Comparison of Normal and Chronic Lymphocytic Leukemia Lymphocyte Surface Ig Determinants Using Peroxidase-labeled Antibodies.

II. Quantification of Light Chain Determinants in Atypical Lymphocytic Leukemia

By Guillermo Dighiero, Jean Yves Follezou, Jean Paul Roisin, Thérèse Ternynck, and Jacques Louis Binet

Five cases of atypical lymphocytic leukemia were investigated with regard to their membrane-associated light chains. Detection and quantitation of antigenic determinants were performed by means of peroxidase-labeled antibodies according to Avrameas et al. The cases studied had clinical and cytotic features in common: an active clinical course, marked splenomegalgy, severe anemia and thrombocytopenia, little or no lymph node enlargement, and very high white blood counts with small mature lymphocytes and poorly differentiated lymphoid cells. Blood lymphocytes of all patients carried a single type of light chain, and 90%-100% of the cells were stained. The average number of antigenic sites per cell was 72,500 (range 40,000-97,500). These results differed from those previously found in typical CLL (mean value 9000) and approached the values of normal peripheral blood lymphocytes (90,000). The criteria investigated in this study could be of value for the diagnosis and prognosis of some atypical forms of lymphocytic leukemia.

Most cases of chronic lymphocytic leukemia (CLL) represent a proliferation of B cells, and monoclonal surface membrane immunoglobulins (SmIg) have been demonstrated on the surfaces of the lymphocytes. Johansson and Klein studied lymphocytes from an atypical form of CLL which possessed large quantities of the same SmIg. Brouet et al. detected a similar monoclonal pattern in blast crises (BC) supervening in CLL. Furthermore, Galton found most cases of prolymphocytic leukemia (PL) to be B-cell proliferations. In a previous paper, we quantified the light chain determinants on the surface of normal and CLL lymphocytes using peroxidase-labeled antibodies, and we found a tenfold decrease in the number of CLL lymphocyte determinants as compared to normal lymphocytes. The purpose of the present study was to emphasize the importance of quantitative determination of SmIg in the classification of lymphocytic leukemia.

MATERIALS AND METHODS

Patients

Five patients (two male and three female), 53-75 yr old, with atypical lymphocytic leukemia were studied. Two were diagnosed as CLL on the basis of May-Grünwald Giemsa smears during blast...
Fig. 1. Blood smear, May–Grünwald–Giemsa stain, two types of lymphocytes are observed (patient 3).

crises supervening 1 and 3 yr after the initial diagnosis, and three as PL in accordance with the clinical and cytologic criteria proposed by Galton. Case 1. The diagnosis of CLL was established in this 67-yr-old female, in November 1969 on the basis of a peripheral lymphocytosis (WBC 25,000/cu mm with 95% lymphocytes) and diffuse bone marrow infiltration by small mature lymphocytes. Neither lymph node enlargement nor splenomegaly were found on physical examination. Hemoglobin level and platelet count were normal. The patient received chlorambucil (4 mg/day) from 1969 to 1972. During this period, the WBC count never exceeded 10,000/cu mm.

In February 1972, the patient was admitted to the hospital for intense asthenia. Clinical findings were confined to pallor and enlargement of the spleen, which extended 7 cm below the costal margin. Laboratory findings included anemia (hemoglobin 8 g/100 ml) and thrombocytopenia (platelet count 50,000/cu mm). The WBC count was 47,000/cu mm, with 80% small mature lymphocytes and 15% lymphoblast-like cells. Treatment by extracorporeal irradiation and splenic irradiation proved to be ineffective. The WBC count rose rapidly to 510,000/cu mm, and the patient died of septicemia in July 1972.

Case 2. This 68-yr-old male was admitted to the hospital in March 1973 because of an abnormal blood picture. The spleen was palpable 3 cm below the left costal margin. Liver size, measured in the midclavicular line, was 16 cm. There were small cervical, axillary, and inguinal lymph nodes. The WBC count was 100,000/mm3, with 90% small mature lymphocytes. Hemoglobin level and platelet count were normal. Treatment with chlorambucil brought the WBC count down to 15,000/cu mm but did not induce any clinical changes.

The patient was readmitted to hospital in September 1974 for edema of the lower extremities. The spleen was palpable 8 cm below the left costal margin. Hemoglobin was 8.5 g/100 ml, platelet count was 40,000/cu mm, and the WBC count was 87,000/cu mm with 75% small lymphocytes and 20% blast-like cells. Despite continuation of chlorambucil, no improvement was recorded. The frequency of peripheral blast cells rose to 60%, and the patient died in October 1974.

Case 3. This 53-yr-old male was admitted to the hospital in April 1972 for severe asthenia. Physical examination revealed marked splenic enlargement (12 cm below the costal margin), hepatomegaly measuring 19 cm, and a few very small cervical and axillary lymph nodes. Hemoglobin level was 7 g/100 ml, platelet count was 40,000/cu mm, and the WBC count was 200,000/cu mm, with 50% small mature lymphocytes and 50% prolymphocytes. Bone marrow smears and sections showed massive infiltration with these same cells, and a diagnosis of PL was made. One month of treatment with chlorambucil was ineffective, as was one course of MOPP, and the patient died in June 1972.

Case 4. This 73-yr-old female presented in September 1973 for asthenia. Physical examination revealed marked splenomegaly (10 cm below the left costal margin) without enlargement of peripheral lymph nodes. Her hemoglobin level was 5 g/100 ml, the platelet count 15,000/cu mm, and the WBC count was 60,000/cu mm, with 70% mature lymphocytes and 20% prolymphocytes. Bone marrow smears showed an identical cytologic picture. On treatment with chlorambucil and
prednisone, the white cell count reverted to normal. A year later, the patient was admitted to hospital because of marked deterioration. The spleen was palpable 15 cm below the costal margin, and there was no enlargement of peripheral lymph nodes. The WBC count was 96,000/cu mm, with 96\% lymphoid cells consisting of small mature lymphocytes and prolymphocytes. No improvement was achieved by treatment with vincristine and prednisone, and the patient died in January 1976.

Case 5. This 70-yr-old female with a history of rheumatoid arthritis was admitted to the hospital in March 1974 for intense asthenia and marked splenic enlargement (14 cm below the left costal margin). There were no palpable peripheral lymph nodes. Hemoglobin level was 7.5 g/100 ml, the platelet count was 70,000/cu mm, and the WBC count was 75,000/cu mm, with 95\% lymphoid cells. Bone marrow smears revealed 90\% of cells identical to those observed in the blood. The cytology of these cells was consistent with the diagnosis of PL. Treatment by splenic irradiation was undertaken and induced a drop in the WBC count to 20,000 and a decrease in spleen size (5 cm below the left costal margin). The patient died of supervening lung infection 2 mo after diagnosis of her disorder.

Methods

The methods used have been previously described in detail\(^7\) and are briefly summarized.

Preparation of anti-kappa and anti-lambda chain antibodies. Anti-kappa and anti-lambda antibodies were isolated from goat antisera using the appropriate light chain immunoadsorbent. They were rendered monospecific for kappa and lambda chains by absorption with an immunoadsorbent of the opposite type, prepared by coupling Bence Jones proteins to glutaraldehyde-treated polyacrylamide beads.\(^7\)

Peroxidase-labeled antibodies. Purified antibodies were coupled to peroxidase with glutaraldehyde, using a two-step procedure.\(^3\)

Purification of peripheral blood lymphocytes. Lymphocytes were purified from heparinized peripheral blood by sedimentation for 1 hr at 37\(^\circ\)C. Cell preparations contained an average of 95\% lymphoid cells, and cell death, determined by trypan-blue exclusion, never exceeded 10\%.

Reaction of cells with enzyme labeled antibody. Samples of 5 x 10\(^6\) cells were incubated either at 4\(^\circ\)C or 37\(^\circ\)C with 0.5 ml of peroxidase-labeled anti-kappa or anti-lambda antibodies or with normal IgG for 2 hr. A concentration of 0.2 mg/ml of labeled antibody was used.

Light and electron microscopy. Aliquots of cells treated at 37\(^\circ\)C were fixed in 4\% formaldehyde in 0.1 M phosphate buffer, pH 7.4, and washed three times by centrifugation. The technique used for preparations subsequently examined by electron microscopy can be summarized as follows: peroxidase staining was performed with a solution of 3-3' diaminobenzidine and hydrogen peroxide for 10 min; cells were then washed, postfixed in osmium chloride, and embedded in Epon after dehydration.

The second procedure, allowing examination only by light microscopy, consisted in staining the cells with a solution of 3-amino-9-ethylcarbazole and hydrogen peroxide for 10 min. One drop of the cell suspension was mounted under a cover slip, and the percentage of labeled versus unlabeled cells was obtained after counting 500 cells.\(^6\)

Measurement of peroxidase activity. Peroxidase activity was determined by adding 2.9 ml of the o-dianizidine reagent to 0.1 ml aliquots containing 5 x 10\(^6\) cells. The reaction was stopped after 15 min with a drop of 6 N HCl. Tubes were then centrifuged at 5000 g for 5 min, and the absorbancy of the supernatant was read at 400 nm. Taking into account the number of cells and the activity found, the quantity of peroxidase fixed per 10\(^6\) cells was calculated by referring to a standard curve of absorbancy versus reaction time for known quantities of peroxidase. Since the mean ratio of peroxidase coupled to antibodies is one to one, the number of molecules of antibody bound per 10\(^6\) cells could be calculated, and the mean number of antigenic sites on the surface of each positive lymphocyte could be determined.

RESULTS

Peripheral blood lymphocytes from the five patients with atypical lymphocytic leukemia were examined. All patients had a certain number of clinical and cytologic features in common (Tables 1 and 2): (1) an active clinical course; (2) marked splenomegaly; (3) little or no lymph node enlargement, except in
Table 1

<table>
<thead>
<tr>
<th>Patients</th>
<th>Case No.</th>
<th>Blood Leukocyte Count</th>
<th>Lymphoid Cells (%)</th>
<th>Stained Cells (%)</th>
<th>No. of Molecules per Cell x 10^3</th>
</tr>
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<tbody>
<tr>
<td>Blastic crisis</td>
<td>1</td>
<td>510,000</td>
<td>100</td>
<td>100</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>87,000</td>
<td>96</td>
<td>90</td>
<td>40</td>
</tr>
<tr>
<td>Prolymphocytic leukemia</td>
<td>3</td>
<td>700,000</td>
<td>99</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>26,000</td>
<td>94</td>
<td>99</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>75,000</td>
<td>95</td>
<td>98</td>
<td>65</td>
</tr>
<tr>
<td>20 Chronic lymphocytic leukemia*</td>
<td>(17,900–450,000)</td>
<td>(81–99)</td>
<td>(26–80)</td>
<td>(3–21.4)</td>
<td></td>
</tr>
<tr>
<td>Normal subjects</td>
<td>5,300–7,000</td>
<td>(39–47)</td>
<td>19.4</td>
<td>93.7</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>(16.4–25.2)</td>
<td>(106)</td>
<td>(4–10)</td>
<td>(32.5–190)</td>
<td></td>
</tr>
</tbody>
</table>

*Eighteen anti-kappa cases, two anti-lambda cases.

one case; (4) severe anemia and thrombocytopenia; (5) a very high blood lymphocyte count (mean 279,000/cu mm, range 36,000–700,000/cu mm) with characteristic morphology on MGG smears: presence of mature lymphocytes and large cells with basophilic cytoplasm, coarsely reticular chromatin, and a large nucleolus (Fig. 1). These large cells appeared as undifferentiated lymphoid cells. No clear cut difference between PL and BC lymphocytes was found. Nevertheless, in cases of BC supervening in CLL, typical prolymphocytes as described by Galton were rarely seen. Undifferentiated cells were distinguished from prolymphocytes by a smaller nucleocytoplasmic ratio and by a coarse reticular chromatin. However, they did not resemble typical lymphoblasts of acute lymphoblastic leukemia. (6) When all these features were present, survival was short (mean 5 mo, range 2–16 mo) and the response to usually effective methods of treatment in CLL and lymphomas (chlorambucil, corticosteroids, splenic irradiation, extracorporeal irradiation, and MOPP) was very poor.

This clinical picture was very different from that seen in 13 previously studied cases of typical CLL and from that observed in 7 new patients. In 17 of these 20 patients, there was marked lymph node enlargement, while splenomegaly was present in only 9 patients. In seven, the spleen was palpable more than 6 cm below the costal margin. Four patients had a hemoglobin level below 10 g/100 ml, and five patients had thrombocytopenia (platelet counts below 100,000/cu mm). In six patients, a few lymphoid cells containing nucleoli were observed. These cells had an immature appearance and represented less than 10% of the total number of lymphocytes. Thirteen patients have survived with a follow-up of 6–84 mo after initial diagnosis. Seven patients have died (mean survival 56 mo, range 24–108 mo).

The spectrophotometric measurements and microscopic observations demonstrated that all of the atypical patients had lymphoid cells bearing a single type of light chain, four of the kappa type, and one of the lambda type (Table 1). The optical density obtained with the antibody to the opposite chain never ex-
<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Lymph Node Enlargement</th>
<th>Spleen (cm Below Costal Margin)</th>
<th>WBC/cu mm</th>
<th>Lymphoid Cells</th>
<th>Hemoglobin g/100 ml</th>
<th>Platelets x 10^3/cu mm</th>
<th>Treatment</th>
<th>Survival (mo)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(BC)</td>
<td>67</td>
<td>F</td>
<td>0</td>
<td>7</td>
<td>510,000</td>
<td>100 15</td>
<td>8</td>
<td>50</td>
<td>Chlorambucil, splenic irrad., ECIt</td>
<td>4</td>
</tr>
<tr>
<td>2(BC)</td>
<td>68</td>
<td>M</td>
<td>Small</td>
<td>8</td>
<td>87,000</td>
<td>96 40</td>
<td>8</td>
<td>40</td>
<td>Chlorambucil</td>
<td>4</td>
</tr>
<tr>
<td>3(PL)</td>
<td>53</td>
<td>M</td>
<td>0</td>
<td>12</td>
<td>70,000</td>
<td>99 50</td>
<td>7</td>
<td>50</td>
<td>Chlorambucil, MOPP</td>
<td>3</td>
</tr>
<tr>
<td>4(PL)</td>
<td>73</td>
<td>F</td>
<td>0</td>
<td>10</td>
<td>76,000</td>
<td>94 60</td>
<td>6</td>
<td>15</td>
<td>Chlorambucil, vincristine, prednisone</td>
<td>6</td>
</tr>
<tr>
<td>5(PL)</td>
<td>62</td>
<td>F</td>
<td>0</td>
<td>14</td>
<td>75,000</td>
<td>95 50</td>
<td>7.5</td>
<td>30</td>
<td>Splenic irrad.</td>
<td>2</td>
</tr>
<tr>
<td>Classical CLL</td>
<td>Mean M 11</td>
<td>17/20</td>
<td>&gt; 6</td>
<td>Mean 7/20</td>
<td>76,000</td>
<td>Mean &lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 100</td>
<td>Chlorambucil in 12, no treatment in 8</td>
<td>—</td>
</tr>
<tr>
<td>Classical CLL</td>
<td>64</td>
<td>F</td>
<td>9</td>
<td>7/20</td>
<td>76,000</td>
<td>87 6/20</td>
<td>4/20</td>
<td>5/20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*After diagnosis of BC or PL.
†Extracorporeal irradiation.
ceeded that obtained with the labeled normal IgG. In the two cases of BC, the lymphoid cells bore the kappa light chain. The percentage of positive cells was, respectively, 100%, and 90%, and the mean number of antigenic sites was 70,000 and 40,000 per cell. In two of the three PL cases, lymphoid cells bore kappa light chains, and in the other case lambda light chains. The calculated number of antigenic sites was, respectively, 75,000, 97,500, and 65,000 per cell, and 98%, 99%, of the cells were positive.

In patients 3 and 5, the first determination was performed before treatment within a month of the onset of presenting symptoms. In patient 3, determination was repeated on two occasions after treatment with similar results for all three determinations (before and after treatment). In patients 1, 2, and 4, quantitation was performed after treatment.

In all cases, mature lymphocytes and less differentiated lymphoid cells showed the same labeling pattern, especially during the blast crisis of CLL where the small mature lymphocytes were heavily stained in contrast to the small lymphocytes of typical CLL (Figs. 2 and 3).

The number of antigenic sites was significantly higher than that found in typical CLL (mean value 9600) and was consistent with the heavy labeling observed under the light microscope. The values were close to those obtained in normal subjects (mean value 90,000). They differed from normal cells, however, by the fact that the latter always showed a polyclonal pattern, and the percentage of labeled lymphocytes never exceeded 35% (16.4% - 25.2% positive for kappa chains and 4% - 10% for lambda chains).

Quantitation of antigenic sites on lymphocytes gave extremely homogeneous results in cytologically typical CLL: even then several of the following features were present: anemia (mean value 11,100 sites—four cases), thrombocytopenia (mean value 8600 sites—five cases), splenomegaly extending more than 6 cm below the costal margin (9300 sites—seven cases), a few immature cells in the blood (mean value 10,600 sites—six cases).
ATYPICAL LYMPHOCYTIC LEUKEMIA

Fig. 3. Section of small peroxidase-labeled lymphocyte of patient 3. Note the more marked dense chromatin zone and the absence of a nucleolus. × 11,170.

In typical CLL (20 cases), determinations were performed either before treatment (mean value 9200 sites—eight cases), after treatment only (mean value 9800 sites—ten cases), or both before and after treatment (two cases) with similar results. We also studied two patients with typical CLL in the course of their disease and during the terminal phase. The intervals between the two determinations were 12 and 24 mo, respectively. Similar results were recorded in both instances in these two patients.

DISCUSSION

The validity of the method used to quantitate antigenic sites has been previously discussed.7 Furthermore, the lymphocytes of patients 2, 3, and 4 were tested three times with similar results.

The main difference between the blast crisis of CLL and PL is the natural history of the disease. Whereas patients with PL initially exhibit the clinical and cytologic pattern described above, patients with BC show this pattern only 1 and 3 yr after the initial diagnosis of CLL.

Compared to typical cases of CLL, we found a marked increase in the number of antigenic sites on the surface of lymphoid cells from both patients with BC and, from the three cases of PL studied. The number of patients studied was too small to draw any conclusions concerning the incidence of such modifications in these disorders. Eskeland2 reported similar findings in an atypical case of CLL. Galton et al.6 in a study of leukemia cell surface markers in PL, found three patients out of four to have B-cell markers, while the remaining patient had T-cell markers. Brouet et al.9 also reported two cases (out of ten studied) of PL in which the cell membrane markers were of the T variety.

Although in two out of our three cases of PL quantitation was performed a very short time after initial diagnosis, and even though the results did not vary during the course of the disease, available data were insufficient to determine whether increased levels were present at the onset of the disorder. Furthermore, it is difficult at present to establish the relationship between blast crisis supervening in CLL and in PL.
The fact that the diagnosis of CLL was established prior to the blast crisis and that MGG smears were reviewed for diagnosis rules out the possibility that BC was in fact PL seen 1 and 3 yr after the onset of the disease.

No explanation can be proposed in terms of lymphoid cell maturation. Nevertheless, we observed that in all cases of lymphoid leukemia with large quantities of SmIg the course and prognosis were poor. It appears therefore, that quantitation of SmIg could be a prognostic factor in CLL.

Do small and large lymphoid cells bear the same quantities of SmIg? Electron microscopic examination after labeling gives the same results, and only quantitation after separation of these two populations can provide an answer to this question (Figs. 2 and 3). The uniformity of these results as compared to typical CLL would tend to indicate that quantitation of antigenic sites might be an additional and useful criterion for classifying a form of leukemia different from CLL and acute lymphocytic leukemia. Cases of ALL with lymphoblasts bearing immunoglobulins are rare, and it would be of interest to examine quantitatively such cases.

The fact that both mature lymphocytes and undifferentiated lymphoid cells were labeled in all cases by the same light chain may be an argument for the clonal origin of the population, although a definite conclusion cannot be reached since other markers were not studied.

Marked splenomegaly with little or no lymph node enlargement raises the problem of its significance and the possible advantage of splenectomy in these disorders with a severe course.

ACKNOWLEDGMENT

We wish to acknowledge the clinical assistance of Dr. Dominique Charron and Dr. Elizabeth Bouvet and the technical assistance of Françoise de Montaut.

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Comparison of normal and chronic lymphocytic leukemia lymphocyte surface Ig determinants using peroxidase-labeled antibodies. II. quantification of light chain determinants in atypical lymphocytic leukemia

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