polymerase chain reaction quantitative kit (AmpliCord Monitor HIV-1 PCR; Roche, Branchburg, NJ) according to the instructions of the manufacturer. Plasma was obtained after centrifugation at 400 g for 7 min, harvested, and immediately stored at -70°C until use.

Statistical analysis. Data points obtained from multiple experiments were reported as means (±SEs). Significance levels were determined by Student’s t test analysis. For comparisons within the group, we used a paired-sample t test.

Results

G-CSF effect on the absolute numbers of CD34 and CD4 cells. The absolute number of circulating CD34 cells increased significantly during G-CSF treatment (figure 1A). On day 0, the mean number of CD34 cells was 1.50 × 10⁹/µL (±0.11). On day 2, the number of CD34 cells had increased significantly (P < .04), and the mean number of CD34 cells peaked on day 5 after initiation of G-CSF treatment, at 20.01 × 10⁹/µL (±0.83, P < .002). By day 8, the number of CD34 cells had almost returned to baseline (figure 1A). In contrast, there was no significant variation in the number of CD34 cells before G-CSF treatment. The number of CD34 cells increased in all 10 patients. The increase in number of CD34 cells was partly due to an increase in white blood cell count from 5.97 × 10⁹/L (±0.48) to 24.45 × 10⁹/L (±2.63).

Before G-CSF treatment, there was only minor variation in the mean CD4 cell count (from 236 to 279/µL, figure 1B). On day 0, the mean number of CD4 cells was 236/µL (±23). On day 1, the CD4 cell count had increased significantly (P < .02), and the number of CD4 cells peaked on day 3 after

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age (years)</th>
<th>CD4 cell count/µL</th>
<th>Virus load (copies/mL)</th>
<th>Clinical symptoms</th>
<th>Antiviral treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>53</td>
<td>269</td>
<td>1867</td>
<td>VZV</td>
<td>D4T, ddI, saquinavir, ritonavir</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>53</td>
<td>235</td>
<td>&lt;200</td>
<td>HIV wasting</td>
<td>3TC, ddI, indinavir</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>48</td>
<td>103</td>
<td>357</td>
<td>Kaposis’s sarcoma</td>
<td>3TC, D4T, indinavir</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>50</td>
<td>242</td>
<td>&lt;200</td>
<td>VZV</td>
<td>AZT, 3TC, indinavir</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>30</td>
<td>288</td>
<td>67,308</td>
<td>Esophageal candidiasis</td>
<td>AZT, 3TC</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>31</td>
<td>246</td>
<td>15,348</td>
<td>Multiple bacterial pneumonias</td>
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</tr>
<tr>
<td>7</td>
<td>M</td>
<td>59</td>
<td>323</td>
<td>42,970</td>
<td>Pneumocystis carinii pneumonia</td>
<td>AZT, 3TC, saquinavir</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>42</td>
<td>300</td>
<td>&lt;200</td>
<td>VZV</td>
<td>AZT, 3TC, indinavir</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>49</td>
<td>237</td>
<td>&lt;200</td>
<td>Pulmonary tuberculosis</td>
<td>AZT, 3TC</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>65</td>
<td>115</td>
<td>&lt;200</td>
<td>Oral candidiasis</td>
<td>AZT, 3TC</td>
</tr>
</tbody>
</table>


NOTE. VZV, varicella-zoster virus. D4T, stavudine; ddI, didanosine; 3TC, lamivudine; AZT, zidovudine.
percentage of CD45RO-positive cells decreased, there was an increase in the absolute number of both CD45RA- and CD45RO-positive cells. Thus, on day 0, the mean numbers of CD4 cells expressing CD45RA and CD45RO were 79 and 155/μL. On day 4, the mean numbers of CD4 cells expressing CD45RA and CD45RO were 154 and 267/μL.

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No evidence of peripheral proliferation. To evaluate if CD4 cells were generated by peripheral proliferation, we measured CD4 cells coexpressing the activation antigens CD69 and CD25. On day 0, 12.4% (±5.5) of CD4 cells expressed CD69, and on day 4 this fraction was reduced to 9.3% (±5.6) (P < .04). In contrast, no differences were found between values for day 0 and day −6, −3, or 8. Furthermore, on day 0 we found that 13.0% (±3.6) of CD4 cells coexpressed CD25. This fraction was not different from the fractions we found on any other day.

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Functionality of mobilized CD4 cells. The functionality of CD4 cells was examined by use of a proliferation assay. All patients responded to PHA, with a mean proliferative response of 110.7 × 10³ cpm on day 0. There were no significant differences between proliferative responses on day 0 and on any other day. In addition, the functionality of the CD4 cells was evaluated by IL-2 production. On day 0, PBMC from 7 patients produced IL-2 in response to stimulation with PHA (mean, 33 pg/mL [±14]). On day 4, PBMC from 8 patients produced IL-2 in response to PHA (mean, 81 pg/mL [±59]). However, no significant differences in IL-2 production in response to PHA were found. Thus, the CD4 cells mobilized by G-CSF treatment appear to have normal functionality.

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Virus load during G-CSF therapy. Virus load during G-CSF treatment was tested by use of HIV RNA polymerase chain reaction. In 6 patients, we detected an increase in copy number per milliliter, in 3 patients the copy number was <200 on day 0 as well as on day 4, and in 1 patient there was a decrease in virus load. On day 0, the mean copy number was 12,885/mL (±7421), and on day 4 it was 16,993 (±11,116). Thus, we found a minor increase in virus load. This increase, however, was not significant (P = .47).

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Discussion

This study demonstrates that G-CSF increases both CD34 and CD4 cells in HIV-infected persons. Furthermore, we found an increase in percentage of naive CD4 cells. These data make G-CSF a candidate for use in gene therapy or to support antiretroviral therapy.

Three events might explain the rise in CD4 cell count: differentiation of CD34 cells, peripheral proliferation, and redistribution of CD4 cells from the lymphatics. Studies in mice have shown that naive CD4 cells exported from the thymus express CD45RA. By contrast, memory CD4 cells derived from peripheral proliferation express CD45RO [6]. However, interconversion between CD45RA and CD45RO has been demonstrated [7], suggesting that isoform expres-
sion may not reflect a unidirectional maturation from naive to memory type cells. Thus, the rise in CD45RA/CD45RO ratio does support differentiation of CD34 cells but does not provide definite proof. The rapid increase in CD4 cell count could indicate that G-CSF had an effect on CD34 cells in the thymus that were already committed to T cell generation.

A recent study concluded that expansion of peripheral lymphocytes in vivo is antigen-driven [8]. Increased CD69 and CD25 expression by lymphocytes activated by antigens has been documented [9, 10]. We did not detect increased expression of either CD69 or CD25. Thus, peripheral proliferation does not seem to explain the increase in CD4 cell count. Finally, redistribution of CD4 cells from the lymphatics might explain the rise in CD4 cell count. The rapid increase in CD4 cell count does support redistribution as being partly responsible. Furthermore, G-CSF has been shown to alter cytokine production in blood, which might result in lymphocyte redistribution as well [11]. However, if redistribution was the sole explanation for the rise in CD4 cell count, the switch toward the naive phenotype would not be expected. Thus, further studies to examine the effect of G-CSF on CD34 cell differentiation are needed.

HIV infection is characterized by a progressive decline in CD4 cell count. A recent study of the effects of protease inhibitors demonstrated that after protease inhibitor therapy, naive CD4 cells increased only if they were present before initiation of therapy [12]. The generation and maintenance of a diverse T cell repertoire are central requirements for immune competence, and it therefore remains uncertain whether even highly efficient antiretroviral therapies will result in restoration of the immune system once damaged. Recently, reports of cytomegalovirus retinitis in HIV-infected patients with CD4 cell counts $>$195 after initiation of protease inhibitor therapy has been published [13]. Such cases of opportunistic infections in patients with high CD4 cell counts could be due to a skewness in CD45RA/CD45RO ratio. In the present study, an increase, although modest, was found in the absolute number of naive CD4 cells during 5 days of G-CSF treatment. It cannot be excluded that a more pronounced effect could be found if HIV-infected patients were treated with G-CSF for a longer period of time. If that is the case, G-CSF might be used in combination with protease inhibitors to maintain a diverse T cell repertoire.

Gene therapy has been suggested as a possible treatment of HIV infection [14, 15]. The recovery of high yields of functionally unimpaired T lymphocytes or progenitor cells is a mandatory prerequisite for such approaches. The present study demonstrates that G-CSF can be used to mobilize functional CD4 cells as well as CD34 cells prior to genetic intervention. Furthermore, it cannot be excluded that the increase in CD4 cell count is partly due to differentiation of CD34 cells. These findings have implications for the design of gene therapy protocols.

Acknowledgments

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References

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