Immunomorphologic Analysis of Bone Marrow Biopsies After Treatment With 2-Chlorodeoxyadenosine for Hairy Cell Leukemia

By Douglas J. Ellison, Robert W. Sharpe, Bruce A. Robbins, John C. Spinosa, John D. Leopard, Alan Saven, and Lawrence D. Piro

Treatment of hairy cell leukemia with 2-chlorodeoxyadenosine (2-CdA) induces complete remissions in 85% of patients. Complete remission has been defined as the absence of hairy cells in the bone marrow after routine morphologic examination. To determine if hairy cells could be detected in complete remission bone marrows using immunohistochemical techniques with antibodies L26 (CD20) and DBA.44, 154 bone marrow biopsies performed between 3 months and 25 months after therapy were studied. Of the biopsies, 50% exhibited staining with L26 and/or DBA.44 in five or more cells with morphologic features of hairy cells. Minimal residual disease was usually less than 1% of the total cellular population. DBA.44-positive cells were demonstrated in 91% of the biopsies, although in 48% of these the morphologic features of the positive cells were not sufficiently distinctive for hairy cells. The proportion of biopsies with residual hairy cells was similar over the 25 months of follow up, indicating a relatively stable amount of residual disease. Immunomorphologic analysis is a more sensitive method for detecting residual hairy cells than morphology alone. Although further follow up is necessary to determine the clinical significance of the L26/DBA.44-positive staining in cells with and without distinctive morphologic features of hairy cells, we conclude that many patients in a stable clinical remission may have residual hairy cells.

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A NEWER PURINE ANALOGUE, 2-chlorodeoxyadenosine (2-CdA), has been found to be a highly effective chemotherapeutic agent for the treatment of hairy cell leukemia (HCL). Complete responses (CRs) are achieved in 85% of patients after a single 7-day course of therapy. CRs are defined by the absence of morphologically evident hairy cells using routine light microscopy within bone marrow biopsies or other tissues of treated patients. Previous experience has shown that immunologic techniques combined with morphology are more sensitive at detecting minimal numbers of neoplastic cells, such as in axillary lymph nodes in patients with breast carcinomas. Previous studies have also used this approach in patients with HCL treated with α-interferon and 2'-deoxycoformycin. There has been no previous large immunomorphologic study with extended follow up of patients treated with 2-CdA for HCL.

Two monoclonal antibodies that stain in routinely processed tissues are useful in directly identifying hairy cells: L26 and DBA.44. L26 (CD20) is a pan-B-cell antibody that is highly lineage-specific and that can be applied to tissue fixed in both formalin and B5. L26 typically stains positively in most cases of HCL. DBA.44 is a B-cell-associated monoclonal antibody of the IgM class that was originally generated against HCL. DBA.44 reacts with a subpopulation of mantle cells and monocytoid B cells in lymph nodes, marginal zone lymphocytes in the spleen, rare isolated small lymphocytes in bone marrow biopsies, and histiocytes. DBA.44 also stains a variety of low-grade and high-grade B-cell lymphomas but only very rarely stains T-cell lymphomas. Previous studies have shown DBA.44 to stain almost all cases of HCL, and these results have been confirmed by preliminary studies in our laboratory. Our initial results using the L26 and DBA.44 antibodies led us to use these two antibodies to study a large series of bone marrow biopsies of patients treated with 2-CdA for HCL.

MATERIALS AND METHODS

Patients treated with 2-CdA for HCL at Scripps Clinic and Research Foundation (La Jolla, CA) had follow-up bone marrow biopsies submitted by outside physicians or performed at this institution. Biopsies were performed at varying intervals after 2-CdA treatment, with recommended times at 3 months, 6 months, 12 months, and 24 months. In 1992, 172 biopsies were studied in which hairy cells could not be identified on routine morphologic evaluations, including review of peripheral blood smears and aspirate smears that were provided in the majority of the cases. Of these 172 biopsies, 166 were available for immunohistochemical staining, and 158 were technically adequate after staining was performed. The biopsies were received from outside institutions fixed in formalin, B-5, Zenker’s, or Bouin’s fixatives; Scripps biopsies were fixed in B-5. Immunohistochemical staining was performed using an indirect method using avidin-biotin complex (Vector, Burlingame, CA) with DAB chromogen. Slides were counter-stained with Harris hematoxylin. The biopsies were stained with L26 and DBA.44 (DAKO, Carpinteria, CA), and with Leu-22 (Beckton Dickenson, San Jose, CA) as a control. The reagents were titrated against tonsillar control tissue fixed in B5. Concurrent staining was performed on bone marrow biopsies from patients with other disorders in order to assess the number and morphologic spectrum of mononuclear cells that would stain with L26 and DBA.44. The control biopsies came from patients with the following diagnoses: iron deficiency anemia (two patients), metastatic breast carcinoma, malignant teratoma, reactive lymphoplasmacytosis, hypereosinophilia syndrome, monoclonal gammopathy of unknown significance, chronic lymphocytic leukemia, plasmacytoid lymphocytic lymphoma/leukemia (two patients), non-Hodgkin’s lymphoma (two patients), Hodgkin’s disease (two patients), multiple myeloma, acute lymphoblastic leukemia, myeloid metaplasia, and chronic myelogenous leukemia (two patients). After immunostaining, evaluation of the biopsies for the number of L26- and DBA.44-positive cells was made on a semiquantitative basis using a scale of negative, indeterminate, rare, less than 1%, and 1%.
1% to 3%, 3% to 5%, or greater than 5% of the cell population. The distinction between the indeterminate group and the group with rare hairy cells was based on the finding of at least five cells with morphologic features of hairy cells that stained positively with either L26 or DBA.44. Also, positive cells were classified as hairy cells, cytoplasmic lymphocytes, or small lymphocytes (see Results for definitions).

RESULTS

Control bone marrows. In 25 bone marrow biopsies from patients without hairy cell leukemia, L26 stained variable numbers of mononuclear cells ranging from less than 1% to 10% of the cellularity. The positive cells were typically small with round to slightly irregular nuclei, clumped chromatin, and scanty amounts of pale cytoplasm. Occasional cells had moderate amounts of cytoplasm. Occasional transformed lymphocytes could be seen in lymphoid aggregates, and these had round vesicular nuclei with multiple nucleoli and moderate amounts of cytoplasm. DBA.44 stained only rare cells, fewer than one per high-powered field and usually only a few (less than 10) cells in an entire biopsy. Staining was more diffuse than with L26, with both cytoplasmic membrane and granular cytoplasmic staining in cells with moderate amounts of cytoplasm. The positive cells typically had small nuclei with clumped chromatin and scanty amounts of cytoplasm, with occasional cells having moderate amounts of cytoplasm (Fig 2A). Histiocytes, occasionally positive, had a granular cytoplasmic pattern of staining. These cells generally had abundant amounts of cytoplasm and ovoid vesicular nuclei and were few in number. Also, rare large cells, with rounded nuclei, vesicular chromatin, multiple nucleoli, and moderate amounts of cytoplasm stained in a granular cytoplasmic pattern. These cells resembled either blasts or transformed lymphocytes. In patients with non-Hodgkin’s lymphomas, increased numbers of L26-positive cells were noted, with a minor subpopulation staining with DBA.44. Staining for DBA.44 was most frequent in the mantle cells of lymphoid aggregates with germinal centers or at the periphery of lymphoid aggregates without germinal centers. Leu-22 (CD43) usually stained granulocytes and lymphocytes and variably stained erythrocytes.

Posttherapy with 2-CdA bone marrows. In 1992, immunohistochemical staining for L26 and DBA.44 was performed on bone marrow biopsies from 42 patients with the
initial diagnosis of hairy cell leukemia, eight patients in morphologically identified relapse, and 15 patients in partial remissions. In these 65 specimens, the hairy cells stained for L26 and DBA.44 in 60 and 62 cases, respectively. The cases that were L26-negative appeared to be technically unsatisfactory with heavy background or no positive small lymphocytes. DBA.44 was truly negative in three cases, but these specimens had few hairy cells within the biopsy.

Posttherapy bone marrow biopsies (PTBMs) were available from 3 to 25 months after 2-CdA treatment. Thirty-eight PTBMs were performed after 3 to 4 months, 28 were performed after 5 to 7 months, 17 were performed after 8 to 10 months, 47 were performed after 11 to 13 months, 10 were performed after 14 to 16 months, and 18 were performed after 17 to 25 months.

Staining of the PTBMs with L26 showed positive staining in 133 cases and with DBA.44 in 144 cases of 158 cases. The greatest numbers of positive cytoplasmic mononuclear cells in each biopsy (L26 or DBA.44) varied from rare in 85 cases, less than 1% in 27 cases, 1% to 3% in 28 cases, 3% to 5% in eight cases, and greater than 5% in four cases.

L26-positive cells were always more numerous than DBA.44-positive cells. L26-positive lymphocytes varied in morphology from small lymphocytes described in the control biopsies to abnormal mononuclear cells with morphologic features of hairy cells with a cytoplasmic membranous staining (Fig 1B). These cells typically had moderate to abundant cytoplasm with frayed nuclear membranes that had strongly positive staining. The nuclear features varied from ovoid nuclei with a reticular chromatin, to bilobed or indented forms with prominent nucleoli. However, there were other forms of lymphocytes that were difficult to characterize as either reactive lymphocytes or hairy cells. These cells had varying nuclear features but usually had small, rounded nuclei with slightly clumped chromatin. However, the nuclei were enlarged over the typical small lymphocytes. Also, they had slightly more cytoplasm and occasionally had thickened cytoplasmic membranes.

DBA.44 stained increased numbers of lymphocytes over control biopsies, although in a typical case they were still rare, generally fewer in number than the L26-positive cells (Fig 2A). These lymphocytes varied in morphology, often having features of small lymphocytes with clumped nuclear chromatin, small

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Fig 2. Bone marrow. (A) Small lymphocytes stained with DBA.44. Note the distinct cytoplasmic membrane positivity without intracytoplasmic staining (original magnification x 1,000). (B) Hairy cell stained with DBA.44. Note the abundant granular cytoplasm positivity (original magnification x 1,000).
nuclei, and scant to moderate amounts of cytoplasm. Cells with the morphologic features of hairy cells, whose nuclear features were described above, typically stained in a diffuse granular cytoplasmic pattern with occasional accentuation of the cytoplasmic membranes. These cells typically had abundant cytoplasm, and when the nuclear features were not obscured by the cytoplasmic staining, were distinguishable from reactive lymphocytes (Figs 2B, 3A and B).

Because of the difficulties in identifying some L26-/DBA.44-positive lymphocytes as either hairy cells or reactive lymphocytes, a bone marrow was considered positive for residual hairy cells when five or more cells with both nuclear and cytoplasmic morphologic features of hairy cells and with a characteristic pattern of staining with L26 and/or DBA.44 were identified. Cells without the typical nuclear features combined with the typical antigen reactivity were considered indeterminant for this analysis. Biopsies were considered negative if they did not contain cells that stained for L26 or DBA.44. Therefore, bone marrow biopsies were placed within three categories: positive, indeterminant, and negative. This last category may represent technical failures, but another antibody (Leu-22) run on this specimen did stain the tissue.

The results of the immunomorphologic analysis of PTBMs are shown in Tables 1, 2, and 3. Table 1 shows that the percentage of PTBMs considered positive for residual hairy cells was similar for all of the time periods studied, with a range of values from 47% to 61%. Tables 2 and 3 indicate that the numbers of L26-/DBA.44-positive cells also remained stable over the time period studied, with no obvious trend toward increasing numbers of hairy cells. In patients

Table 1. Immunomorphologic Findings in CR PTBM Detection of Residual Disease

<table>
<thead>
<tr>
<th>Months Posttherapy</th>
<th>Categories* (%)</th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>3-4</td>
<td>18 (47)</td>
</tr>
<tr>
<td>5-7</td>
<td>17 (61)</td>
</tr>
<tr>
<td>8-10</td>
<td>9 (53)</td>
</tr>
<tr>
<td>11-13</td>
<td>20 (43)</td>
</tr>
<tr>
<td>14-16</td>
<td>15 (40)</td>
</tr>
<tr>
<td>17-25</td>
<td>10 (56)</td>
</tr>
<tr>
<td>Total</td>
<td>79 (50)</td>
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</table>

* See Results for definitions of categories.
with multiple biopsies, only 4 of 18 showed an increase in the number of hairy cells as determined by immunomorphology. Two of these four had substantial numbers of residual hairy cells 3 and 6 months after therapy.

**DISCUSSION**

This study demonstrated that the immunomorphologic approach to detecting residual hairy cells in patients after therapy with 2-CdA is a more sensitive technique than light microscopy alone. The results also indicate residual hairy cells were less than 1% of the cellularity in 112 of 158 biopsies. In these cases, detection of hairy cells by routine light microscopy is difficult and would explain why previous studies indicated high CR rates. In the biopsies with greater numbers of hairy cells, especially those with greater than 3% hairy cells, the aspersion smears were usually suboptimal or not available. Hairy cells would probably have been detected in adequate smears in some of these cases. In the indeterminate biopsies, putative hairy cells were usually present but in an insufficient number to meet our criteria for residual disease. The data should not be interpreted as indicating that these biopsies do not have hairy cells, but rather that there is inconclusive immunomorphologic evidence that they are present. Many of these biopsies contained rare cells that met the criteria for hairy cells or had very rare DBA.44-positive cytoplasmic cells suspicious for hairy cells. The majority of the indeterminate biopsies were, therefore, abnormal, and so one must be guarded about the interpretation of these findings. The criteria for minimal residual disease of five cells with morphologic features of hairy cells and with positivity with L26 and/or DBA.44 was chosen to ensure no false-positive results. No control case had five cells that could be mistaken for hairy cells. However, in some of the cases with low-grade non-Hodgkin’s lymphomas, a rare cell could be mistaken for a hairy cell. The problem arises because there is some morphologic overlap between hairy cells that are not sectioned near the maximum diameter of the cell, where its distinctive size and nuclear morphology is evident, and cytoplasmic lymphocytes seen in other disorders. Although five cells with immunomorphologic features of hairy cells were not determined necessary for positivity, in this study, we expect that some of the cases that had one to four hairy cells by immunomorphologic analysis are true positives. In this regard, a recent study, using the polymerase chain reaction and clone-specific probes derived from heavy chain immunoglobulin genes found minimal residual disease in seven of seven patients who achieved a CR after one course of 2-CdA. This molecular genetic data would seem to support our immunomorphologic findings.

One could question whether the infrequent L26-/DBA.44-positive mononuclear cells are actually hairy cells. Supporting this interpretation is the fact that the L26-/DBA.44-positive cells stained in a similar pattern to hairy cells seen in bone marrows before therapy, and in PTBMs with routine morphologic evidence of minimal residual disease. Therefore, it seems reasonable to conclude that these rare cells are, indeed, hairy cells. The L26-/DBA.44-positive mononuclear cells that have some features of hairy cells, but did not meet our morphologic criteria for hairy cells, may represent hairy cells altered by therapy or a regenerating B-cell population in patients receiving 2-CdA.

This study is the first to observe HCL patients after 2-CdA therapy for up to 25 months after therapy. The results indicate that the amount of residual disease seems to remain stable over a prolonged period of time in the majority of patients. There appears to be no clear trend in the data over time in regard to the proportion of biopsies considered diagnostic for residual disease, or in the amount of residual disease. Other studies have found residual hairy cells detectable by immunologic methods. A single previous study of 2-CdA-treated patients applied L26 and UCHL-1 (CD43) to PTBMs to detect hairy cells in patients in CR. They used different criteria requiring both the presence of L26-positive cells with morphologic features of hairy cells, and that these L26-positive cells were greater in number than UCHL-1 (T cells)-positive cells. They reported 21% of patients in CR to have immunologically detectable hairy cells. These criteria would seem to be less sensitive than those in the present study, and, therefore, these patients with immunohistochemically detected residual disease would have greater numbers of hairy cells than the patients in this study. In a follow-up study, Dyrda et al correlated their level of

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>No. of L26/DBA.44-Positive Cells by Month Posttherapy</th>
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<tbody>
<tr>
<td>1</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>2</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>&lt;1%</td>
</tr>
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<td>6</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>NA</td>
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<td>8</td>
<td>NA</td>
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<tr>
<td>9</td>
<td>Rare</td>
</tr>
<tr>
<td>10</td>
<td>NA</td>
</tr>
<tr>
<td>11</td>
<td>Neg</td>
</tr>
<tr>
<td>12</td>
<td>3%-5%</td>
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<tr>
<td>13</td>
<td>Neg</td>
</tr>
<tr>
<td>14</td>
<td>1%-3%</td>
</tr>
</tbody>
</table>

Abbreviations: NEG, negative; NA, not available.

* Positive by routine morphology.
minimal residual disease with morphologic relapse. Of 4 of 18 patients with minimal residual disease at 3 months and 1 at 1 year, 3 showed morphologic relapse, while 13 patients without minimal residual disease have not morphologically relapsed. Clinical parameters on these patients are not reported in the abstract. These results are similar to the results in our study if one only considers the patients with relatively numerous residual hairy cells: patients 10 and 12 of the 14 (14%). It is important to remember that morphologic relapse is dependent not only on the number of hairy cells present, but on the quality of the material for evaluation. Good smears may show hairy cells before they are morphologically evident in biopsy sections. Therefore, the finding of morphologic relapse should be qualified by the actual tumor burden for meaningful comparison of disease progression. From these two studies, it seems that relatively frequent hairy cells detected by immunohistochemistry are associated with morphologic relapse. However, in distinction from the previous studies, it seems likely that large numbers of patients may have low numbers of residual hairy cells that are relatively stable over time. Because hairy cells have a very low proliferative rate, relapses may take years, especially when the tumor burden is extremely low. In this regard, it is also important to note that some patients who have developed only partial responses to therapy but have greater than 5% hairy cells in the marrow do require retreatment. These patients have increasing numbers of hairy cells with time easily evident by morphology alone in biopsy sections. Therefore, it seems that the results and interpretations from these two studies are reflective of the immunomorphologic criteria for positive results.

A technical consideration in the follow up of these patients was the finding that L26 more frequently did not stain formalin-, Zenker’s-, or Bouin’s-fixed decalcified tissues than DBA.44. It is, therefore, useful to have both antibodies available, as the patterns of staining of the abnormal mononuclear cells is different and lends a complementarity to the antibodies.

Continued follow up is necessary to determine the significance of the residual hairy cells and of the L26/DBA.44-positive cells that were found in those cases without identifiable hairy cells. This latter group may ultimately prove to represent residual hairy cells in all patients, or perhaps an unusual population of B cells. Also to be determined is if minimal residual disease has an impact on survival or if clinical relapse will occur.

REFERENCES
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