Cutaneous T-Cell Lymphoma: Neoplasm of T Cells With Helper Activity

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Neoplastic lymphocytes from 3 patients with aleukemic cutaneous T-cell lymphoma were studied to determine if their neoplastic T cells had helper activity, i.e., ability to promote immunoglobulin synthesis by normal human B cells. Homogeneous populations of neoplastic T cells were isolated from involved lymph nodes and in two of three instances were shown to be of monoclonal origin by karyotypic analysis. Mononuclear leukocytes from control peripheral blood were separated into T- and B-cell-enriched fractions by E-rosette formation and subsequent Ficoll-Hypaque flotation. Pokeweed-mitogen-stimulated normal and malignant T cells were co-cultured with normal B cells. Cytocentrifuged cell preparations were stained with fluorescein-conjugated goat antihuman immunoglobulin to quantitate immunoglobulin-producing B cells. Addition of control T-cell fractions to control B-cell fractions increased the number of labeled cells from 8 to 34. Neoplastic T cells increased the numbers of normal B cells containing cytoplasmic immunoglobulin to a mean of 125, demonstrating exceptional helper activity. These results support a relationship between the Sézary syndrome, previously demonstrated to be a leukemia of helper T cells, and aleukemic cutaneous T-cell lymphoma and suggest that malignant helper T cells have a distinct affinity for the skin.

During the past 5 yr it has become clear that mycosis fungoides and the Sézary syndrome are part of the broader spectrum of cutaneous T-cell lymphoma. These neoplastic processes are characterized by widespread cutaneous infiltration, frequently associated with extensive visceral involvement, and they are further distinguished by the regularity with which their malignant cells possess membrane features typical of thymus-derived (T) lymphocytes.

Broder et al. have demonstrated that the leukemic T cells of the Sézary syndrome are functionally "helper T cells," since they facilitate normal B-cell differentiation into immunoglobulin-secreting plasma cells. This observation has been confirmed by Siegal and Siegal. The present studies were designed to determine if aleukemic cutaneous T-cell lymphoma is also a neoplasm of helper T cells. In 2 patients the availability of homogeneous populations of tissue-localized malignant T cells shown to be of monoclonal origin provided a special opportunity to investigate this possibility.

Materials and Methods

Source of Cells

Portions of lymph nodes, all with complete effacement of normal architecture, were obtained from 3 patients with aleukemic cutaneous T-cell lymphoma. One patient, E.O. (Table 1), had histologically...
Table 1. Diagnosis and Source of Cells From Patients With Cutaneous Lymphoma*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Source of Cells/Histology</th>
<th>Karyotype</th>
<th>Percentage E-Rosette-Forming Cells</th>
<th>Percentage EAC-Rosette-Forming Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.O.</td>
<td>Aleukemic cutaneous T-cell lymphoma</td>
<td>Lymph node: architecture effaced by sheet of neoplastic cells</td>
<td>No normal karyotypes; monoclonal</td>
<td>56</td>
<td>3</td>
</tr>
<tr>
<td>T.C.</td>
<td>Aleukemic cutaneous T-cell lymphoma</td>
<td>Lymph node: architecture effaced by sheet of neoplastic cells</td>
<td>No normal karyotypes; monoclonal</td>
<td>83</td>
<td>14</td>
</tr>
<tr>
<td>D.R.</td>
<td>Aleukemic cutaneous T-cell lymphoma</td>
<td>Lymph node: architecture effaced by sheet of neoplastic cells</td>
<td>No normal karyotypes</td>
<td>26</td>
<td>33</td>
</tr>
<tr>
<td>R.J.</td>
<td>Sézary syndrome</td>
<td>Peripheral blood: &gt;80% of lymphocytes showing nuclear indentations of Sézary cells</td>
<td>Not done</td>
<td>76</td>
<td>4</td>
</tr>
<tr>
<td>Controls</td>
<td>B-cell chronic lymphocytic leukemia (mean of 3 subjects)</td>
<td>Peripheral blood</td>
<td>Not done</td>
<td>8 (± 3.6)</td>
<td>73 (± 11.3)</td>
</tr>
<tr>
<td>Mean of 20 normal subjects</td>
<td>—</td>
<td>Peripheral blood</td>
<td>—</td>
<td>63 (± 7.7)</td>
<td>15 (± 5.3)</td>
</tr>
</tbody>
</table>

*Abbreviations: E represents sheep erythrocytes; EAC represents complexes of E with IgM anti-Forsmann antibody and murine complement.

confirmed cutaneous mycosis fungoides with characteristic Pautrier’s microabscesses in the epidermis. Two patients, T.C. and D.R., had cutaneous T-cell lymphoma not histologically classifiable as mycosis fungoides. These 2 patients had widespread cutaneous nodules characterized microscopically by dense dermal infiltration by atypical mononuclear cells that spared the overlying epidermis. The fourth patient, R.J., had the Sézary syndrome, with more than 80% of his peripheral blood lymphocytes containing the deep nuclear indentations characteristic of Sézary cells and exfoliative erythroderma with dermal infiltration by similar atypical mononuclear cells.

Control blood was obtained from normal volunteers. Fifty to 100 cc of heparinized (20 units heparin/cc, Bel-Mar Laboratories, Inwood, N.Y.) venous blood were drawn for testing. Informed consent was obtained from all volunteers, and approval for blood drawing procedures was obtained from the Columbia University Human Investigation Committee.

In preliminary experiments comparisons were made between mononuclear leukocyte populations obtained from minceates of histologically nonmalignant lymph nodes and cell populations obtained from normal peripheral blood. Cells from these two sources exhibited statistically identical helper activities and responses to mitogens. We therefore decided to use mononuclear cell populations obtained from normal peripheral blood as controls in all future experiments, since these cells were more accessible on a routine basis than were normal human lymph node cells.

Leukocyte Isolation

Portions of lymph nodes excised for diagnostic purposes were finely minced with scalpels in Hanks buffered salt solution (HBSS, GIBCO, Grand Island, N.Y.) and passed through 40-μm sieves to obtain cell suspensions. Peripheral blood specimens were layered on Ficoll-Hypaque (Gallard-Schlessinger, Carle Place, N.Y.), and the interface containing the mononuclear leukocyte fraction was collected and washed twice in HBSS. Aliquots of the neoplastic cell suspensions were reserved for future testing by freezing at −70°C in 15% dimethyl sulfoxide/RPMI-1640-10% fetal calf serum (FCS) (GIBCO), a procedure previously shown not to interfere with the following studies in our laboratory.

Chromosome Analysis

Slides were prepared from 3-day cultures of mononuclear leukocytes from the lymph nodes of all 3 patients. Doses of the mitogens concanavalin A (40 μg/cc) (Sigma, St. Louis, Mo.), phytohemagglutinin (5 μg/cc) (Sigma), and pokeweed (5 μg/cc) (Sigma) were predetermined (for each studied cell population) to induce optimal DNA synthesis, as indicated by tritiated-thymidine scintillation counting. Direct preparations were made from the lymph node of patient T.C. Chromosome banding of
metaphases was performed using a trypsin-Giemsa technique. The direct preparation from patient T.C.'s lymph node was chromosome banded with quinacrine. The nomenclature used to describe the karyotypes was that established by the Paris Conference.

T- and B-Cell Enrichment

All cell suspensions were counted in a Coulter counter (model Zf, Coulter Electronics, Wayne, N.J.) and adjusted to a concentration of 5 x 10^6 cells/cc in RPMI-1640/20% FCS. Aliquots of mononuclear leukocyte suspensions at a cell concentration of 1 x 10^6/cc were maintained at 4°C prior to assessing helper activity. Portions of each mononuclear leukocyte population were fractionated further. Mononuclear leukocytes at a cell concentration of 5 x 10^6/cc were incubated with an equal volume of a 1% solution of neuraminidase-treated sheep red blood cells (E) on a tube rotator at 37°C for 10 min. The cells were then centrifuged (800 g for 10 min), the supernatant fluid was replaced with FCS, and the cells were refrigerated overnight. To determine if maximum numbers of T cells had been rosetted, standard E rosetting by the method of Weiner et al. was performed using a concentration of 0.5% neuraminidase-treated sheep erythrocytes.

The rosetted and nonrosetted cell populations were separated by a second flotation on Ficoll-Hypaque. After centrifugation (600 g for 45 min), the supernatant fluid was discarded, and the interface and pelleted cells were collected. The cells remaining at the interface of the Ficoll-Hypaque constituted the T-cell-depleted fraction, characterized further as described below.

The presence of receptors for the split product of the third component of complement (C3) on the surface of these mononuclear leukocytes was determined using the technique of Bianco et al. Briefly, the interface cells were incubated with sheep red blood cells coated with the IgM fraction of rabbit antibody to sheep red blood cell stroma and with the split product of the third component of mouse complement. In determinations on preparations from normal peripheral blood, a mean 72% of these Ficoll-Hypaque interface cells (after E-rosette enrichment) had identifiable C3 receptors, and only 7% formed rosettes with sheep red blood cells. This interface fraction constituted the B-enriched fraction, since C3 receptors are characteristic of B-cell populations. The T-enriched fraction was pelleted beneath the Ficoll-Hypaque and was enriched for E-rosetting lymphocytes (68% E-rosetting lymphocytes and only 2% C3-receptor-bearing cells). The pelleted T cells were freed of adherent sheep red blood cells by lysis with NH4Cl (0.155-M NH4Cl-0.01-M KHCO3/10% FCS). The T- and B-cell-enriched fractions were then washed twice in HBSS and resuspended to a concentration of 1 X 10^6 cells/cc in RPMI-1640/20% FCS.

Cell Culture

The lymphocyte culture method used to assay for pokeweed-induced polyclonal helper activity was based on the technique of Siegal and Siegal. Unseparated mononuclear leukocyte suspensions and T- and B-enriched fractions were cultured for 7 days with an equal volume of RPMI-1640/20% FCS or with pokeweed mitogen (10 μg/cc in the same medium). Co-cultures consisting of recombinations of normal T- and B-enriched fractions or of T-enriched fractions from the patients' neoplasms added to normal B cells were incubated under the same conditions. The cells were cultured at a 1:1 T-to-B-cell ratio. All cultures were maintained in an incubator under a 5% CO2 atmosphere at 37°C.

After incubation, the cells were centrifuged (300 g for 10 min) and washed twice in phosphate-buffered saline (PBS) (GIBCO). The cells were returned to their initial volume in 3% bovine serum albumin/PBS, and cytocentrifuge (Shandon Southern Instruments, Sewickle, Pa.) slides were prepared for quantification of results by immunofluorescence.

Immunofluorescence

The slides were fixed in 5% acetic acid/methanol at -20°C for 10 min, rinsed twice in PBS, and then incubated in a moist chamber with a 1:4 dilution of polyvalent fluorescein-conjugated goat antihuman immunoglobulin (anti-IgG, -IgM, and -IgA, Hyland Laboratories, Costa Mesa, Calif.) for 30 min. After incubation, the slides were washed twice in PBS and examined by light and fluorescence microscopy with a Leitz Ortholux II microscope equipped with an epifluorescent illuminator (Leitz, Rockleigh, N.J.). The number of brightly stained cells containing diffuse intracytoplasmic fluorescence present in 500 randomly encountered cells was determined (Fig. 1). Cultures that contained no pokeweed mitogen were used to determine background fluorescence, and the number of fluorescing cells counted in these cultures was subtracted from that present in pokeweed-mitogen-stimulated cultures.
Non-specific staining of nonviable cells by the fluorescein-conjugated reagent was ruled out by studies in which suspensions of nonviable frozen and thawed cells were incubated with fluorescein-conjugated goat antihuman immunoglobulin. In all instances, nonviable intact lymphocytes failed to stain with this labeled antihuman immunoglobulin.

**Lymphocyte Stimulation**

Mononuclear leukocyte preparations from all patients and from controls were evaluated for responsiveness to mitogens. The cell suspensions in RPMI-1640 were added to the wells of Microtiter plates (Flow Laboratories, Rockville, Md.) at concentrations of $2 \times 10^5$ cells per 0.1 cc per well. The plant lectins phytohemagglutinin (5 μg/cc) (Sigma), pokeweed mitogen (5 μg/cc) (Sigma), and concanavalin A (40 μg/cc) (Sigma) were prediluted in RPMI-1640 containing 30% autologous plasma and added to the cultures in 100-μl aliquots. Mitogen-containing cultures were incubated for 3 days at 37°C in a 5% CO₂ atmosphere. After incubation, and 4 hr prior to harvesting, the cultures were pulsed with 1 μCi of tritiated thymidine (specific activity 6.7 Ci/mmole) (New England Nuclear, Boston, Mass.) delivered in RPMI-1640.

The plates were harvested with a Mash II harvester (Microbiological Associated, Bethesda, Md.) and counted in a liquid scintillation counter (Mark II, Searle, Elmsford, N.Y.) using an omnifluor (New England Nuclear toluene scintillation cocktail).

**Stimulation Ratio**

The stimulation ratio was calculated by dividing the counts per minute (cpm) incorporated by mononuclear leukocytes cultured with appropriate stimulant by the (cpm) incorporated by unstimulated cells from the same source cultured in parallel. The percentage of control stimulation ratio was obtained by dividing the patient’s stimulation ratio by the control stimulation ratio.

**Statistical Evaluation**

The data were evaluated for statistical significance using the standard Student’s t test.

**RESULTS**

**Homogeneity of Neoplastic Cells**

Involved lymph nodes from each of the 3 aleukemic patients (E.O., T.C., and D.R.) provided large numbers of lymphocytes (E.O. $4.0 \times 10^8$, T.C. $2.4 \times 10^8$, D.R. $1.1 \times 10^8$). In all three instances normal lymph node architecture was completely effaced by homogeneous sheets of morphologically atypical mononuclear cells, with no residual identifiable remnants of follicular structures.
More definite indication of monoclonality of the cells from E.O. and T.C. was obtained through karyotypic analysis. First, no normal karyotypes were identified in lymphocytes obtained from involved lymph nodes in any of these patients. Second, all proliferating cells had distinguishing chromosomal abnormalities.

All 10 karyotyped cells obtained from the lymph node of patient E.O. contained identical abnormalities: deletion of the long arm of chromosome 1 at band q42, a single chromosome 2, a single chromosome 14, absence of chromosomes 11 and 12, and a triplet chromosome 3. In addition, there were 8–9 marker chromosomes of undetermined origin in each cell. Two of these were long submetacentric chromosomes, the size of a normal chromosome 2, with distinctive banding patterns that could be identified in every cell examined. These consistent chromosomal changes indicated a monoclonal origin for all of these cells. All five metaphases studied from the direct preparations of the lymph node of patient T.C. revealed absence of chromosome 16 and a translocation between the long arms of chromosomes 4 and 14 (band points 4q35 and 14q13). The presence of these chromosomal abnormalities in every cell indicated a monoclonal origin of the neoplastic cells.

As described in greater detail elsewhere, neoplastic cells from D.R. and T.C. frequently had receptors for both sheep erythrocytes and the third component of complement. This phenomenon has been previously identified in a patient in the
leukemic phase of cutaneous T-cell lymphoma, as has diminished E-rosette formation (E.O. and D.R.) in patients with highly anaplastic T cells.

**Assay for Helper Cell Function**

The results of the helper activity assay are presented in Fig. 2. Control T-enriched and B-enriched fractions cultured individually contained means of 16 and 8 fluorescein-labeled cells. Recombination of control T-cell fractions and control B cells induced an increase to a mean of 34 fluorescein-labeled cells. This increase was greater than would be produced by a simple additive effect, thus indicating synergism between the two normal lymphocyte populations.

When T-cell-enriched fractions from the patients with various manifestations of cutaneous T-cell lymphoma were added to normal B cells, an increase occurred in the numbers of normal B cells that produced identifiable intracytoplasmic immunoglobulin far exceeding that seen in co-cultures of normal T and B cells. The numbers of fluorescein-labeled B cells present in pokeweed-mitogen-stimulated co-cultures of patients' T cells added to control B cells were E.O. 122, T.C. 217, D.R. 80, demonstrating a substantial increase over the control T-plus-B-cell mean of 34 fluorescein-labeled cells.

The peripheral blood T-cell-enriched fraction from patient R.J. with the Sézary syndrome increased immunoglobulin-containing B cells to 82 labeled cells. Therefore the co-cultures of T-cell-enriched fractions from the patients with aleukemic
cutaneous T-cell lymphoma enhanced the differentiation of normal B cells into immunoglobulin-containing cells at least as well as did that from the patient with the Sézary syndrome and substantially more than did normal B lymphocytes.

Figure 3 directly relates the helper activity of the malignant and normal T-cell populations. The T-enriched fractions from the patients with aleukemic cutaneous T-cell lymphoma increased normal B-cell differentiation by factors of approximately 4, 7, and 1.75, as compared with the helper activity normally present in peripheral blood.

**Functional Responses**

To determine the capacity of these mononuclear leukocytes to respond to standard stimulants, populations of unfractionated cells from the same sources were studied. The proliferative responses by malignant T cells to mitogens are compared with those of controls in Fig. 4. With the exception of the PHA response by lymphocytes obtained from patient T.C., cell populations extracted from the lymph nodes of patients with aleukemic cutaneous T-cell lymphoma did not respond to standard mitogenic stimuli by increasing scheduled DNA synthesis.

The proliferative responses to phytohemagglutinin, pokeweed mitogen, and concanavalin A by neoplastic cells from all 3 patients (with the exception of patient T.C.'s phytohemagglutinin response) were significantly depressed ($p \leq 0.01$) as compared with that of controls. The response to phytohemagglutinin in patient T.C. was not significantly different from control values ($p \geq 0.05$). The peripheral blood mononuclear leukocytes from patient R.J. with the Sézary syndrome showed a significant depression in response to phytohemagglutinin and concanavalin A ($p \leq 0.001$), but the response to pokeweed mitogen was significantly increased above that of controls ($p \leq 0.001$).
DISCUSSION

The recognition that helper and suppressor T-cell subpopulations normally exert an immunoregulatory effect has provided new insight into the functioning of the immune system. T helper cells promote B-cell immunoglobulin synthesis, whereas suppressor T cells inhibit antibody synthesis and can act to limit other nonhumoral cellular immune responses. The amplification of these phenomena in T-cell neoplasms may be of substantial clinical significance. Broder et al. have reported suppressor T-cell activity by the neoplastic T cells in 1 patient with acute lymphoblastic leukemia with associated hypogammaglobulinemia and have also demonstrated that a majority of studied cases of the Sézary syndrome were neoplasms of cells with helper function.

The demonstration here that each of 3 cases of aleukemic cutaneous T-cell lymphoma is also a malignancy of T cells with helper activity extends the observations of Broder et al. and supports the suggestion that the leukemic Sézary syndrome is closely related to these aleukemic processes. Some clinicians have considered the Sézary syndrome to be distinct from mycosis fungoides and other cutaneous lymphoproliferative disorders, whereas others have considered the Sézary syndrome to be the leukemic phase of cutaneous T-cell lymphoma. Similar functional properties of the malignant cells in the leukemic and aleukemic processes described in this report support the latter view.

The lymph nodes obtained from these 3 cases of cutaneous T-cell lymphoma presented special opportunities to study the aleukemic phases of cutaneous T-cell lymphoma. Whereas leukemic lymphocytes are readily available from the peripheral blood, it is difficult to extract large numbers of viable lymphocytes from the skin, and tissue cells are usually contaminated by significant numbers of normal cells. The heavily infiltrated lymph nodes obtained from these 3 patients presented an opportunity to study homogeneous populations of neoplastic cells. In such studies it is important to determine that primarily abnormal lymphocytes are being studied. The effacement of normal architecture in the lymph nodes used as sources of cells, the absence of cells with normal karyotypes responding to mitogens, and the direct evidence of monoclonality by karyotypic analysis in 2 patients constitute strong evidence that the observations presented here directly reflect the properties of the malignant T cells.

The extraordinary helper activity demonstrated by these T cells suggests that the studied examples of cutaneous T-cell lymphoma represent monoclonal expansion and dissemination of neoplastic T cells with helper activity. This observation may help to explain the maintenance of normal or elevated levels of immunoglobulin synthesis in these patients despite the presence of marked absolute reductions in circulating B cells. These residual circulating normal B cells may be hyperstimulated by the large number of neoplastic T cells with helper activity.

The finding that the malignant T cells from these patients with cutaneous T-cell lymphoma are hyporesponsive to standard stimulants and still express helper activity is important for at least two reasons. First, it underscores the demonstration by Insel and Merler that T-cell proliferation is not required for expression in vitro of helper activity in the pokeweed mitogen system. Second, despite diminished capacity to proliferate in response to standard T-cell mitogens, these malignant cells retain significant helper function. This suggests that as helper T cells undergo...
malignant change, the capacity to continue to function as helper cells may be lost relatively late in the process of dedifferentiation.

The homogeneous populations of neoplastic T cells with helper activity present in patients with cutaneous T-cell lymphoma provide significant investigative opportunities. Heteroimmunizations using human helper T cells may lead to production of specific antibodies, which would be invaluable probes in the dissection of human lymphocyte interactions. An anti-helper-T-cell-specific reagent may also provide a therapeutic agent for the management of cutaneous T-cell lymphoma that is more refined than the antithymocyte globulin already shown to be a promising therapeutic agent in the management of this disorder.21

The presence or absence of helper activity in the neoplastic T cells of cutaneous T-cell lymphoma may correlate with the clinical status of the patient. Leukemic T cells isolated from 1 patient (unpublished results) in terminal acute lymphoblast crisis were shown to have no helper function. Detection of loss of helper activity in a patient previously shown to have neoplastic T cells with helper function may be a poor prognostic sign and an aid to early diagnosis of the patient’s deteriorating clinical status. In addition, the presence of humoral abnormalities such as autoreactive antibodies in cutaneous T-cell lymphoma22 may be linked to the helper T-cell etiology of the disease.

Most provocatively, this study suggests a direct helper-T-cell/skin interaction. The affinity that the malignant cells of cutaneous T-cell lymphoma display for the skin becomes apparent as clinically evident infiltration. This infiltration may simply represent a neoplastic amplification of an otherwise undetected phenomenon: a similar interaction between normal helper T cells and the skin. Elucidation of the basis of such a postulated phenomenon could prove useful in our understanding of the basic functioning of the immune system.

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REFERENCES

9. Breg WR: Quinacrine fluorescence for identifying metaphase chromosomes, with special
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