Decrease and Altered Distribution of Human T Antigen on Chronic Lymphatic Leukemia Cells of T Type, Suggesting a Clonal Origin

By E. Thiel, H. Rodt, D. Huhn, and S. Thierfelder

B- and T-cell markers were studied in a patient with chronic lymphocytic leukemia and erythroderma. The absence of immunoglobulin, complement receptor, and Fc receptor, and the presence of sheep erythrocyte receptor and T-cell antigen on the membrane of the leukemic cells classified them as thymus derived. Using quantitative microphotometric immunonuclear autoradiography, surface antigen densities were measured at the cellular level with the following results: (1) The density of T-antigenic sites was less on leukemic cells compared to normal T lymphocytes. (2) The T-antigen densities of leukemic lymphocytes varied less from cell to cell forming a homogeneous peak in histograms. (3) An Ig density of normal B lymphocytes was demonstrated on the residual T-antigen-negative cells. The results were qualitatively confirmed by direct immunofluorescence and electron microscopy with peroxidase-labeled antibodies. Furthermore, the surface antigens were quantitatively measured at the cell population level by a quantitative microcomplement fixation test which revealed reduced binding of anti-T-cell antibodies and complement, and no antiglobulin fixation on the leukemic lymphocytes. Since lymphocytes with normal T-antigen concentration could not be found among the leukemic T lymphocytes, a lack of normal T cells was assumed. The findings that there was a decrease and altered distribution of surface markers on chronic lymphatic leukemia cells of the B- and T-cell type are discussed as further arguments referring to their clonal origin.

Although there has been a multitude of studies dealing with B- and T-cell markers in lymphoid proliferations, reports on chronic lymphatic leukemias with a thymus-derived cell surface nature remain extremely rare. First references to the possible existence of thymus-derived chronic lymphatic leukemias (T-CLL) have been based on the absence of any detectable surface immunoglobulin. Since no direct proof of T-cell markers has been obtained in these studies, the nature of these leukemias remains dubious. Reports of few if any immunoglobulins on otherwise proven B leukemia cells make their nature completely uncertain. Later reports of T-CLLs deal with the existence of T-cell markers in the absence of surface immunoglobulin. A reliable though unexplained marker for human T lymphocytes is provided by rosette formation with sheep erythrocytes or, more important, by the cytolysis by antihuman thymocyte sera. It should be mentioned, however, that most heteroantisera “specific” for human T cells have been, until now, only relatively specific and useful only in cytotoxicity tests. Using a highly specific antihuman T-cell globulin, we have measured autoradiographically the percentage of T-antigen-bearing cells and the cellular T-antigen density on normal lympho-

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cytes and on the leukemia cells of a T-CLL. Other cell surface markers (Slg, E rosettes, HuEAC rosettes, FL-Agg) have also been investigated. The determination of the concentration of T-cell antigens by photometric quantitative immunoautoradiography has revealed characteristic differences of the T-cell antigen densities on normal and leukemic lymphocytes.

MATERIALS AND METHODS

Case Report

A 77-yr-old man was transferred to the I Medical Clinic, University of Munich, in October 1974. His anamnestic data was: erythroderma and chronic bronchitis for several years; a 6-kg weight loss, severe pruritus, high number of blood lymphocytes (60,000/cu mm), and treatment with prednisone (10 mg daily) for the past 3 mo. On admission, he suffered from bronchopneumonia and severe pruritus. Lymph nodes and spleen were enlarged moderately. He had a leukocytosis of 15,000 25,000/cu mm (>90% lymphocytes), a thrombocytopenia of 40,000 50,000/cu mm, and a moderate anemia of 10 g/100 ml hemoglobin without signs of hemolysis. Histologic bone marrow investigation revealed a dense infiltration with lymphoid cells displacing normal blood cell precursors. A skin biopsy was compatible with the diagnosis of leukemic infiltration. Immunoglobulins of the blood were within normal levels; no traces of a monoclonal Ig were found in serum or urine. The pulmonary infection improved under tetracycline therapy, and the patient was discharged without specific therapy. Because of a deterioration of his general condition, along with pruritus and pneumonia, he was readmitted 3 mo later. The number of lymphatic cells had remained at 20,000/cu mm, but normal blood cells and immunoglobulins had permanently declined. Cytostatic agents, prednisone, and irradiation of the skin were applied, without success, to mitigate the tormenting pruritus. In spite of intensive therapy with antibiotics and substitution of immunoglobulins, the patient died 2 mo later from septicemia after increase of pulmonary and skin infections. Autopsy was refused by the next of kin.

Cytochemistry

The following cytochemical reactions were used: acid phosphatase, periodic acid-Schiff (PAS), naphthol-AS-acetate-esterase (NAS) and suppression by sodium fluoride.

Rosette Formation (E and HuEAC)

After separation of mononucleated cells by Ficoll-Isopaque gradient centrifugation according to Böyum, assays for lymphocytes that formed spontaneous rosettes with sheep erythrocytes (SRBC) or with sensitized human erythrocytes (HuEAC) were performed as described by Jondal et al. Mouse complement was used for sensitizing. Monocytes were differentiated by phagocytosis of latex particles added on incubation after gradient centrifugation.

Immunofluorescence (Surface Ig and Aggregated Ig Binding)

Surface membrane bound immunoglobulin (Slg) was studied by a direct immunofluorescence method according to Pernis et al. using a polyclonal rabbit anti-human Ig serum (anti-IgM, anti-IgG, anti-IgD) conjugated with fluorescein isothiocyanate (FITC), the F/P ratio being 2.0. A heat aggregated FITC-labeled IgG (FL-Agg) was used as described. At least 500 cells were scored using a Leitz Ortholux microscope with epi-illumination.

Quantitative Microcomplement Fixation Test

A microcomplement fixation test adapted to human lymphocytes as antigens was performed as described. In principle, the amount of complement fixed by lymphocytic antibodies was measured by per cent hemolysis of sensitized sheep erythrocytes.

Radiolabeling of Antisera

A highly specific anti-human T-cell globulin (ATCG), prepared by immunization of rabbits with human thymocytes and absorbed on a pool of CLL cells and different human B-cell lines, was
radioiodinated by the chloramine-T method with a specific activity of 2 mCi/mg as described. A purified anti-Ig antibody, prepared as described, was also iodinated with a specific activity of 0.5 mCi/mg.

**Quantitative Immunol autoradiography**

Cell labeling, autoradiography, and microphotometry of immunol autoradiographs were performed as described. For absolute quantitation, radioiodinated human erythrocytes were exposed simultaneously on the same slide as a reference. Microphotometry of grain densities on single cells was performed using the MPF 2 (Leitz, Germany). By virtue of the linear relationship between the number of silver grains measured and the amount of light recorded, the photometer readings could be taken as equivalent to the number of grains. Determination of the real labeling index and conversion of photometric data into radioactivity was conducted as described. The data were statistically analyzed and plotted in histograms using a data processing system (Interdata, U.S.A.).

**Electron Microscopy**

Peripheral blood cells and skin biopsies were investigated by electron microscopy after fixation with glutaraldehyde, embedding in Maraglas and staining with lead hydroxide and uranyl acetate.

**Electron Microscopic Immunohistochemistry**

Indirect labelings of peripheral lymphocytes were performed using peroxidase-conjugated sheep anti-rabbit IgG globulin after preincubation with ATCG as described. Preincubation with normal rabbit globulin served as a control. Membrane-bound immunoglobulin was demonstrated by direct labeling with peroxidase-conjugated anti-human Ig globulin of the rabbit. At least 400 lymphocytes of each trial were differentiated, and their fine structure was evaluated.

**RESULTS**

**Cytochemistry**

All lymphocytes of patient K.P. showed a moderate to strong acid phosphatase activity. The reaction product appeared granular and was located paranuclearly. Granular deposits of a PAS-positive material were demonstrated in 77% of the peripheral lymphocytes. Activity of NAS was weak and was not suppressed by sodium fluoride.

**Determination of Surface Immunoglobulins (Slg), F, Receptor (FL-Agg), and Complement Receptor (HuEAC)**

It was not possible to demonstrate any Slg on the large majority of the patient's lymphocytes. Direct immunofluorescence and autoradiographic methods applying high anti-Ig antibody concentrations as well as indirect methods using peroxidase- or FITC-labeled sheep anti-rabbit globulins failed after preincubation with our own or commercial (Behring, Germany) antoglobulin antisera (anti-κ, -λ, -μ, -δ, -γ, -α). Only 2.5% of the lymphocytes carried readily demonstrable immunoglobulins of a polyclonal origin and bound aggregated Ig (FL-Agg). The amount of purified anti-Ig antibodies bound on these cells was comparable to that of normal B lymphocytes (see Table 1).

Only 0.5%–1% of the patient's lymphocytes formed HuEAC rosettes. Normal values of HuEAC-forming rosettes (CRL) in our laboratory are between 10% and 15% lymphocytes.
Table 1. T- and B-Cell Marker Densities on Normal and Leukemic Lymphocytes Evaluated by Photometric Quantitative Immunomunautoradiography at the Cellular Level

<table>
<thead>
<tr>
<th></th>
<th>Anti-Ig Antibody (125I)</th>
<th>Anti-T Globulin (125I)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lympho Labeled No. of Molecules per Cell x 10^5 (Mean ± SD)</td>
<td>Slg Staining Labeled Mean Label Density ± SD (%)</td>
</tr>
<tr>
<td>Normal subjets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7,800</td>
<td>33 10.5 328 ± 164</td>
</tr>
<tr>
<td>2</td>
<td>6,900</td>
<td>29 8 264 ± 217</td>
</tr>
<tr>
<td>3</td>
<td>8,100</td>
<td>34 13.3 240 ± 157</td>
</tr>
<tr>
<td>4</td>
<td>7,400</td>
<td>33 7.6 289 ± 164</td>
</tr>
<tr>
<td>5</td>
<td>5,900</td>
<td>39 12 292 ± 235</td>
</tr>
<tr>
<td>CLL &amp; type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>80,000</td>
<td>97 88.5 83 ± 58</td>
</tr>
<tr>
<td>2</td>
<td>240,000</td>
<td>99 76 75 ± 24</td>
</tr>
<tr>
<td>3</td>
<td>65,000</td>
<td>88 85 750 ± 128</td>
</tr>
<tr>
<td>CLL T-type (K.P.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>18,000</td>
<td>93 2.5 245 ± 205</td>
</tr>
</tbody>
</table>

Spontaneous Rosette Formation

During the patient’s hospitalization, E-rosette binding tests were done several times. A considerable fluctuation of the percentage of rosette-forming lymphocytes was observed (Table 2), whereas the values of the same normal test person varied only to a limited degree. Rosettes formed by the patient’s lymphocytes appeared less densely loaded than those of the test person.

Microcomplement Fixation Tests

Quantitative binding of complement by anti-T and anti-Ig antibodies reacting with the T leukemia lymphocytes, with normal blood lymphocytes, with thymus cells and with lymphocytes from a typical B-CLL, is shown in Fig. 1. The results of a direct demonstration of Ig-bearing lymphocytes showing only 2.5% of cells with Slg are in good agreement with the low amount of complement bound by anti-Ig antibodies when incubated with the patient’s cells. The complement fixed by anti-T antibodies incubated with the patient’s lymphocytes was below that of normal persons but markedly above that of the B-CLL.

Quantitative Immunomunautoradiography (qulA)

Saturating conditions for anti-human T globulin (ATCG) were established in the following manner: equal cell numbers of the same donor were incubated with increasing dilutions of labeled antiserum. The photometric data reflected the label density on the lymphocytes of a normal person and of the chronic

Table 2. Percentage of Rosette-forming Lymphocytes of the T-Cell Leukemic Patient and a Normal Test Person on Different Occasions

<table>
<thead>
<tr>
<th>Date</th>
<th>Patient K.P.</th>
<th>Test Person</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-31-74</td>
<td>29.0</td>
<td>54</td>
</tr>
<tr>
<td>11-5-74</td>
<td>24.0</td>
<td>49</td>
</tr>
<tr>
<td>11-13-74</td>
<td>71.5</td>
<td>55</td>
</tr>
<tr>
<td>11-20-74</td>
<td>57.5</td>
<td>57</td>
</tr>
<tr>
<td>11-28-74</td>
<td>69.0</td>
<td>—</td>
</tr>
<tr>
<td>1-29-75</td>
<td>39.5</td>
<td>58</td>
</tr>
<tr>
<td>2-4-75</td>
<td>52.0</td>
<td>51</td>
</tr>
</tbody>
</table>
leukemic patient K.P. in relation to the ATCG concentration used for incubation shown in Fig. 2. Under these conditions all surface T antigens were saturated by ATCG at a concentration of 155 µg/ml. More than 90% of the lymphocytes of five B-CLL cases bound no ATCG at this concentration.

The labeling index (96.5%) of the T leukemia cells (see Fig. 2, values written over the abscissa) reached its plateau one titer below the saturating concentration.
The number of antiglobulin antibodies bound on single cells as a function of the antibody concentration was also measured using labeled standard cells as a reference source as described. The photometrically evaluated data of five normal persons, three CLL with Ig-bearing lymphocytes and the T-CLL are shown in Table 1. The label density of ATCG on the remaining T cells (4%–11%) of the three CLL corresponds to that found on T cells of normal persons. This finding may indicate a normal T-cell population in patients with chronic lymphatic leukemia of the B-cell type. Conversely, lymphocytes with normal values of anti-Ig antibodies bound per cell could be found in the patient with T-cell leukemia.

Normal Ig densities on B cells were also present in the three CLL cases. These cells were not recorded because they were far below 1%. In two cases of CLL, the labeling density of anti-Ig antibodies on the chronic lymphatic leukemia cells was markedly below that on B cells from normal persons. The anti-T labeling density on T leukemia cells was also reduced at a concentration of 34% less than that found in the five normal donors indicated in Table 2. This difference was not due to cell size since comparable cell diameters of T leukemia cells and normal T lymphocytes were measured.

The standard deviations of the label density on leukemic lymphocytes were below that of normal lymphocytes after incubation with radioiodinated anti-Ig antibodies. Also, the CLL with high label density showed a lower variability in labeling densities when compared to B cells of normal donors. Correspondingly, the T-antigen concentration on the leukemic T cells varied less than that on normal T lymphocytes. This difference is demonstrated in Fig. 3. The histograms of T leukemia cells and normal T lymphocytes are plotted by a data processing unit according to their label density. Its uniform distribution on leukemia cells is in contrast to the widely scattered histogram of normal T cells. A similar difference could be shown between Ig-bearing lymphatic leukemia
Fig. 3. Histograms of label density on normal T lymphocytes (A) and leukemia cells (B) after incubation with radiolabeled anti-I globulin at saturating conditions. The values of 500 cells are plotted using a data processing unit. The peaks on the left represent the fraction of unlabeled cells.

cells and normal B cells. This more homogeneous label density may serve as a further argument in favor of the monoclonal origin of CLL.

Electron Microscopy

The fine structure of the patient's blood lymphocytes did not differ from lymphocytes in other cases of typical chronic lymphocytic leukemia (Fig. 4). There were areas of condensed chromatin along the nuclear membrane. The nucleoli were prominent. The cytoplasm contained deposits of glycogen; mitochondria were numerous and large; a few short strands of rough-surfaced endoplasmic reticulum were observed. Lymphatic cells in a focal array were located
in the corium from a biopsy of the skin. These cells sometimes resembled blood lymphocytes, while sometimes they were like lymphoblasts.

Electron Microscopic Immunohistochemistry (EMI)

T-cell antigen was demonstrated on 77%, of the lymphatic cells in the blood (Fig. 4). The peroxidase reaction product covered parts of the cell surface and infoldings of the cell membrane (Fig. 5). Near the peroxidase-positive deposits, the cytoplasm sometimes exhibited a finely fibrous structure; 2.3%, of the lymphocytes demonstrated membrane-bound immunoglobulins after incubation
with anti-Ig globulin; 1.5% of the controls showed weak deposits of peroxidase-positive material after preincubation with normal rabbit globulin.

DISCUSSION

Convincing evidence has been obtained in recent years that chronic lymphatic leukemia is a malignancy of B lymphocytes. Membrane-bound immunoglobulin, complement (C3) receptor, Fc receptor, and absence of SRBC receptor and thymus surface antigen have provided evidence for the B nature of the neoplastic cells. The restriction of the Ig to a single light-chain class and its idiotypic uniformity make the clonal nature of this type of leu-
leukemia evident. This concept has also recently been confirmed in leukemias where IgM and IgD are demonstrated on the same cell by similar idio
typic specificity for the membrane-bound IgM and IgD. The observation that Ig density on single cells varies less in CLL than on normal B cells, when measured by quantitative immunoautoradiography, may be used as a further argument for the clonal nature of CLL.

The application of quantitative immunoautoradiography (qulA) to the expression of surface antigens on single cells resulted in two observations in the case of a T-CLL: (1) the density of T-antigenic sites per cell was lower on neo
tropic cells when compared to normal T cells; (2) the cellular distribution pattern of T antigens was more uniform in CLL (Fig. 3). These observations comply with our findings of Ig expression on B lymphatic leukemia cells where the cellular Ig density was generally decreased and varied less among the neo
tropic cell population. Though leukemias with high Ig densities do occur, the variation of cellular Ig expression remained at a lower level (Table 1).

The decrease in the content of T antigens on the lymphatic leukemia cells was also measured by a quantitative complement fixation test recently described by us. This test revealed a high T-cell antigen concentration on thymus cells and a minor degree of complement binding by a typical B-CLL after incubation with ATCG. Peripheral lymphocytes of a normal person incubated with ATCG fixed more complement than lymphocytes of the T-CLL (Fig. 1). The opposite result was found with anti-Ig globulin: no binding of complement on thymus and T leukemia cells in contrast to marked complement fixation by B-CLL and normal peripheral lymphocytes.

The decrease of T antigenic sites on the T leukemia cells may explain the lower value of 77% labeled cells in electron microscopy immunohistochemistry (EMI) compared to 96.5% in immunoautoradiography, since the probability of no antigenic site lying in the section plane increased in the former case. The T-cell labeling indices of 82% of normal peripheral lymphocytes in EMI corre
spond well to the 83% found with qulA (average of ten normal persons). The demonstration of Ig-bearing cells revealed the same result (2.3% or 2.5%) in both test systems. The qulA showed that the Ig density on these cells was the same as that on B lymphocytes from normal persons suggesting a residual population of normal B lymphocytes diluted by the neoplastic T cells. They gave almost normal values for the B lymphocytes when converted into absolute numbers. This finding may explain the normal serum levels of immunoglobulins at the onset of our observations. Unfortunately, subsequent evaluations in the preterminal stage were not performed when the serum level of Ig had fallen markedly.

As shown in Table 1, a low percentage of lymphocytes with roughly normal T-antigen densities was also found among the B-type leukemic lymphocytes. This observation is in accordance with recent findings demonstrating the presence of a normally reactive T-cell population in patients with CLL. In addition to this normal T-cell pool, we also found some lymphocytes with normal Ig densities among the prevailing CLL cells carrying a low amount of surface Ig. The label distribution pattern of leukemic cells formed a uniform peak on histograms of cellular label density, whereas the former cells were found out-
side this peak (Thiel, unpublished observations). The findings indicate that at least three kinds of lymphoid cell populations can be found in CLL: (1) the neoplastic lymphocytes frequently carrying a lower surface antigen concentration\textsuperscript{28,39} and a reduced variation of antigen density from cell to cell;\textsuperscript{28} (2) normal T lymphocytes diluted by leukemic cells in average\textsuperscript{37} or even increased\textsuperscript{38} absolute cell numbers; (3) normal B lymphocytes.

In the case of B-CLLs we found evidence of all three cell populations, even though relatively low numbers of B cells with normal Ig density were scored. In the T-CLL, the compartment of normal B cells could be easily demonstrated, whereas a pool of T lymphocytes carrying normal T-antigen densities was not found. When the data of various autoradiographic investigations were plotted by a data processing unit, all histograms showed the same peak and no cells with the higher label density of normal T lymphocytes (Fig. 3). Correspondingly, no thymidine was incorporated by the patient's lymphocytes after stimulation with concanavalin A. The reduced response to phytohemagglutinin and only moderately depressed stimulation by pokeweed mitogen will be referred to in detail elsewhere. A weak and delayed response to phytohemagglutinin (PHA) was also found in three T-CLL cases,\textsuperscript{8,11} whereas normal PHA responsiveness was observed in one case.\textsuperscript{3} Our results in the T-CLL can be explained by a lack of normal T cells.

The spontaneous rosette-forming ability of the neoplastic T cells fluctuated markedly when tested on different occasions (Table 2). It should be emphasized that high or normal rosette values were scored (normal range in our laboratory 50\%-60\%) on occasions (November 28, 1974 and February 4, 1975) when low T-antigen expression per single cell was measured. Since the number of indicator cells bound to the leukemia cells was found to be smaller, the SRBC binding affinity may have been reduced, causing these varying results. With the low rosette values at the beginning of our observations, the leukemia's T cell nature would have easily been overlooked. The great advantage of a specific antihuman T-cell globulin need not be emphasized. The titration to a plateau (Fig. 2) at the cellular level confirmed the findings in other test systems\textsuperscript{19} and complied with recently defined serologic criteria.\textsuperscript{40} Further proof of specificity on mixed and pure T- and B-cell populations demonstrating reciprocity or coincidence with other markers was provided successfully in other test systems.\textsuperscript{19} Additional proofs of specificity such as double-label experiments are currently under study. Preliminary results with peroxidase-labeled antiglobulin reagents and radioiodinated ATCG demonstrated that ATCG did not label immunoglobulin-bearing cells. Likewise, aggregated Ig-binding cells were not stained by ATCG except for about 1\% of cells with both B- and T-cell markers.

The clinical importance of T-marker demonstration was recently shown in childhood acute leukemia.\textsuperscript{41} The presence or absence of a T-cell marker (sheep erythrocyte receptor) permitted a distinction between two forms of acute lymphatic leukemia, each with a distinct distribution in terms of age and sex as well as other characteristic clinical features. In comparison, clinical T-CLL characteristics are still difficult to define because of the rarity of the disease. Also, only a few clinical data have been reported so far. Interestingly, three cases—including our own—of the 12 patients with T-CLL reported so far
suffered from erythroderma with pruritus. This symptom makes it necessary to draw a line between the T-CLL and the Sézary syndrome, a disease with erythroderma and proliferation of atypical T lymphocytes. Our T-CLL case differs from the Sézary syndrome by the absence of the cerebriform aspect of the nuclear profile, typical for the large Sézary cell, and the lack of a grooved pattern of the nucleus, a feature of the small cell variant of the Sézary cell. Furthermore, the marked infiltration of the bone marrow is in contrast to the Sézary syndrome where the degree of marrow involvement is remarkably limited. A further peculiarity of T-CLL appears to be the relatively moderate enlargement of lymphatic tissues associated with a marked bone marrow infiltration.

A better insight into lymphoid proliferation may be expected with the help of the methods described. Quantitative immunoautoradiography should help to decide whether a lymphoid proliferation is caused by a tumor cell arising from a malignant clone, or if it is the product of chronic immunologic stimulation. This question has not been solved in diseases such as the Sézary syndrome until now.

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