Clonal Origin of a T Cell Lymphoproliferative Malignancy

By Pierre A. Stryckmans, Louise Debusscher, Christiane Heyder-Brückner, Rodolph Heimann, Israel M. Mandelbaum, and Joseph Wybran

A woman with a T cell lymphoproliferative malignancy and heterozygosity at the X chromosome-linked locus for glucose-6-phosphate dehydrogenase (G-6-PD) isoenzymes was studied to find the clonal origin of her circulating neoplastic T cells. The red blood cells, polymorphonuclear cells, whole mononuclear cells, and T cell-depleted mononuclear cells contained both A and B isoenzymes of G-6-PD. In contrast, the tumor cells, separated by using their capacity to form rosettes with sheep red blood cells, contained only the B isoenzyme of G-6-PD. This observation strongly suggests the monoclonality of this T cell malignancy.

The clonal origin of B lymphocyte-derived malignancies can be established readily. Idiotype studies on B lymphocyte-derived malignancies indicate that the neoplastic cells synthesize immunoglobulins with a single VH region, a single VL region, and a single light chain of either κ or λ type, attesting to their clonal origin.

In the last three years the malignant cells of several types of human leukaemia and lymphomas were found to have characteristics of thymus-derived cells. In two reported series of childhood acute lymphoblastic leukemia 25% and 64% of the cases were considered to be T cell derived. Generally 1% of chronic lymphocytic leukemias (CLL) in adults are considered to be T cell derived. In T cell-type CLL a clonal origin of the disease has been suggested. However, at present a clonal origin cannot be established in T lymphocyte-derived malignancies by using cell surface marker methods as easily as in B lymphocyte-derived malignancies.

Linder and Gartler in 1965 introduced the use of an enzymatic marker for investigation of the clonal origin of a tumor. This approach is applicable to patients heterozygous for the X chromosome-linked locus of the glucose-6-phosphate dehydrogenase (G-6-PD) isoenzymes A (Gd-A) and B (Gd-B). Assuming Lyon’s hypothesis of the inactivation of one X chromosome in the female is correct, then heterozygous patients for G-6-PD possess in each tissue cells with phenotype Gd-A and cells with phenotype Gd-B. The presence of only one isoenzyme in the neoplastic cells of such patients strongly supports
the clonal origin of the neoplasm. Fialkow et al. showed by this method the clonal origin of chronic myeloid leukemia. Later, the same method made it possible to extend the concept of monoclonality to polycythemia vera and to many solid tumors. Practically, however, this method is applicable only if the patient is a black female, since these women are the only known heterozytes, and if the sample of tumor analyzed is free of contaminating normal cells. These conditions, fulfilled in a patient with a T cell lymphoproliferative malignancy, made it possible to show the monoclonality of this neoplasm.

CASE REPORT

The patient, a 34-yr-old black female from Guyana, was well until September 1975, when a chest x-ray showed diffuse interstitial infiltrates in both lungs. In January 1976 she was referred to the Institut Jules Bordet for evaluation of dyspnea and hepatomegaly. An open lung biopsy showed Pneumocystis carinii pneumonitis. The liver biopsy showed diffuse infiltration by cleaved lymphoid cells. The white blood cell (WBC) count was $34.2 \times 10^9$/liter with $80^\circ$ atypical mononuclear cells, many with cloverleaf-shaped nuclei. All atypical cells formed rosettes in the presence of sheep red blood cells (SRBC). The bone marrow was normocellular and contained $25^\circ$ atypical cells similar to those found in the peripheral blood. The following tentative diagnosis was made: non-Hodgkin lymphoma, T cell type, stage IV. Classification by the usual pathologic systems was not possible.

The pneumocystis pneumonitis was treated with a sulfamethoxazole-trimethoprim combination. Rapid resolution of both clinical and roentgenologic signs of infection occurred. However, by March 1976 the WBC count had progressively increased to $100 \times 10^9$/liter with concomitant increase in serum calcium to 16 mg/dl. Bone lesions were not found by radiographic or isotopic examination. Two cycles of a standard combination of cyclophosphamide, vincristine, prednisone, and procarbazine were administered, but only partial remission was obtained. In May 1976 high-dose corticosteroid therapy and $^{60}$Co irradiation (1500 R to the liver) were administered. The WBC count and differential returned to near normal but the hypercalcemia persisted. In June 1976 the patient died from the complications of hypercalcemia resistant to all forms of therapy. Autopsy showed involvement by neoplastic cells of most lymph nodes of the liver (2390 g), the spleen (390 g), and the bone marrow.

MATERIALS AND METHODS

Characterization of the peripheral blood tumor cells. The neoplastic cells in the peripheral blood were examined by light and electron microscopy. For light microscopy May-Grünwald-Giemsa stain was used. For electron microscopy aliquots of cell suspensions were fixed for 10 min at 22°C in 2.5% glutaraldehyde buffered with 0.2 M cacodylate pH 7.4 and were postfixed with 2%, osmium tetroxide buffered with 0.1 M cacodylate pH 7.4. The pelleted cells were embedded in Epon 812. Sections were stained with lead citrate. Other aliquots were filtered on silver membranes, dehydrated in alcohol and acetone, and processed in the Sorvall critical point drying system according to a method described in detail elsewhere. After being coated with gold, the preparations were examined in a Jeol 35 scanning electron microscope (SEM).

Total and active T cells were determined by E-rosette assays described elsewhere. EAC-rosette assays were also performed. The percentage of surface immunoglobulin-bearing cells was determined using a polyclonal anti-human globulin antiserum according to the method described by Pernis et al. Isolation and preparation of the different cell populations of the blood. Heparinized blood was centrifuged at 500 g for 10 min and the buffy coat was removed. The leukocyte-poor red blood cells (RBC) were then washed three times in isotonic saline and lysed in a hypotonic solution containing mercaptoethanol ($7.1 \times 10^{-3}$ M, Fluka), nicotinamide adenine dinucleotide phosphate ($1 \times 10^{-5}$ M, Boehringer), and EDTA ($2.7 \times 10^{-4}$ M, Merck). The supernatant obtained by centrifugation (10 min, 800 g) was used for the determination of G-6-PD enzymatic activity and isoenzymes.
After sedimentation of whole blood, the RBC-depleted WBC were layered on top of Ficoll-Hypaque (density 1.077 g/ml) and centrifuged. The mononuclear cells were then collected from the interface and the polymorphonuclear cells (PMN) from the pellet. The T cells were isolated using a modification of the method previously described by Wybran et al. Briefly, mononuclear cells suspended in 0.25 ml RPMI-1640 medium were added to 0.25 ml fetal calf serum and 0.25 ml of 50% sheep red blood cells (SRBC) suspension in normal saline. They were centrifuged for 5 min at 400 g, left for 1 hr at room temperature, and then gently resuspended in 5 ml Hanks' balanced saline solution. The cells were thereafter layered over Ficoll-Hypaque and centrifuged for 30 min at 400 g. The cells in the interface and in the pellet were collected separately and washed. The percentage of T cells in the T cell rich pellet and in the T cell-poor interface were determined by an E-rosette assay.

Leukolysates were obtained by freezing and thawing the cells in acetone-dry ice. The cellular debris were removed by centrifugation at 10,000 g, and the supernatant was tested for G-6-PD enzymatic activity and isoenzymes. G-6-PD activity and isoenzymes. G-6-PD activity was determined by the method of Beutler. The enzymatic activity was expressed as μM/min/g hemoglobin for RBC and μM/min/g protein for WBC. Isoenzymes were studied by the method of Sparkes et al. Briefly, aliquots of supernatant were electrophoresed on cellulose acetate gels for 75 min at 300 V. The gels were stained by a dye precipitation method and photographed.

RESULTS

Morphologic studies. Light microscopy of the peripheral blood smear revealed some of the cells to have classical lymphocyte morphology—round nuclei with condensed, darkly staining chromatin and scant to moderate amounts of cytoplasm. However, most cells had complex “cloverleaf” nuclei (Fig. 1) and moderate amounts of cytoplasm. Electron microscopy of these cells (Fig. 2) showed heavily condensed peripheral chromatin, poorly developed Golgi apparatus, sparse rough endoplasmic reticulum, abundant mitochondria, and absence of cytoplasmic granules. These findings are consistent with a cell of lymphoid origin. A scanning electron micrograph of the cells is shown in Fig. 3.

Immunologic studies. B and T cell identification of peripheral blood mononuclear cells showed 7% of cells to have membrane-bound Ig, 88% of cells to form E rosettes, 18% of cells to form active E rosettes, and 10% of cells to form EAC rosettes (Table 1). The percentage of T cells in the peripheral blood and in the T cell-enriched preparation is also shown in Table 1. All morphologically abnormal cells formed E rosettes.

Enzymatic studies. The G-6-PD activity in the different blood cell populations is shown in Table 2. The electrophoresis of G-6-PD in the various cell populations before treatment is shown in Fig. 4; it shows approximately equal staining of the two isoenzymes (A and B) in the RBC, in the isolated peripheral blood mononuclear cells, in the polymononuclear cells, and in the T cell–poor lymphocyte population. In contrast, heavy staining of Gd-B and only traces of Gd-A can be seen in the T cell–rich population. After treatment of the patient, equal staining of both isoenzymes was seen in all cellular populations, including the T cell–rich population. (Unfortunately, this electrophoresis was lost and cannot be presented.) The G-6-PD activity of the SRBC was low. Moreover, its electrophoretic migration was considerably different from that of human G-6-PD. Therefore sheep G-6-PD certainly did not interfere with the present study.
DISCUSSION

The lymphoid nature of the circulating tumor cells in this patient was shown by morphologic studies with light and electron microscopy. These cells were identified as T cells by E-rosette assay. When the patient's peripheral WBC count reached $95 \times 10^9$/liter, $88\%$ of the cells ($83 \times 10^9$/liter) formed E rosettes.

The classification of this disease was extremely difficult. Three pathologists,* experts in the field of lymphoma, reviewed the clinical and autopsy material. They agreed on a diagnosis of non-Hodgkin lymphoma involving lymph nodes, bone marrow, blood, and liver. However, the presence of $25\%$ malignant lym-

*Professor K. Lennert, Pathologisches Institut der Universität, Kiel, West Germany; Dr. H. Noel, Université Catholique de Louvain, Belgium; Professor J. A. M. van Unnik, Rijksuniversiteit Utrecht, The Netherlands.
Fig. 2. Transmission electron micrograph of representative atypical lymphoid cell characterized by deeply indented nucleus. × 18,000.

Fig. 3. Scanning electron micrograph of lymphoid cells in peripheral blood. Some cells are provided with large lamellipodes and microvilli. × 4400.
Table 1. Cell Identification

<table>
<thead>
<tr>
<th>Date</th>
<th>Treatment</th>
<th>WBC (x 10^9/liter)</th>
<th>Lymphoid Cells in the Blood (%)</th>
<th>T Lymphoid Cells (%)</th>
<th>Ig-bearing Lymphoid Cells (%)</th>
<th>T Cells in Isolated T Cell-rich Population (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/17/76</td>
<td>Before</td>
<td>95</td>
<td>64</td>
<td>88</td>
<td>7</td>
<td>92</td>
</tr>
<tr>
<td>5/24/76</td>
<td>After</td>
<td>3.7</td>
<td>21</td>
<td>70</td>
<td>—</td>
<td>89</td>
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Table 2. G-6-PD Activity

<table>
<thead>
<tr>
<th>Blood Cell Subpopulation</th>
<th>G-6-PD Content Patient's Cells</th>
<th>Normal (Mean ± SEM)</th>
</tr>
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<tbody>
<tr>
<td>RBC</td>
<td>3.2 μM/g hemoglobin/min</td>
<td>5.4 ± 1.4</td>
</tr>
<tr>
<td>WBC</td>
<td>78 μM/g protein/min</td>
<td>—</td>
</tr>
<tr>
<td>PMN</td>
<td>445 μM/g protein/min</td>
<td>—</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>29 μM/g protein/min</td>
<td>—</td>
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</tbody>
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Fig. 4. G-6-PD isoenzyme patterns of (A) red blood cells, (B) whole mononuclear blood cells, (C) polymorphonuclear cells, (D) T cell-poor population, and (E) T cell-rich population.
phocytes in the bone marrow at the time of diagnosis, a peripheral leukocytosis of $95 \times 10^9$/liter with 88% atypical mononuclear cells in the differential, and even the age of the patient (30 yr) were compatible as well with the diagnosis of adult T cell leukemia. The complex nuclear chromatin folding was also compatible with this diagnosis.

G-6-PD is a useful marker to investigate when attempting to show the clonal origin of a neoplasm in female patients heterozygous at the G-6-PD locus, i.e., patients with two distinct cell populations, cells with B and cells with A isoenzymes of G-6-PD. In such heterozygous patients, the presence of only one isoenzyme (either A or B) in the neoplastic cells is a strong argument for their monoclonality. However, the electrophoretic finding of only one isoenzyme in a neoplasm in an heterozygous patient requires also that the neoplastic cells examined be completely or almost completely depleted of normal cells. Any contamination by normal cells would provide both isoenzymes. In the present case, the capacity of the neoplastic cells to form rosettes with SRBC was useful not only in making the diagnosis of a T cell malignancy but also in allowing the separation by centrifugation of the neoplastic from the normal cells.

The observation that the G-6-PD was almost exclusively of the B type in the circulating neoplastic cells strongly suggests the monoclonal origin of this T lymphocyte-derived malignancy. Theoretically, however, normal heterozygous individuals (Gd-A/Gd-B) could have only the B type of G-6-PD in their T lymphocytes. This possibility could not be ruled out in the present study owing to the failure to find control Gd-A/Gd-B subjects. However, arguments for the coexistence of A and B isoenzymes containing T lymphocytes were provided by the patient herself. The first argument is the existence of traces of A isoenzyme in her initial tumor population, which suggests the presence of a few A-type G-6-PD normal T lymphocytes. However, this observation could alternatively be explained by contamination of the sample by normal B lymphocytes. A second argument for the coexistence of T lymphocytes with A and B isoenzymes was the finding in our patient of an almost equal proportion of the two isoenzymes in the isolated T cells after treatment when the peripheral blood leucocyte count and smear had returned to normal.

ACKNOWLEDGMENT

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REFERENCES

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