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Persistent Abnormalities in Lymphoid Tissues of Human Immunodeficiency Virus–Infected Patients Successfully Treated with Highly Active Antiretroviral Therapy

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Effective highly active antiretroviral therapy (HAART) for human immunodeficiency virus type 1 is associated with virus suppression and immune reconstitution. However, in some patients, this reconstitution is partial or incomplete because CD4+ cell counts do not increase significantly. This may be due to damage in the microenvironment of lymphoid tissues (LTs), where CD4+ T cells reside. To test this hypothesis, LT samples were obtained from 23 patients enrolled in a prospective trial that compared 3 different HAART regimens. Analysis of LT architecture and CD4+ T cells populations revealed abnormalities in 100% of the LT samples, especially in the follicles, with 43% showing absence, 14% showing regression, and 43% showing hyperplasia. CD4+ T cell populations were abnormal in 16 (89%) of 18 tissue samples, with 7 (39%) of 18 decreased by >50% of normal levels. These data are consistent with the hypothesis that persistent abnormalities in the microenvironment can influence immune reconstitution and document persistent LT abnormalities with HAART not detected by measures of peripheral CD4+ T cell count.

In 1996, the clinical introduction of the protease inhibitor (PI) class of antiretrovirals, designed to inhibit replication of the human immunodeficiency virus (HIV), provided the first opportunity to demonstrate a significant impact on the amount of detectable virus in both blood plasma and lymphoid tissue (LT), the principal site of HIV replication and storage [1–3]. It has now become standard practice to initiate highly active antiretroviral therapy (HAART) with combinations of PIs and other antiretrovirals to more completely and durably suppress viral replication.

Subsequent studies have demonstrated conclusively that this reduction in HIV RNA load in peripheral blood and concomitant increase in peripheral CD4+ T cell count are associated with a significant survivor benefit and fewer opportunistic infections [4–7]. However, some patients experience only a small or insignificant increase in CD4+ T cells as therapy begins, despite successful suppression of plasma HIV-1 viremia. Because of the importance of the microenvironment in T cell homeostasis, we hypothesized that persisting pathological abnormalities might be responsible for the differing rates and extent of CD4+ T cell increase and undertook LT analysis to test this hypothesis. The Atlantic study, begun in 1997, prospectively compared the clinical and virologic benefit to antiretroviral-naïve patients assigned to receive 3 nucleoside analogues (NAs; mono-class therapy), 2 NAs plus 1 nonnucleoside analogue (NNA), or 2 NAs plus 1 PI (dual-class therapy) and provided an opportunity to investigate the relationships of these different treatment regimens to CD4+ T cell population and LT architecture. We expected that patients with good recovery of the peripheral CD4+ T cell count or with a high baseline CD4+ T cell count that was maintained over the course of follow-up would have a more normal LT architecture and CD4+ T cell repopulation than would patients with poor recovery or low peripheral CD4+ T cell count at the time of biopsy.

Patients and Methods

Patient population. All patients enrolled in the Atlantic Study from 3 clinical sites (Chicago, Illinois; Barcelona, Spain; and Warsaw, Poland) with plasma HIV RNA loads <500 copies/mL were approached to have a lymph node biopsy performed ~1 year after the initiation of therapy. The type of procedure (inguinal LT excision or tonsil biopsy) was left to the discretion of the patient and the doctors at the clinic. Demographic data, peripheral CD4+ cell counts, plasma HIV RNA load, and clinical stage of infection were
Abnormalities in the formation of secondary follicles, which were seen in virtually every lymphoid tissue sample examined. Tissues were serially examined with hematoxylin-eosin (HE) and antibodies to CD20 and Ki67. We used CD20 to look for presence of B cells and Ki67 to look for activated cells to confirm the presence of secondary follicles when interpretation of the HE was ambiguous. Panels A and B show results from a single patient, demonstrating hyperplastic secondary follicle formation. A, HE stain; B, stain for CD20. Panels C and D show results from a different patient. C, HE showing that the tissue has no discernible secondary follicle formation; D, the same tissue stained with antibodies to Ki67 to look for collections of activated cells that may not have been obvious by either HE or CD20 antibody staining. In this example, there were only a few cells staining positive for Ki67, suggesting a complete absence of secondary follicle formation. E, Normal accumulation of Ki67+ cells in the B cell follicle.
recorded for all patients from each site and compared with data from all patients from that site same who agreed to undergo lymph node biopsy.

LT collection and fixation procedures. Tissues were biopsied using standard surgical techniques for each respective clinic. Tissues biopsied at the clinics in Chicago and Warsaw were immediately placed into fixative and shipped to the University of Minnesota. Tissues biopsied at the clinic in Barcelona were immediately frozen at −70°C and thawed in fixative after arrival at the University of Minnesota. Five-micrometer sections were cut for routine histologic examination and immunohistochemistry. These tissue preparation protocols have been published elsewhere [8].

Histologic analysis of follicle formation. All tissues were subjected to routine hematoxylin-eosin staining, and 2 observers independently performed the microscopic analysis. In addition, 5-μm-thick sections were stained with antibody to CD20 and Ki67 to ascertain the presence of secondary follicle formation that might not have been apparent by routine histologic analysis of hematoxylin-eosin staining. Each sample was classified as normal or as containing follicular hyperplasia, follicular regression, or follicular depletion (no evidence of secondary follicle formation). These changes are illustrated in figure 1.

Detection of HIV p24 antigen. All tissues were stained by immunohistochemistry (IHC) using antibodies to HIV-1 p24 antigen. The quantity of p24 antigen in the tissues was graded on a 5-point scale (0–4), with 0 equivalent to no p24 antigen detected on interfollicular areas. Immunohistochemistry staining for CD4+ T cells was performed on the paraffin-embedded sections with an anti-CD4+ antibody (clone IF6; Novacastra) to characterize the quantity and distribution of CD4+ cells in all LT samples. Tissues were graded as normal, 50%–75% of normal, 25%–50% of normal, or <25% of normal (severely depleted). Sections of lymph nodes uninvolved by any malignant neoplasm or infectious process were used for comparison.

Results

Description of cohort. A total of 23 individuals enrolled into the Atlantic trial underwent biopsy: 8 from Chicago, 11 from Barcelona, and 4 from Warsaw. There were no significant differences in peripheral CD4+ T cell counts and plasma HIV-1 RNA loads at baseline (prior to initiation of HAART) between patients who underwent biopsies and patients who were enrolled but who did not undergo biopsies at each site (table 1). Overall, 11 patients were randomly assigned to receive mono-class therapy, consisting of didanosine (ddI), stavudine (d4T), and lamivudine (3TC); 12 patients were assigned to receive dual-class therapy, with 4 receiving a PI (ddI, d4T, or indinavir) and 8 receiving an NNA (ddI, d4T, or nevirapine). There were differences in the duration of therapy among the clinics: the 11 individuals from Barcelona underwent biopsy a median of 48 weeks (range, 36–84 weeks) after randomization, whereas the patients from Chicago and Warsaw underwent biopsy a median of 123 and 118 weeks (range, 84–156 and 116–123 weeks, respectively) after randomization; however, there were no differences in the level of virus suppression or the time to suppression of peripheral viremia. Of the 23 patients who underwent biopsy, 17 (74%) had a tonsil biopsy, and 6 (26%) had an inguinal lymph node biopsy. There were no significant differences in the CD4+ cell count, duration of treatment, or plasma HIV RNA load at the time of biopsy between patients who had an inguinal biopsy and those who had a tonsil biopsy.

Histologic analysis and characterization of follicle formation. Overall, 21 (91%) of 23 LT samples were appropriately fixed and stained to grade presence, size, and location of both primary and secondary follicles. Large quantities of HIV-1 are stored in association with the FDC network, the site where B and T cells and antigen interact to generate humoral immune responses [3, 12–15]. This network is largely destroyed during the course of protracted HIV-1 infection but can be regenerated, albeit slowly, with HAART [12]. The follicular architecture thus is one marker of the LT microenvironment that can be evaluated.

We found that, despite a median of 84 weeks (range, 36–156 weeks) of HAART with complete suppression of peripheral viremia, all 21 patients had abnormal histologic test results, with 9 (43%) showing a complete absence of secondary follicle
Figure 2. CD4⁺ T cell depletion abnormalities in the quantity and distribution of CD4⁺ T cells in lymphoid tissue (LT). These abnormalities were observed in 16 of 18 patients with LT samples available for analysis. We found a marked decrease among those receiving mono-class therapy. A, Patient with severe CD4⁺ T cell depletion (<25% of normal) in LT whose peripheral CD4⁺ T cell count at entry was 428 cells/mm³ at entry and increased by 111 cells/mm³ over the course of follow-up. B, Patient with 25%–50% of a normal LT population whose peripheral CD4⁺ T cell count at entry was 510 cells/mm³ and decreased by 219 cells/mm³ over the course of follow-up. C, Patient with 50%–75% of a normal LT population whose peripheral CD4⁺ T cell count was 369 cells/mm³ at entry and increased by 223 cells/mm³ over the course of follow-up. D, Patient with a relatively normal distribution and quantity of CD4⁺ T cells in LT whose peripheral CD4⁺ T cell count was 296 cells/mm³ at entry and increased by 157 cells/mm³ over the course of follow-up.

formation, 3 (14%) showing regression of secondary follicles, and 9 (43%) showing follicular hyperplasia (figure 1). Of interest, we found that 60% (6/10) of patients receiving triple NA therapy had hyperplastic LT, whereas only 27% (3/11) of LT samples from patients assigned to receive either an NNA- or PI-containing regimen were hyperplastic.

Detection of HIV p24 antigen in LT samples. HAART greatly reduces the quantity of HIV-1 immune complexes bound
Table 2. Demographic and lymphoid tissue (LT) characteristics of all 21 patients, compared with peripheral CD4+ T cell count at time of biopsy and change in peripheral CD4+ T cell count during therapy.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at biopsy, years</th>
<th>Months treated</th>
<th>Regimen</th>
<th>CD4+ cell count at biopsy, cells/mm3</th>
<th>Change in CD4+ cell count, cells/mm3</th>
<th>Secondary follicles</th>
<th>LT type</th>
<th>LT CD4+ cell count, % of normal</th>
<th>p24 Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45</td>
<td>21</td>
<td>ddI/d4T/3TC</td>
<td>473</td>
<td>113</td>
<td>Hyperplastic</td>
<td>LN</td>
<td>&lt;25</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>57</td>
<td>21</td>
<td>ddI/d4T/NVP</td>
<td>291</td>
<td>−219</td>
<td>Hyperplastic</td>
<td>Tonsil</td>
<td>25–50</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>46</td>
<td>30</td>
<td>ddI/d4T/3TC</td>
<td>624</td>
<td>171</td>
<td>Hyperplastic</td>
<td>Tonsil</td>
<td>50–75</td>
<td>−</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>30</td>
<td>ddI/d4T/NVP</td>
<td>424</td>
<td>133</td>
<td>Hyperplastic</td>
<td>Tonsil</td>
<td>50–75</td>
<td>−</td>
</tr>
<tr>
<td>5</td>
<td>39</td>
<td>31</td>
<td>ddI/d4T/3TC</td>
<td>915</td>
<td>484</td>
<td>Hyperplastic</td>
<td>Tonsil</td>
<td>50–75</td>
<td>−</td>
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<tr>
<td>6</td>
<td>37</td>
<td>36</td>
<td>ddI/d4T/3TC</td>
<td>641</td>
<td>285</td>
<td>Hyperplastic</td>
<td>Tonsil</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>41</td>
<td>39</td>
<td>DdI/d4T/NVP</td>
<td>592</td>
<td>223</td>
<td>Hyperplastic</td>
<td>LN</td>
<td>100</td>
<td>−</td>
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<tr>
<td>8</td>
<td>47</td>
<td>32</td>
<td>DdI/d4T/IDV</td>
<td>957</td>
<td>425</td>
<td>Hyperplastic</td>
<td>Tonsil</td>
<td>50–75</td>
<td>−</td>
</tr>
<tr>
<td>9</td>
<td>45</td>
<td>21</td>
<td>ddI/d4T/IDV</td>
<td>553</td>
<td>257</td>
<td>Hyperplastic</td>
<td>Tonsil</td>
<td>100</td>
<td>+</td>
</tr>
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<td>41</td>
<td>9</td>
<td>ddI/d4T/3TC</td>
<td>473</td>
<td>34</td>
<td>Depleted</td>
<td>Tonsil</td>
<td>ND</td>
<td>−</td>
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<td>11</td>
<td>34</td>
<td>12</td>
<td>ddI/d4T/3TC</td>
<td>800</td>
<td>341</td>
<td>Hyperplastic</td>
<td>Tonsil</td>
<td>25–50</td>
<td>+</td>
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<tr>
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<td>9</td>
<td>ddI/d4T/3TC</td>
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<td>111</td>
<td>Hyperplastic</td>
<td>Tonsil</td>
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<td>Hyperplastic</td>
<td>Tonsil</td>
<td>&lt;25</td>
<td>+++++</td>
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<td>14</td>
<td>29</td>
<td>9</td>
<td>ddI/d4T/3TC</td>
<td>507</td>
<td>212</td>
<td>Depleted</td>
<td>Tonsil</td>
<td>50–75</td>
<td>−</td>
</tr>
<tr>
<td>15</td>
<td>37</td>
<td>9</td>
<td>ddI/d4T/NVP</td>
<td>315</td>
<td>38</td>
<td>Depleted</td>
<td>Tonsil</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>16</td>
<td>32</td>
<td>12</td>
<td>ddI/d4T/NVP</td>
<td>651</td>
<td>139</td>
<td>Depleted</td>
<td>Tonsil</td>
<td>50–75</td>
<td>−</td>
</tr>
<tr>
<td>17</td>
<td>31</td>
<td>12</td>
<td>ddI/d4T/IDV</td>
<td>455</td>
<td>125</td>
<td>Depleted</td>
<td>Tonsil</td>
<td>50–75</td>
<td>−</td>
</tr>
<tr>
<td>18</td>
<td>39</td>
<td>29</td>
<td>ddI/d4T/NVP</td>
<td>619</td>
<td>143</td>
<td>Depleted</td>
<td>LN</td>
<td>50–75</td>
<td>−</td>
</tr>
<tr>
<td>19</td>
<td>43</td>
<td>31</td>
<td>ddI/d4T/NVP</td>
<td>547</td>
<td>−214</td>
<td>Depleted</td>
<td>LN</td>
<td>50–75</td>
<td>−</td>
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<tr>
<td>20</td>
<td>36</td>
<td>29</td>
<td>ddI/d4T/IDV</td>
<td>548</td>
<td>−57</td>
<td>Depleted</td>
<td>LN</td>
<td>25–50</td>
<td>−</td>
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<tr>
<td>21</td>
<td>36</td>
<td>30</td>
<td>ddI/d4T/IDV</td>
<td>587</td>
<td>521</td>
<td>Depleted</td>
<td>LN</td>
<td>25–50</td>
<td>+++++</td>
</tr>
</tbody>
</table>

NOTE. ND, not determined.

a 3TC, lamivudine; d4T, stavudine; ddI, didanosine; IDV, indinavir; NVP, nevirapine.

b LN, inguinal lymph node; tonsil, palatine tonsil.
c +, Present; −, absent.

to FDCs by p24 complexes. We examined the relationship between the follicular abnormalities and persistent p24 IHC staining. Overall, 18 (78%) of 23 LT samples were adequate to perform HIV p24 antigen staining to examine for the presence of HIV p24 antigen in the FDC meshwork. We detected p24 antigen in 6 (35%) of 17 samples: 2 were inguinal LT, and 4 were tonsil. Of interest, we found that, despite plasma HIV RNA levels <500 copies/mL, 56% (5/9) of persons assigned to receive triple NA therapy had p24 antigen present, whereas only 11% (1/9) of individuals assigned to either an NNA- or PI-containing regimen had detectable p24 antigen in LT. All 5 patients with persistent antigen and who were in the triple NA group also had follicular hyperplasia.

Analysis of CD4+ T cell population in LT samples. In total, 18 (78%) of 23 LT samples were adequately fixed to stain for antibodies against CD4+ T cells. Each section was analyzed by 2 individuals and characterized for quantity and distribution of CD4+ T cells. All but 2 LT samples showed evidence of a depleted T cell population, with 3 samples being completely depleted, 4 with <25% of normal cell population, and 8 with 50%–75% of normal cell population (figure 2). We grouped the results by those with either <50% of normal CD4+ T cell population and those with normal counts or ≥50% of normal counts. Of the 7 samples with <50% of normal CD4+ T cell population, the median duration of treatment was 84 weeks (range, 36–121 weeks), and the median CD4+ T cell count was 548 cells/mm3 (range, 291–800 cells/mm3), compared with 120 weeks (range, 36–156 weeks) and 619 cells/mm3 (range, 424–957 cells/mm3), respectively, in the samples with ≥50% of a normal CD4+ T cell population. In the samples with <50% of a normal CD4+ T cell population in LT, 5 (71%) of 7 had follicular hyperplasia, compared with 4 (36%) of 11 in the second group.

Discussion

We analyzed LT samples from patients receiving HAART who had effective suppression of viral replication for at least 1 year. We wanted to determine the impact of HAART on the overall architecture and size of the CD4+ population in LT, the principal site of HIV replication. We hypothesized that patients with undetectable plasma HIV-1 RNA loads and significantly higher peripheral CD4+ T cell counts would have a more normal architecture, with a greater CD4+ T cell population in LT, suggesting a significant reconstitution of immune function. This result was expected because multiple studies have documented immunologic improvement associated with suppression of viral replication and increases in peripheral CD4+ T cell count. Surprisingly, we found no such correlation. In fact, every tissue specimen examined demonstrated a significant abnormality. By any clinical measure, these patients would have been considered to have achieved therapeutic success at the time of biopsy. All but 2 had plasma HIV-1 RNA loads <50 copies/mL (patients 4 and 5 in table 2, with 94 and 267 copies/mL, respectively), and most (81%) had peripheral CD4+ cell counts that had increased over baseline. The median increase in peripheral CD4+ T cell count was 171 cells/mm3 (range, 34–121 cells/mm3), with...
having an increase of $>200$ cells/mm$^3$ and 15 (71%) having an increase of $>100$ cells/mm$^3$. We found no significant relationship or trend toward restoration of normal architecture or CD$^+$ T cell population. The follicular hypoplasia or depletion at one year after initiation of HAART is consistent with the slow and variable regeneration in the FDC network documented elsewhere [12]. The follicular hyperplasia and the underlying basis for the correlation between lower repopulation of LTs with CD$^+$ T cells, follicular hyperplasia, and triple therapy is not at all clear, nor do we understand why there would be more p24 antigen left in the follicles of these patients. However, this residual antigen is the likely explanation for follicular hyperplasia, which is caused by mechanisms that interfere with restoration of the interfollicular CD$^+$ T cell population that could involve competition for space and growth factors, bystander activation, increased activation-induced cell death, and altered trafficking. It is possible that the prolonged and persistent viral replication in CD$^+$ T cells of the interfollicular space, coupled with local innate and cellular attempts at immunologic control, cause a loss of tissue structure such that the interfollicular space is no longer capable of sustaining a population of CD$^+$ T cells. It is also possible that the tissues require a longer period of virus suppression for remodeling to occur to the extent that a population of CD$^+$ T cells can be maintained.

In conclusion, we have shown that significant abnormalities in the follicle structure and low CD$^+$ T cell counts persist in LT samples obtained from patients who, by any clinical measure, have met the common goals of HAART (i.e., virus suppression and improving peripheral CD$^+$ T cell count). The persistent abnormalities might be secondary to a limited duration of infection or therapy (i.e., a longer duration of therapy is needed) or persistence of viral replication in LT that is not detected by peripheral measures of viral replication. It is not possible to answer this question with this data set because we made only one observation per patient after therapy began and made no observations before. Prospective, longitudinal trials with frequent sampling of LT will be required to answer these questions, preferably among patients with a known date of HIV-1 seroconversion who enroll at variable intervals from the time of seroconversion.

Acknowledgments

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References