Immunohistologic Study of Bone Marrow Involvement in B-Chronic Lymphocytic Leukemia

By G. Pizzolo, M. Chilosi, A. Ambrosetti, G. Semenzato, L. Fiore-Donati, and G. Perona

Bone marrow trephine biopsies from 17 patients with B-chronic lymphocytic leukemia (B-CLL) were studied by immunohistologic techniques in order to investigate the cellular phenotypes of both neoplastic (B-lymphoid) and reactive (T-lymphoid) infiltrates. For this purpose, several heteroantisera and monoclonal antibodies against human Ig isotypes, HLA-DR antigens, and T-cell subpopulations were used in immunofluorescence. The findings were analyzed in relationship to the histologic pattern of involvement, as well as to the immunologic data of cell suspensions from peripheral blood. In all cases, the dominant lymphoid population within the bone marrow infiltrates showed identical phenotypic characteristics of B-CLL cells from the blood (HLA-DR+, μ+, most frequently δ+, κ+, or λ+, and weakly Fc'e1). The infiltration by these malignant B cells was diffuse in 5 cases and nodular plus interstitial in 12. The number of T cells (UCHT1+, RFA-1+, μ+) was variable (5%–25%) in the different samples, but the values were high when compared to the proportion of T cells in normal bone marrow and in the blood of most patients studied. Furthermore, a clear predominance of T cells exhibiting the inducer phenotype (Leu-3+) was observed in all bone marrow samples, which is in contrast with the findings from peripheral blood, where T cells with the suppressor/cytotoxic phenotype (Leu-2+) were dominant. These data suggest a different blood and tissue distribution of inducer and suppressor/cytotoxic cells in B-CLL, which may have important pathophysiologic significance.

The availability of monoclonal antibodies may open up new opportunities for regulating immune responses and influencing the growth of malignant cells. It is therefore important to further investigate the interrelationship of T-lymphoid cells and B-lymphoid malignancies. Immunohistologic studies in follicular lymphoma have already shown a conspicuous T-cell infiltration in the malignant follicles, but similar detailed immunopathologic studies have not yet been performed for the analysis of various cell types in the bone marrow during chronic lymphocytic leukemia of B-cell type (B-CLL). Instead, in this disease, the peripheral blood (PB) most frequently has been studied, and it has been shown in various laboratories that there is an increased percentage and absolute number of T cells bearing Fc'e (Tg)5,7 and OKT810 phenotypes. In addition, functional analysis of these cells demonstrated increased suppressor in vitro activities, suggesting that abnormalities in immunoregulatory T cells may be involved in the pathogenesis of impaired immunoglobulin synthesis in B-CLL patients.6,7,9,11

The main purpose of this study was to investigate the cellular phenotypes of both neoplastic (B-lymphoid) and reactive (T-lymphoid) infiltrates in bone marrow sections of patients with B-CLL and to analyze the relationship of these findings to the histologic pattern of bone marrow involvement. For this reason, we have evaluated the proportions and the distribution of T-cell subsets, referred to as inducer type and suppressor/cytotoxic type cells (defined as Leu-3+ and Leu-2+ cells, respectively) among the neoplastic B cells. Bone marrow cryostat sections from patients with well characterized B-CLL were studied, using a previously described immunohistologic technique, and were compared to the findings in the peripheral blood.

MATERIALS AND METHODS

Patients

Seventeen patients with B-CLL were investigated. The diagnosis and classification were established according to Rai's staging system. At the time of the study, the patients were either untreated (13 cases) or had not received treatment for at least 6 mo (4 cases). The relevant clinical and immunologic data are listed in Table 1.

Studies on Cell Suspension From Peripheral Blood

Mononuclear cells were separated by Ficoll-Hyphaque density gradient from freshly drawn heparinized peripheral blood and then washed three times in sterile phosphate-buffered saline (PBS). Adherent cells were removed by incubation at 37°C for 45 min in plastic Petri dishes. Conventional immunologic characterization was performed as previously described, at the same time that bone marrow and lymph node biopsies were obtained. T lymphocytes were purified from non-T-cells by rosetting with neuraminidase (Sigma Chemical Co., St. Louis, MO) treated sheep red blood cells (nSRBC) and separated on a Ficoll-Hyphaque gradient, as previously described in detail. In brief, the rosette-forming cell fractions were purified further by repeated centrifugations (sometimes more than five) in order to obtain optimal purification. T cells were recovered after lysis of nSRBC with 0.83% ammonium chloride and were resuspended in RPMI 1640 (Grand Island Biological Co., Grand Island, NY). The purified fractions...
always contained more than 97% and 90% (in 30 controls and in B-CLL patients, respectively) T cells, while the contamination with surface immunoglobulin-positive cells never exceeded 2%. Remaining cells could represent T cells that had lost the rosetting ability following repeated centrifugations. The viability was assessed by trypan blue exclusion test (over 96%). Purified T cells were stained by indirect immunofluorescence with Leu-3 and Leu-2; monoclonal antibodies (from Becton Dickinson, Sunnyvale, CA). The immunologic phenotyping of purified T cells was concomitant with the bone marrow biopsies in 10 of 17 patients. In fact, the first 7 cases were analyzed before the advantage of comparing peripheral blood and bone marrow T-cell subpopulations became evident. For this reason, in these 7 cases, purified T cells from peripheral blood were investigated several weeks after bone marrow biopsies were obtained.

**Immunohistologic Studies**

Preliminary studies on bone marrow cell suspensions from patients with B-CLL gave contradictory results, due to the dilution of the neoplastic tissue with the residual normal bone marrow populations. This was particularly evident in cases with nodular involvement.

Immunohistologic studies were performed on bone marrow cryostat sections from all cases and also on lymph nodes from patients 6, 8, and 13. Bone marrow biopsies were obtained under local anesthesia with an 11-gauge Jamshidi needle from the posterior iliac spine. The cores were divided into two parts: one of them used for conventional histology and the other snap-frozen in liquid nitrogen and stored at -70°C until sectioning, as described previously.12,13 The histologic pattern of involvement was judged as diffuse or nodular, as recently suggested.14-16 Cryostat sections (8-10 μm) were stained with monoclonal antibodies and conventional antisera using an immunofluorescence technique.17,18,19 Briefly, after fixation in cold ethanol and rehydration in PBS, the sections were covered with monoclonal antibodies (0.1-0.2 μg in 10-20 μl fluid) or diluted antisera (1:20-1:40) for 30 min, and washed in PBS. Rabbit and goat antisera to human Ig isotypes (from Dako and Nordic) were directly conjugated to fluorescein isothiocyanate (FITC, green) or tetra-ethylrhodamin isothiocyanate (TRITC, red) and used in various combinations (e.g., anti-κ-TRITC/anti-λ-FITC).

Monoclonal antibodies were used in a three-step indirect immunofluorescence, so that a rabbit anti-mouse Ig antisera (Nordic) was used as the second layer and a goat anti-rabbit Ig antisera labeled with FITC or TRITC (Nordic) as the third layer. Each step was followed by quick washing in PBS. The three-step indirect system to detect monoclonal antibodies was used instead of the classic two-layer procedure in order to amplify the fluorescence intensity. In fact, preliminary experiments using two layers showed weak immunostaining with some monoclonal antibodies (in particular Leu-3 and Leu-2), which often made a proper evaluation of the preparations difficult. Monoclonal antibodies used in this study were: UCHT1 (OKT3-like, from Dr. P. Beverley, London22-23); RFA-1 (OKT1- and Leu-l-like, from Prof. G. Janossy, London); and Leu-3, Leu-2, and anti-HLA-DR (from Becton Dickinson). Double immunostaining was performed as follows: UCHT1/anti-Ig, UCHT1/anti-κ, UCHT1/anti-λ, RFA-1/anti-Ig, Leu-2 or Leu-3/anti-κ. In each combination, one reagent was labeled with FITC and the other with TRITC. The sections were finally mounted with a drop of glycerol with some monoclonal antibodies (in particular Leu-3 and Leu-2) and anti-HLA-DR (from Becton Dickinson). Double immunostaining was performed as follows: UCHT1/anti-Ig, UCHT1/anti-κ, UCHT1/anti-λ, RFA-1/anti-Ig, Leu-2 or Leu-3/anti-κ. In each combination, one reagent was labeled with FITC and the other with TRITC. The sections were finally mounted with a drop of glycerol and coverslip and examined under a Leitz Dialux microscope equipped for epifluorescence.

In cases with a nodular pattern, all bone marrow nodules (3-12 in the different cases) were carefully examined and the proportions of T-cell subpopulations were determined by counting on double-
stained sections (e.g., anti-IgM-TRITC/Leu-3-FITC) the number of T cells among the B neoplastic population. The proportion of T cells on sections from lymph nodes and bone marrow in cases with a diffuse involvement was determined similarly, and at least 8 different areas were examined for each case.

RESULTS

The lymphoid infiltrates in the bone marrow exhibited a diffuse pattern in 5 cases and a nodular pattern in 12 cases (Table 2). This was established by the histologic appearance on conventionally processed samples and on peroxidase-stained cryostat sections. Interstitial lymphoid infiltrates could also be observed in 4 cases with a nodular pattern, while the scattered malignant B cells were apparently absent in the other 8 cases (but see the results with immunologic tests below).

Analysis of Malignant B Cells

In all cases, the bone marrow biopsies contained lymphoid infiltrates which, when studied by immunofluorescence staining in sections, showed the membrane phenotype HLA-DR+, μ+, and κ+ (11 cases; Fig. 1E) or λ+ (6 cases). The staining intensity for HLA-DR was bright, and for μ, κ, or λ, it was weaker but easily detectable (Fig. 1, B and E, and Fig. 2B). In 13 cases, the δ chain could also be detected, the intensity of which was weak. Furthermore, these HLA-DR+, μ+ B lymphocytes were also weakly stained with the RFA-1 antibody (Figs. 1A and 2E).

Table 2. T- and B-Cell Subsets in the Bone Marrow of B-CLL as Analyzed by Immunohistology

<table>
<thead>
<tr>
<th>Cases</th>
<th>Bone Marrow Involvement*</th>
<th>Percentage of T Cells†</th>
<th>Leu-3/Leu-2 Ratio</th>
<th>B Cells‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nodular</td>
<td>23±</td>
<td>8</td>
<td>μ+ δ+ λ+</td>
</tr>
<tr>
<td>2</td>
<td>Nodular</td>
<td>8</td>
<td>7</td>
<td>μ+ δ+ κ+</td>
</tr>
<tr>
<td>3</td>
<td>Nodular</td>
<td>18</td>
<td>6</td>
<td>μ+ δ+ κ+</td>
</tr>
<tr>
<td>4</td>
<td>Nodular</td>
<td>11</td>
<td>4</td>
<td>μ+ δ+ λ+</td>
</tr>
<tr>
<td>5</td>
<td>Nodular</td>
<td>17</td>
<td>4</td>
<td>μ+ δ+ κ+</td>
</tr>
<tr>
<td>6</td>
<td>Nodular</td>
<td>12</td>
<td>2</td>
<td>μ+ δ+ κ+</td>
</tr>
<tr>
<td>7</td>
<td>Nodular</td>
<td>15</td>
<td>2</td>
<td>μ+ δ+ κ+</td>
</tr>
<tr>
<td>8</td>
<td>Nodular</td>
<td>8</td>
<td>3</td>
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<tr>
<td>9</td>
<td>Nodular</td>
<td>20</td>
<td>4</td>
<td>μ+ δ+ λ+</td>
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<tr>
<td>10</td>
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<td>5</td>
<td>4</td>
<td>μ+ δ+ κ+</td>
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<tr>
<td>11</td>
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<td>15</td>
<td>3</td>
<td>μ+ δ+ κ+</td>
</tr>
<tr>
<td>12</td>
<td>Nodular</td>
<td>20</td>
<td>10</td>
<td>μ+ δ+ κ+</td>
</tr>
<tr>
<td>13</td>
<td>Diffuse</td>
<td>10</td>
<td>3</td>
<td>μ+ δ+ κ+</td>
</tr>
<tr>
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<td>Diffuse</td>
<td>18</td>
<td>6</td>
<td>μ+ δ+ λ+</td>
</tr>
<tr>
<td>15</td>
<td>Diffuse</td>
<td>11</td>
<td>5</td>
<td>μ+ δ+ λ+</td>
</tr>
<tr>
<td>16</td>
<td>Diffuse</td>
<td>8</td>
<td>3</td>
<td>μ+ δ+ κ+</td>
</tr>
<tr>
<td>Mean values</td>
<td>13.35</td>
<td>4.53</td>
<td></td>
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*Established with conventional histology.
†In cases with nodular involvement, the percentage refers to the UCHT1+ T cells inside the nodules. The percentage was lower outside the nodules (usually <5%). Similar percentages were strongly reactive with the RFA-1 antibody (Figs. 1A and 2E).
‡The values underlined represent higher figures than the percentages of T cells in the peripheral blood detected by E rosettes (see Table 1). These B cells weakly reacted with RFA-1 (Figs. 1A and 2E).

This is in contrast to the RFA-1 staining in control lymph node samples, where T lymphocytes were almost exclusively stained in the paracortical area and germinal centers, but no RFA-1+ B lymphocytes were seen in the lymphocyte corona. This reagent has the same reactivity as OKT1 and Leu-1. In suspension, the B-CLL cells had the same phenotype as the malignant (monoclonal) B cells of the bone marrow. The phenotypic characteristics of these B-CLL cells in the bone marrow and blood were therefore identical (HLA-DR+, μ+, most frequently δ+, κ+, or λ+, and weakly RFA-1+).

An additional observation in the bone marrow was that interstitial infiltrates of RFA-1+ B cells could also be detected outside the nodules in all cases. That these cells most probably belonged to the malignant B-cell clone is suggested by the fact that similar RFA-1+ B cells are not found in normal bone marrow.

Analysis of Infiltrating T Cells in the Bone Marrow

The reagent RFA-1 stained some BM cells strongly within the areas of B-cell infiltration. When studied in double-marker assays in sections of bone marrow, the strongly RFA-1+ cells were μ+ (Fig. 1, A and B). The number and distribution of the RFA-1+/μ+ population were similar to the features of T cells identified in adjacent bone marrow sections with the UCHT1 (OKT3-like) antibody (Fig. 2, A and E).
Fig. 1. Immunohistology of T- and B-cell subpopulations in patient 3, with B-CLL showing a nodular infiltration of bone marrow. Adjacent sections were double-stained with different fluorochromes and photographed with selective filters for FITC and TRITC. (A) RFA-1 and (B) anti-IgM (μ-specific); (C) Leu-3; (D) Leu-2; (E) anti-α; and (F) anti-λ. The staining with RFA-1 reveals two populations that are strongly (T cells) and weakly (B cells) stained. Magnification 250×.
Fig. 2. Immunohistology of T- and B-cell populations in patient 13, with B-CLL diffusely infiltrating the bone marrow. (A) UCHT1 (OKT3-like) and (B) anti-lgM (μ-specific); (C) Leu-3; (D) Leu-2; (E) RFA-1. Magnification 250×.
The numbers of strongly RFA-1⁺, UCHT1⁺ and μ⁻ cells were variable (5%–23%) in the different samples (Table 2), and these numbers were often higher than the proportion of the E-rosette-positive T cells in the blood. In fact, in 10 of 17 cases, the blood had lower proportions of T cells than the bone marrow (compare Tables 1 and 2). An additional observation was that the numbers of strongly RFA-1⁺, UCHT1⁺, μ⁻ cells were higher inside the nodules of malignant B cells than outside the nodules in the residual normal bone marrow with minimal B-cell involvement. These T cells were fairly evenly scattered in these nodules, and small clusters were seen only occasionally (Fig. 1A).

When the bone marrow sections were stained for T-cell subsets with Leu-3 (helper type) and Leu-2 (suppressor/cytotoxic cell type) reagents, a dominance of Leu-3⁻ cells was seen in every case (Fig. 1C). Small clusters were seen only occasionally (Fig. 1A). Again, the Leu-3⁺ cells were most frequently inside the nodules (Fig. 1C), but a similar accumulation was also observed in cases with diffuse B-cell infiltrate (Fig. 2C). A further important point was that the Leu-3/Leu-2 ratio in the circulating blood was much lower (mean 0.73, Table 1) than the ratio observed in the bone marrow.

Analysis of Lymph Nodes in B-CLL

Taking lymph node biopsies in this disease is not always justifiable on medical grounds. For this reason, only three patients were studied. Nevertheless, the observations in these cases fully confirmed the findings in bone marrow. In particular, the monoclonality (κ⁺ in two and λ⁺ in the other case), as well as weak RFA-1 staining of the B-cell population, could be demonstrated among the diffuse infiltrates. In addition, the percentages of UCHT1⁺, strongly RFA-1⁺ T cells were 15%, 20%, and 25% of the lymphoid population with the Leu-3/Leu-2 ratios of 5, 12, and 15, respectively.

DISCUSSION

In this study, the following two points could be elucidated from the immunohistologic analysis of bone marrow biopsies taken from patients with B-CLL: first, the membrane phenotype and tissue distribution of neoplastic infiltrates and, second, the distribution of normal T-cell subsets among these monoclonal B cells.

In all the cases studied, B cells in the bone marrow showed the typical membrane phenotype of B-CLL: weakly Ig⁺, HLA-DR⁺, and reacting with the Leu-1-type monoclonal antibody against an antigen of 67,000 daltons. In this study, the antibody used against this antigen was RFA-1, a reagent available as a culture supernatant that provides good histologic staining. This phenotype corresponds to a minute subset of normal B cells that forms mouse-erythrocyte rosettes and can be found in lymph nodes and tonsil but not in normal bone marrow.

The findings of the phenotypically identical population in the blood, bone marrow, and (in the three cases studied) within the involved lymph node, confirm our earlier report which applied mouse-erythrocyte rosetting and histochemical criteria to the comparative analysis of B-CLL cells in the blood and bone marrow. Taken together, these observations indicate that the circulating and tissue-seeking malignant B-cell populations in CLL have a similar, if not identical, phenotype (weak Ig⁺, RFA-1⁺, mouse-erythrocyte⁺). These findings are at variance with another report, which used mouse-erythrocyte rosetting and found low values in the bone marrow and nodes and high values in the blood. The explanation for this discrepancy is unclear.

An additional finding is that the immunohistologic analysis was more sensitive than conventional histology for identifying isolated neoplastic cells among the interstitial infiltrates outside the nodules. This was possible by using the staining combination of RFA-1 and anti-IgM with which the distinction between RFA-1⁺ (strong), μ⁻ T cells and RFA-1⁺ (weak), μ⁺ neoplastic B cells is feasible, as was pointed out by Caligaris-Cappio and his colleagues. Normal cells of the latter type are restricted to the germinal center of peripheral nodes and are not normally found in the bone marrow, hence the apparent leukemia specificity of this particular marker combination.

The main finding about the T-cell population was the identification of an increased number of T lymphocytes with inducer phenotype in the bone marrow and lymph nodes. This seems to be a positive accumulation for the following three reasons. First, the proportion of T cells was higher in these tissues than T cells in normal bone marrow and also higher, in 10 of 17 patients, than the proportion of T cells in the circulating blood (compare Tables 1 and 2). Second, the Leu-3⁻ (OKT4⁺) T cells are rare in normal bone marrow, where the T lymphocytes mainly belong to the suppressor/cytotoxic subset and similarly, in patients with B-CLL, the majority of circulating T cells is frequently of T8⁺ (Leu-2⁺) type. This was also the case in our patients. Finally, this positive accumulation of Leu-3⁻ T cells in the bone marrow was apparently restricted to the neoplastic nodules and...
could not be seen in the surrounding bone marrow with mostly hemopoietic cells.

The last point represents an intriguing bone finding that may have important pathophysiological significance. In fact, previous reports based on cell suspension studies from the blood suggested that the fairly constant increase in the absolute number of T cells of suppressor/cytotoxic type may contribute to the development of hypogammaglobulinemia in B-CLL. Nevertheless, a strict correlation between T-cell subset imbalance in the peripheral blood and hypogammaglobulinemia has never been convincingly provided. Our results do not contradict this possibility, but rather suggest, in accord with other authors, that other parameters should be taken into account. The differential homing of the various T-cell populations demonstrated in our study creates a more complex situation than was expected. Areas infiltrated with B-CLL seem to attract the inducer-type T cells, which in turn may help the proliferation of malignant B cells. At the same time, areas in the body and the residual normal lymphoid tissue may be depleted of this cell type, leading, on balance, to immunosuppression.

ACKNOWLEDGMENT

We wish to thank Prof. G. Janossy, London, for critical review of the manuscript. We are also grateful to Mariestella Bonaldi for typing the manuscript, and to Licia Montagna and Carlo Vincenzi for excellent technical support.

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