Immunologic Characterization of a Helper T-Cell Lymphoma

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The lymphocytes of a patient with a T-cell non-Hodgkin's lymphoma with peripheral blood involvement and polyclonal hypergammaglobulinemia were characterized in terms of surface markers and immunologic functions. Using the fluorescence-activated cell sorter and employing various monoclonal antibodies against T-cell surface antigens, it was shown that almost all of the patient's peripheral blood lymphocytes were positive for OKT4 and 9.3, antibodies that recognize helper T-cell subset. The circulating lymphoma cells had typical characteristics for T cells; they formed spontaneous rosettes with sheep erythrocytes and stained with the pan-T-cell antibodies 9.6 and 10.2, but did not react with other anti-T-cell monoclonal reagents such as OKT3, UCHT-1, and 3A1. The cells appeared to be mature by the fact that they did not stain with OKT6, and terminal deoxynucleotidyl transferase was undetectable. Functionally, they were able to provide "help" for antibody production, and they could be stimulated to produce moderate amounts of interleukin-2, while unable to proliferate in response to mitogens. Morphologically, some of the lymphocytes showed a deeply cleaved nucleus.

In order to obtain a better understanding of the heterogeneity of lymphoreticular disorders, attempts have been made to match the morphologically oriented classification with cell surface markers and/or functional immunologic findings with regard to T-, B-lymphocyte, or macrophage origin of the malignant cells. Recent advances, such as the development of more sophisticated functional test systems for lymphocytes, as well as monoclonal antibodies for specific lymphocyte subsets, allow us to study leukemias and lymphomas in more detail.

Reactivity with monoclonal antibodies has been helpful for the classification of acute lymphoblastic leukemia (ALL), the rare T-cell form of chronic lymphocytic leukemia (CLL), and Sézary syndrome/mycosis fungoides.

In this article, we describe a patient with an advanced, diffuse poorly differentiated lymphocytic lymphoma (Rappaport classification) with blood involvement. Her peripheral blood lymphocytes were found to have a unique pattern of surface markers and could be characterized as having the antigenic and functional features of helper T cells. The investigation of such malignant cells provides greater insight into their ontogenetic derivation and increases our knowledge concerning the differentiation pathways of lymphocytes.

Clinical Information

Patient History

The patient, E.M., was an 80-yr-old black female who was hospitalized because of intermittent fever, headaches, and the inability to eat due to swollen, tender lips and gingival ulcerations. Approximately 2 wk before admission she had developed a drainage and fissuring of her lips with crust formation and bleeding. No medication was taken. Physical examination revealed bilateral multiple, enlarged, firm, non-tender submandibular, supraclavicular, axillary, and inguinal lymph nodes. There was no detectable enlargement of the liver or spleen and no skin lesions, aside from the swollen and focally ulcerated lips. Chest x-rays showed no abnormality.

Laboratory Data and Histology

Lactic dehydrogenase (LDH) in the serum was elevated at 1044 mU/ml (normal range 100–200 mU/ml). Serum electrophoresis showed a polyclonal hypergammaglobulinemia, and immunoglobulins were determined quantitatively as 2030 mg/dl for IgG (normal range 710–1490 mg/dl), 900 mg/dl for IgA (normal 160–400 mg/dl), and 200 mg/dl for IgM (normal 80–270 mg/dl). Hematologic studies revealed a hemoglobin of 13.3 g/dl, hematocrit 40.3%, platelet count 182,000/cu mm, 2% reticulocytes, and a white blood cell count of 18,000/cu mm. A differential on the blood smear showed 71% lymphocytes, 25% neutrophils, 2% eosinophils, and 2% monocytes. Ninety percent of the lymphocytes were atypical, with half of them showing indented, clefted, or attenuated nuclei and varying amounts of blue cytoplasm (Fig. 1A). Bone marrow aspirate smears revealed decreased cellularity, with infrequent lymphocytes. Sections of the marrow biopsy (Fig. 1B) showed small foci of poorly differentiated lymphocytic cells, with small numbers of plasmacytoid cells singly dispersed or in small aggregates within or abutting the nodules. The biopsy of the axillary lymph node showed diffuse, poorly differentiated, malignant lymphoma (Fig. 1C). Biopsy of the lip showed absence of epithelium due to necrosis and infiltration with poorly differentiated lymphoid cells.

Materials and Methods

Cell Preparation and Cryopreservation

Mononuclear leukocytes from the peripheral blood (PBML) of the patient and healthy subjects serving as normal controls were obtained by means of a Ficoll-Diatrizoate density gradient and cryopreserved as previously described. After storage in a liquid
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nitrogen freezer, cells were rapidly thawed, washed, and then used in the tests described below.

Electronmicroscopy

PBML were washed twice in Hanks' buffer and fixed in 4% glutaraldehyde for 24 hr at 4°C, then washed twice in Dulbecco's phosphate-buffered saline A and postfixed in 2% osmium tetroxide for 1 hr in the cold. After dehydration with ethanol and propylene oxide, the cells were embedded in an Epon-Araldite mixture. Thin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100C transmission electron microscope (JEOL, Peabody, Mass.).

Terminal Deoxynucleotidyl Transferase (TdT)

The enzyme TdT was detected by indirect immunofluorescence with specific rabbit anti-TdT antisera as described by Bollum using a test kit of the Bethesda Research Laboratories (Rockville, Md.).

Rosette Assays

The spontaneous binding of lymphocytes to sheep red blood cells (SRBC) was tested with 2-aminoethylisothiouronium bromide (AET) treated SRBC to determine the total T-cell population and with untreated SRBC and incubation at 29°C for 1 hr to detect T cells with high affinity to SRBC. Stable rosettes were detected by incubation at 37°C. The T lymphocytes bearing receptors for the Fe portion of IgG or IgM (Tc or Ts) were evaluated by rosetting with bovine erythrocytes coated with purified rabbit anti-bovine erythrocyte IgG or IgM.

Surface Immunoglobulin

Surface immunoglobulin was detected by rosetting with Immuno-beads that were labeled with purified rabbit anti-human immunoglobulin (heavy and light chains) (Bio-Rad laboratories, Richmond, Calif.).

Monoclonal Antibodies

The reagents OKT3, OKT4, OKT5, OKT6, OKT8, and OKM1 (generous gifts of Dr. P.C. Kung) have been characterized previously in detail. Antibodies 9.3, 9.6, 10.2, and 7.2 (kindly provided by Dr. J.A. Hansen) have been raised and described by Hansen and co-workers. The antibodies 3A1 and 4F2 have been developed by Haynes et al. UCHT-1 is a reagent characterized by Callard and colleagues (courtesy of Dr. P. Beverly).

Fluorescence-Activated Cell Sorter (FACS) Analysis

The reactivity of monoclonal antibodies against specific surface determinants was performed as previously described. Cells were analyzed for quantitative immunofluorescence utilizing a FACS-II (Becton Dickinson, Sunnyvale, Calif.) and a digital decagraphic computer (Digital Equipment Co., Maynard, Mass.), as described previously. Percentages of positive cells were calculated from histograms of 256 linear channels by subtracting the negative control from the curve obtained with the test reagents. Furthermore, for calculation, a cut-off channel for reactivity was chosen. Ten thousand cells were analyzed for fluorescence intensity. Percentages of positivity given in the tables are obtained in several experiments with PBML of the patient and different normal controls.

Stimulation Assays

The PBML were stimulated with 0.5 and 1 μg/ml phytohemagglutinin (PHA) (purified, Wellcome, Beckenham, England), with 10 μg/ml concanavalin A (Con-A), or with pokeweed mitogen (PWM) in a 1:100 final dilution, always using standard procedures. After incubation for 3 days, cells were pulsed for 4 hr with 1H-thymidine (6.7 Ci/m mole) and harvested with a MASH-harvester on paper strips and counted in a β-scintillation counter. Results of proliferative assays are expressed in counts-per-minute (cpm) ± standard deviation (SD) or standard error of the mean (SE).
Table 2. FACS Analysis With Monoclonal Antibodies Against "Pan"-T-Cell Surface Determinants*

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Patient Percent Positive Cells</th>
<th>Normal Control Percent Positive Cells</th>
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<tbody>
<tr>
<td>9.6</td>
<td>98%</td>
<td>72%</td>
</tr>
<tr>
<td>10.2</td>
<td>97%</td>
<td>65%</td>
</tr>
<tr>
<td>OKT3</td>
<td>6%</td>
<td>66%</td>
</tr>
<tr>
<td>UCHT-1</td>
<td>7%</td>
<td>65%</td>
</tr>
<tr>
<td>3A1</td>
<td>5%</td>
<td>73%</td>
</tr>
</tbody>
</table>

*Antibodies that have been reported to react with ≥85% T lymphocytes.

Interleukin-2 (IL) (T-Cell Growth Factor) Production

Lymphocytes were incubated with 0.5 μg/ml PHA or with PHA and the B-lymphoblastoid cell line (LCL) RMI for 3 days in 16-mm well tissue culture plates (Costar, Cambridge, Mass.), as described in detail. The supernates were then harvested and frozen until testing for IL-2 activity in a proliferative assay system with human cultured T cells (CTC) as indicator cells.

Helper Cell Assay

The capability of T cells to "help" was tested as described previously. Briefly, varying numbers of T cells were mixed with 2.5 × 10^5 T-cell-depleted allogeneic PBML and incubated for 12 days with PWM as a stimulant. Production of IgG in each culture was then measured using the enzyme-linked immunosorbent assay (ELISA).

Natural Killer (NK) Cell Activity

NK activity was measured in a 4-hr chromium release assay. PBML were used as effectors against the ^65^Cr-labeled K562 cell line. Reactions were performed in triplicate with different effector-to-target cell ratios. The specific NK-cytotoxicity was calculated as previously described.

RESULTS

The light microscopical picture of the patient’s peripheral blood smear, as described above, showed approximately 90% atypical lymphocytes, 50% with indented or cleaved nuclei and moderate to abundant cytoplasm. Ultrastructurally (Fig. 2), these heterogeneous peripheral lymphocyte populations were comprised of cells with a round nucleus and cells that had deeply cleaved nuclei and showed, in certain views, a prominent nucleolus. In the latter cells the heterochromatin was prominently clumped and situated mainly around the periphery of the nucleus. An ultrastructural morphology resembling the "cerebriform" appearance of a highly convoluted nucleus typical of a Sézary cell was not found.

Testing for rosette formation (Table 1) revealed that 86% of the cells were able to bind AET-treated SRBC. Most of the T cells had a high affinity for SRBC, since 63% were able to form rosettes at 29°C. But only 7% of the patient’s lymphocytes formed stable rosettes at 37°C—normally a characteristic of activated cells, thymocytes, and some T-ALL lymphoblasts. The percentage of T-lymphocytes bearing Fc-IgG or Fc-IgM receptors was very low.

The conclusion that T lymphocytes were predominant in this leukemic lymphoma was further suggested by bright staining by FACS analysis of fluorescence intensity with the anti-pan-T-cell (SRBC receptor) antibody 9.6. Furthermore, the anti-T-cell antibody 10.2 reacted strongly, although it detects a surface determinant also expressed on B-CLL cells. In contrast, three other T-cell reagents, OKT3, UCHT-1, and 3A1, were consistently negative, none reacting with more than 7% of the cells (Table 2 and Fig. 3). A subpopulation of normal 9.6 T cells, however, lacks these markers: at least 15% do not react with 3A1, 10% are negative with UCHT-1, and some apparently do not react with OKT3 (Hansen, personal communication).

To characterize further the T-cell population in the peripheral blood of this lymphoma patient, monoclonal antibodies were used that have been shown to react with functionally defined T-cell subsets (Tables 3 and 4). As can be seen from Fig. 3, nearly all of the patient’s T-cell population reacted with 9.3 and OKT4, with a low-density antigen expression for both surface markers. Both antibodies, detecting different anti-
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Fig. 3. FACS profiles generated with 9.3 and 9.6 antibodies (A) and with OKT3 and OKT4 (B). While almost all of the patient's lymphocytes reacted strongly with 9.6, very few were stained by OKT3. Monoclonal anti-helper-cell antibodies 9.3 and OKT4 showed a low density binding with an unusually homogeneous staining pattern. Supernates of the P3 × 63Ag8 myeloma cell line served as control (C) for background staining.

OKM1, which is directed against antigenic determinants on monocytes, granulocytes, null, and some Tγ cells, reacted with only a few of the patient's peripheral blood mononuclear cells. Another antibody, 4F2, which binds to monocytes, a subset of activated T lymphocytes, as well as Sézary cells and T-ALL lymphoblasts, was brightly positive. Finally, the patient's lymphocytes appeared to be mature, since they lacked the enzyme TdT, a marker lost during differentiation in the thymus.

Eleven percent of the cells were B lymphocytes detected as positive for surface immunoglobulin; 7% bore HLA-DR (Ia-like) antigens as revealed by antibody 7.2.

In addition to the surface marker studies, functional tests were performed. The patient's PBML did not proliferate in response to PHA, Con-A, or to PWM (Table 5). However, in a PWM-driven test system where the capability of T lymphocytes to provide "help" to an allogenic T-cell-depleted fraction containing B cells and monocytes is measured, the patient's cells induced immunoglobulin synthesis of B cells, thus providing helper-cell function (Fig. 4).

Although the patient's lymphocytes did not proliferate significantly in response to PHA, they produced detectable amounts of IL-2. As shown in Table 6, the IL-2 production compared to normal controls was somewhat low. The B-lymphoblastoid cell line, RM1, could, as described for healthy individuals, enhance the production of IL-2, leading to increased proliferation of the test CTC even at the higher dilutions of the supernatant.

Table 3. FACS Analysis With Monoclonal Antibodies Directed Against T-Cell Subsets

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Reported Specificity on Peripheral Blood Mononuclear Cells</th>
<th>Percent Positive Cells</th>
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<tbody>
<tr>
<td>9.3</td>
<td>Helper/cytotoxic T cells</td>
<td>94% 40%</td>
</tr>
<tr>
<td>OKT4</td>
<td>Helper T cells</td>
<td>93% 38%</td>
</tr>
<tr>
<td>OKT5</td>
<td>Suppressor/cytotoxic T cells</td>
<td>2% 15%</td>
</tr>
<tr>
<td>OKT8</td>
<td>Suppressor/cytotoxic T cells</td>
<td>2% 22%</td>
</tr>
</tbody>
</table>

Table 4. FACS Analysis With Monoclonal Antibodies Directed Against Miscellaneous Cell Surface Structures

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Reported Specificity</th>
<th>Patient</th>
<th>Normal Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>OKT6</td>
<td>Subset of thymocytes</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>OKM1</td>
<td>Monocytosis, granulocytes, null, and some Tγ cells</td>
<td>7% 26%</td>
<td></td>
</tr>
<tr>
<td>4F2</td>
<td>Monocytosis, subset of activated T cells, thymocytes</td>
<td>90% 13%</td>
<td></td>
</tr>
<tr>
<td>7.2</td>
<td>Human Ia (common structure of the HLA-DR molecule)</td>
<td>7% 14%</td>
<td></td>
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</tbody>
</table>

Table 5. Lymphoproliferative Response to Lectins

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Final Concentration</th>
<th>^3H-Thymidine Incorporation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Patient</td>
</tr>
<tr>
<td>PHA</td>
<td>0.5 µg/ml</td>
<td>1.514 ± 393</td>
</tr>
<tr>
<td></td>
<td>1 µg/ml</td>
<td>1.204 ± 273</td>
</tr>
<tr>
<td>Con A</td>
<td>10 µg/ml</td>
<td>54 ± 20</td>
</tr>
<tr>
<td>PWM</td>
<td>1:100</td>
<td>110 ± 29</td>
</tr>
<tr>
<td>Medium</td>
<td></td>
<td>179 ± 124</td>
</tr>
</tbody>
</table>

*cpm ± SE.
Finally, the patient's PBML were negative for natural cytotoxicity against the myeloid cell line K562 (Table 7).

**DISCUSSION**

The present study describes a case of a leukemic malignant non-Hodgkin's lymphoma that was of mature T-cell origin, displaying functional and cell surface antigen characteristics of helper T lymphocytes. The T-cell origin was indicated by the ability of most PBML to form spontaneous rosettes with SRBC and to react with the monoclonal pan-T-cell antibodies 9.6 and 10.2. The staining with the anti-helper-cell antibodies 9.3 and OKT4, together with the evidence from the functional assays, allowed us to finally characterize the malignancy as a true helper T-cell lymphoma.

This is the first report of a case of 9.6+/OKT3/3A1 T-cell non-Hodgkin's lymphoma. Two possible explanations for this unusual phenotype are conceivable. First, the surface structures detected by the reagents OKT3, UCHT-1, and 3A1 may have been lost during the malignant transformation. Second, a small subset of T lymphocytes, normally lacking these markers, may have been clonally expanded. Although it might be argued that due to a genetic polymorphism this patient never expressed the missing antigens, this can be ruled out by the fact that a small percentage of cells, positive with these reagents, was found in the patient's blood. The lymphoma cells described here might be considered immature, since they lack markers such as OKT3 expressed only on mature T cells. However, these malignant cells, being TdT negative and lacking OKT6, appeared to be mature T cells.

Further insight into the nature of these lymphoma cells was provided by the finding that they stained with OKT4 and 9.3. This made them distinct from the subpopulation of T cells with suppressor (OKT5/8−) or cytotoxic (OKT5/8+/9.3+) activity and from NK cells (9.6+/OKT3+/OKM1+). Compatible with the function normally related to the OKT4+/9.3+ subset, the patient's T lymphocytes were able to induce immunoglobulin secretion in B cells from normal donors. This was consistent with the clinically observed polyclonal hypergammaglobulinemia. Although the malignant T cells were able to provide help in antibody responses, they were unable to respond to mitogens in proliferation assays. This finding, which has been reported in cutaneous T-cell lymphomas like Sézary syndrome, may be due to an intrinsic blastic unresponsiveness of the cell that is dissociated from its ability to help in B-cell responses.

In Sézary syndrome/myosis fungoides, malignant T cells have been reported to possess the helper-cell phenotype and function. Despite these common features in the case described here, the clinical picture, the histology, and the electron microscopical view were not typical. Although Sézary cells were reported to lack the antigen detected by 3A1, they were always OKT3 positive. The ability to produce normal amounts of IL-2 upon PHA stimulation has been reported in a case of Sézary syndrome, suggesting a relation of the helper cell containing subset to the IL-2 producer cell. The lymphocytes from the lymphoma patient described here did produce some IL-2 but considerably less than normal controls.

**Table 6. Interleukin-2 Production**

<table>
<thead>
<tr>
<th>Stimulus for IL-2 Production</th>
<th>Dilution of the Supernate</th>
<th>IL-2 Activity in the Supernate* Produced by PBML of</th>
<th>Normal Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA</td>
<td>1:2</td>
<td>58,598 ± 2,295</td>
<td>135,350 ± 5,434</td>
</tr>
<tr>
<td>PHA + B-LCL RM1</td>
<td>1:6</td>
<td>137,020 ± 14,147</td>
<td>162,486 ± 11,209</td>
</tr>
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</table>

*3H-thymidine incorporation in cpm ± SE by human cultured T cells (CTC) used as responder cells. Medium alone was 419 cpm; PHA 0.5 μg/ml gave 11,822 cpm.
Recently, Popppema et al. in a report on T-cell subgroups in human lymph nodes, showed that the OKT4+ T cells were located in the medulla, the paracortical area, and in a crescentic accumulation on the capsular side of the germinal center of the secondary follicle. The cleaved cells seen in half of our patient's lymphoid cells are similar to those described as follicular center cells (FCC). It is an intriguing possibility, considering the recent data about helper T cells in the germinal center, that the patient's lymphoma may have originated in this region of the lymph node, notwithstanding the fact that the majority of FCC lymphomas are of B-cell origin.

The characterization of this non-Hodgkin's lymphoma reveals a close relation between surface marker expression of the malignant cell and functional activity. This extends and confirms that such associations in lymphoproliferative diseases exist as reported previously in T-cell CLL and Sézary syndrome and supports the view that these diseases relate to distinct stages of normal lymphocyte differentiation. The question whether, in our case, some surface characteristics on the malignant cells were lost or a normally existing subset was clonally expanded, remains open.

In the near future, the characterization of lymphoproliferative malignancies with advanced immunologic techniques may help to clarify further current classifications, which hopefully, might have an impact on diagnosis, prognosis, and therapeutic efforts.

REFERENCES

Immunologic characterization of a helper T-cell lymphoma

M Gramatzki, MF Dolan, AS Fauci, JA Maples, GD Bonnard and DM Strong