Mitogen Requirements for the In Vitro Propagation of Cutaneous T-Cell Lymphomas

By Adi F. Gazdar, Desmond N. Carney, Paul A. Bunn, Edward K. Russell, Elaine S. Jaffe, Geraldine P. Schechter, and John G. Guccion

We attempted to generate continuous in vitro cultures from patients with mycosis fungoides and the Sezary syndrome (cutaneous T-cell lymphomas, CTCL). Using conventional culture techniques without mitogen stimulation, multiple attempts (32 specimens from 25 patients) failed to replicate T cells, but 6 B-lymphoblastoid cultures were established. Athymic nude mice injected by a variety of routes with CTCL cells from 13 patients failed to develop tumors; however, the B-lymphoblastoid cultures were tumorigenic. Lymphocytes from 6 healthy donors and CTCL cells from 7 patients were seeded in growth medium supplemented with one of the following mitogens: phytohemagglutinin (PHA), concanavalin A (Con-A), lymphocyte-conditioned medium (LyCM), pokeweed mitogen (PWM), and staphylococcal protein-A. Normal lymphocytes failed to replicate for more than a few days except in LyCM, where viability and replication remained absolutely dependent on the continued presence of the mitogen. In contrast, mitogen-induced proliferation of CTCL cells was variable, and 1 or more of 4 mitogens induced replication in 4 CTCL cultures. There was partial correlation between the ability of mitogens to stimulate CTCL cells in lymphocyte transformation assays and their ability to act as growth factors. CTCL cells were more resistant to the toxic effects of mitogens than lymphocytes from normal donors, permitting the use of mitogens as long-term growth factors. Two long-term CTCL cultures were established, one after stimulation with Con-A, and the other with LyCM. The cultures required mitogens only for initial growth and have proliferated without mitogens for over a year. The cultures retained many of the features of the CTCL cells present in the patients from whom they were derived, but differed from each other in morphology, E-rosette formation, DNA content, and tumorigenicity. Cytochemical studies provided further evidence of their T-cell origin. Both cultures released macrophage inhibitory factor.

The majority of human lymphoid malignancies are of B-lymphocyte origin. Well characterized T-lymphocyte malignancies include some cases of acute and chronic lymphocytic leukemias, lymphoblastic lymphomas, and the cutaneous T-cell lymphomas (CTCL). The latter disorders include mycosis fungoides (MF), Sezary syndrome (SS), and the related cutaneous disease lymphomatoid papulosis. CTCL cells have characteristic convoluted nuclear morphology, membrane features of T lymphocytes, a tendency to infiltrate the skin, and in SS, the blood.

While numerous Epstein-Barr (EB) virus-infected cultures have been established from normal and malignant B cells, relatively few T-cell cultures exist. Growth of normal T cells is induced for short periods by many mitogens, but long-term exposure is usually toxic. Peripheral blood mononuclear cells (PBMCs) from normal human donors stimulated with certain mitogens release a T-cell blastogenic factor. T cells from normal donors can replicate for long periods in the presence of such lymphocyte-conditioned medium (LyCM). However, these T cells remain absolutely dependent on the continued presence of LyCM for viability and growth. We now report that mitogens may induce prolonged proliferation of CTCL cells; that the proliferative responses to different mitogens are variable; that LyCM does not invariably induce proliferation in CTCL cultures; that long-term cultures of CTCL cells may be established using mitogens as growth factors; and that CTCL cultures require mitogenic stimulation only for initial growth.

Materials and Methods

Specimens and Culture Methods

Thirty-two specimens were received from 25 patients: 15 with SS, 8 with MF, and 2 with lymphomatoid papulosis. Cells from 21 lymph nodes and 2 visceral lesions were disaggregated by passage through a cytosieve. PBMCs were obtained from 9 heparinized blood samples by Ficoll-Hypaque density gradient centrifugation. For culture, 10⁶ cells/ml were seeded into 25 or 75 cm² plastic flasks in RPMI-1640 medium (GIBCO, Grand Island, N.Y.) containing 20% heat-inactivated fetal bovine serum (growth medium). Cells were fed every 7 days and passed whenever a tenfold increase in cell concentration occurred. Replicate flasks of the last 7 specimens were supplemented with the addition of single mitogens. Cultures were tested for mycoplasma contamination by Microbiological Associates, Bethesda, Md.

Lymphocyte Morphology and Cell Markers

Fresh and cultured tumor cells were characterized morphobiologically and by cell surface markers. Blood smears and cytocentrifuge preparations were stained with Wright-Giemsa. Sections of formalin-fixed specimens were stained with hematoxylin-eosin (HE). For electron microscopy, tumor specimens and cell pellets ofuffy coats...
and cultures were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon 812. Ultra-thin sections were stained with lead citrate and uranyl acetate, and examined in an AEI 801 electron microscope. Previously published methods were used for the detection of E rosettes, complement (C'), and EB-virus-associated nuclear antigen (EBNA).

Surface immunoglobulin (SIg) was detected by a direct fluorescent method using fluorescein-labeled goat anti-human immunoglobulin (Melody Labs., Springfield, Va.).

Tumoricogenicity

For testing tumoricogenicity, 10⁷ cells were injected intracranially (i.c.) into 4-10 athymic nude mice (nu/nu), BALB/c background (A. R. Schmidt, Madison, Wisc.). If cell numbers permitted, additional mice were injected subcutaneously, intraperitoneally, and intradermally. Mice were examined weekly for development of tumors or neurologic signs (ataxia, paralysis). Autopsies were performed on mice sacrificed electively and on those found dead, and the major organs sampled for histologic examination.

Mitogens

The mitogens used were as follows: phytohemagglutinin-M (PHA, 0.2%-5%, GIBCO); concanavalin A (Con-A, 0.2-20 µg/ml Pharmacia, Piscataway, N.J.); staphylococcal protein-A (SPA, 10-500 µg/ml, Pharmacia); pokeweed mitogen (PWM, 1%-5%, GIBCO); and LyCM, prepared by a modification of the method of Morgan et al.⁴⁶ (5%-40%). PBMCs from normal donors were pooled and stimulated with PHA, and culture fluids collected 3 and 6 days later. Stock solutions of mitogens were kept frozen at -20°C.

Lymphocyte Transformation Assays (LTAs)

Mitogen responses of PBMCs were measured by LTAs. For assays, 2 x 10⁵ cells in 0.2 ml of mitogen-supplemented growth medium were seeded into each of 6 replicate wells of microtiter plates. The mitogen concentrations tested were as mentioned above. After 3-day incubation, 1 µCi of ³H-thymidine (6.7 Ci/mole) was added to each well. Cells were harvested onto glass-fiber filter papers 6 hr later, using a multisample cell collector (Brandell, Rockville, Md.), disrupted with washes in distilled water, and their radioactivities determined. Stimulation indices (SI) were calculated as follows:

\[
SI = \frac{\text{Mean cpm of stimulated cells}}{\text{Mean cpm of unstimulated cells}}
\]

DNA Content and Chromosome Analyses

Fresh and cultured cells were analyzed for DNA content by flow microfluorometry (FMF) after staining with the fluorochrome propidium iodide.⁴⁸ The frequency distribution of fluorescent emission per cell (relative DNA content) was measured by a 128-channel TPS-1 cell sorter (Coulter Electronics, Hialeah, Fla.), and is displayed on the "x" axis of the resultant DNA histograms. The numbers of cells analyzed varied from 3 x 10⁵ to 1 x 10⁷ and is displayed on the "y" axis. The percentages of cells in the various cell compartments (G1/G0, S, and G2/M) were calculated by planimetric integration.⁴⁷ PBMCs from normal donors were used as internal and external standards. Peripheral blood smears of select patients were stained with Feulgen, and the DNA content of 100 lymphocytes analyzed with an integrated microdensitometer (Artek Systems, Farmingdale, N.Y.). Methods for chromosome studies are detailed elsewhere.⁴⁹

Assays for Migration Inhibition Factor (MIF)

MIF assays were performed by a previously published capillary tube method.⁵⁰ Hartley strain guinea pig macrophages from mineral oil stimulated ascitic fluid were suspended in RPMI-1640 medium with 10% homologous serum: Clarified test samples were diluted 1:5 in growth medium with 10% guinea pig serum. Negative control (medium with 10% guinea pig serum) and positive control (supernatant fluid from Con-A-stimulated human donor lymphocytes) were also tested.

Percent migration inhibition (%MI)

\[
\text{Percent migration inhibition} = \frac{100 \times \text{Mean area of test sample}}{\text{Mean area of negative control}}
\]

A sample was considered positive for MIF activity if the %MI was greater than 20%.

Cytochemical Staining Techniques

Cyto centrifuge preparations of cell cultures were air-dried and stained within 48 hr for the following cytoplasmic enzymes by previously published techniques:⁵⁰ α acid naphthyl acetate esterase (ANAE), beta glucuronidase (BG), acid phosphatase (AP), tartrate-resistant acid phosphatase (AP-T), α acid butyrate esterase (B.EST), and alkaline phosphatase (ALP).

RESULTS

Lack of Tumoricogenicity of Fresh CTCL Cells

Thirteen fresh CTCL specimens were injected i.c. into 102 nude mice. Six of the specimens were also injected into 42 additional mice by other routes. All of the mice remained tumor free during observation periods of 3-6 mo. Microscopic examination of the tissues of electively sacrificed mice did not reveal occult tumors.

Establishment and Characterization of B-Lymphoblastoid Cultures

Thirty-two CTCL specimens from 25 patients were cultured without the addition of mitogens. Lymphoid cells in most of the cultures died within the first 2 wk (fibroblast proliferation was observed in most of the lymph node and tumor specimens). After latent periods of 30-45 days, progressive lymphoid cell growth was observed in cultures from 6 patients. All 6 cultures had characteristics of B-lymphoblastoid cell lines,⁵ and were positive for Slg, C' receptors, and EBNA. Morphologically, the cells were large (18-25 μ) with round nuclei and prominent nucleoli. The cells had diploid DNA contents and chromosome numbers (although one became aneuploid after long-term culture), and they did not form E rosettes. Intracranial injection of these cells into nude mice induced meningeal and invasive tumors after latent periods of 42-100 days. The B-lymphoblastoid cells differed in many ways from the CTCL cells present in the patients from whom they were derived—the CTCL cells having convoluted nuclei, forming E rosettes, lacking C' receptors and Slg, having aneuploid DNA contents and chromosome numbers, and being nontumorigenic.
Mitogen-Initiated Long-Term Proliferation of CTCL Cultures

Because T cells failed to replicate in unsupplemented growth medium, one or more mitogens were added to replicate flasks. PBMCs from patient 1 with SS replicated after the addition of Con-A (10 µg/ml), and lymph node cells from patient 2 with MF proliferated in the presence of LyCM (20%). Properties of the cultures, named HUT 78 and HUT 102, respectively, are presented below. While mitogen-induced proliferation was immediate, growth rates varied, with periods of vigorous growth (tenfold increases in cell numbers/week) alternating with periods of slow growth and cell death (crises). Cells surviving the second crisis (after approximately 12 wk of culture) have proliferated vigorously and continuously for more than 52 wk without requiring mitogen supplementation; however, there was an absolute requirement for the mitogens during the first 10–12 wk.

Correlation Between LTAs and Mitogen-Induced Proliferation

The mitogen responses of cells from the last 5 CTCL patients (all with SS) were tested by LTAs and compared to the ability of mitogens to induce prolonged proliferation. The results, and those of patients 1 and 2, are summarized in Table 1. The results of PBMCs from 6 healthy donors are also presented. While all 5 mitogens stimulated normal PBMCs in LTAs (SIs of 45–150), only LyCM induced proliferation of more than 2-wk duration. The LyCM cultures increased tenfold in number every 5–7 days and consisted of 70%–83% E-rosetting cells. The cultures were discontinued after 4–6 wk. The other mitogens were toxic to normal lymphocytes, and B-lymphoblastoid cells eventually grew from these cultures.

![Fig. 1. DNA histograms of unstimulated and mitogen-stimulated PBMCs.](image)

Unresponsive patients are those whose cells had stimulation indices (SIs) of less than 10 with all 5 mitogens. The cells of patient 7 were hyperresponsive to SPA (SI = 830).

The mitogen concentrations used for proliferation (PHA, 1%–2%; Con-A, 1–10 µg; LyCM, 10%–20%; SPA, 50 µg; and PWM, 1%) were selected from the results of the LTAs.

*E-lymphoblastoid cells eventually grew from these cultures.

†ND, not done.

### Table 1. Duration of Mitogen-Initiated Proliferation

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Disease</th>
<th>Specimen</th>
<th>Summary of LTAs</th>
<th>Duration of Mitogen-Initiated Proliferation (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (6)</td>
<td>-</td>
<td>PBMC</td>
<td><strong>PHA &gt; Con-A &gt; LyCM</strong></td>
<td>None &lt;2 &lt;2 &lt;2 &gt;4 &lt;2 &lt;2 &lt;2</td>
</tr>
<tr>
<td>1</td>
<td>SS</td>
<td>PBMC</td>
<td>ND</td>
<td>&lt;2 &lt;2 &lt;2 &gt;4 &lt;2 &lt;2 &lt;2</td>
</tr>
<tr>
<td>2</td>
<td>MF</td>
<td>Node</td>
<td>ND</td>
<td>&lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2</td>
</tr>
<tr>
<td>3</td>
<td>SS</td>
<td>PBMC</td>
<td>Unresponsive</td>
<td>&lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2</td>
</tr>
<tr>
<td>4</td>
<td>SS</td>
<td>PBMC</td>
<td>Unresponsive</td>
<td>&lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2</td>
</tr>
<tr>
<td>5</td>
<td>SS</td>
<td>PBMC</td>
<td>Normoresponsive</td>
<td>&lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2</td>
</tr>
<tr>
<td>6</td>
<td>SS</td>
<td>PBMC</td>
<td>Normoresponsive</td>
<td>&lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2</td>
</tr>
<tr>
<td>7</td>
<td>SS</td>
<td>PBMC</td>
<td>SPA &gt; Con-A &gt; PHA</td>
<td>&lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2 &lt;2</td>
</tr>
</tbody>
</table>

Peripheral blood mononuclear cells from 6 healthy donors and 6 patients with SS, and lymph node cells from a patient with MF were cultured with and without the addition of mitogens. A summary of the mitogen responses, as tested by lymphocyte transformation assays (LTAs), are also presented.
thymidine incorporation and cell numbers decreased after 7–11 days. The mitogen responses of CTCL cells were more variable than those of normal lymphocytes. However, all of the CTCL samples were more resistant to the toxic effects of long-term exposure to mitogens, enabling the latter to be used as growth factors. The cells of two of the patients (3 and 4) were relatively unresponsive to all 5 mitogens (SIs of less than 10), and mitogens failed to induce replication for more than a few days. While cells of patient 5 responded normally to mitogens, none of the mitogens, including LyCM, induced growth for more than 14 days. The mitogen responses of patient 6 were also within the normal range, and PHA and LyCM induced replication of PBMCs for 4–6 wk. The cells of patient 7 were hyperresponsive to PHA, Con-A, and SPA (SIs of 255, 260, and 830, respectively) and poorly responsive to LyCM and PWM (SIs of 20 and 15, respectively). PHA induced replication for 4 wk and SPA for 16 wk. The SPA-stimulated cells, HUT 110, remained absolutely dependent on the continued presence of SPA during the entire period of proliferation. As mentioned previously, mitogen-initiated cultures were established from patients 1 and 2; however, their cells were not tested in LTAs. Thus, one or more mitogens induced proliferation of more than 14 days duration in 4 of 7 CTCL specimens.

FMF studies (Fig. 1) indicated that the vast majority of circulating normal and CTCL cells are in the G0/G1 phase of the cell cycle. In some samples the markedly aneuploid CTCL cells could readily be distinguished from the nonmalignant PBMCs (Fig. 1C). FMF data confirmed that mitogens induced DNA synthesis in both nonmalignant and aneuploid CTCL cells (Figs. 1B and 1D).

Properties of Mitogen-Initiated Cultures

A summary of the properties of mitogen-initiated cultures is presented in Table 2, along with relevant clinical data. The properties of LyCM-stimulated lymphocytes from normal donors are also presented. Surface marker studies were performed at several passages. All cultures were consistently negative for Slg, C' receptors, and EBNA. The cultures formed E rosettes whenever tested, except for HUT 78 cells which formed them only during early passages. The CTCL cultures varied in morphology, but bore a general resemblance to the CTCL cells present in the patients from whom they were derived (Fig. 2). HUT 78 and HUT 110 cells had large moderately convoluted nuclei with nucleoli, while the patients’ cells were hyperconvoluted and seldom had nucleoli. HUT 102 cells, and the CTCL cells present in patient 2’s node and bone lesions, appeared blast-like, with rounded nuclei and large nucleoli.

While initial growth rates of HUT 78 and HUT 102 were variable and factor dependent, after approximately 12 wk the cultures grew vigorously without

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### Table 2. Properties of Mitogen-Initiated Cultures

<table>
<thead>
<tr>
<th>Clinical data</th>
<th>Controls</th>
<th>HUT 78</th>
<th>HUT 102</th>
<th>HUT 110</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient No.</td>
<td>—</td>
<td>1</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Age (year)</td>
<td>—</td>
<td>53</td>
<td>26</td>
<td>44</td>
</tr>
<tr>
<td>Race/sex</td>
<td>—</td>
<td>White male</td>
<td>Black male</td>
<td>White male</td>
</tr>
<tr>
<td>Disease</td>
<td>—</td>
<td>SS</td>
<td>MF</td>
<td>SS</td>
</tr>
<tr>
<td>Extent</td>
<td>—</td>
<td>Skin, blood, nodes, liver</td>
<td>Skin, nodes, bone</td>
<td>Skin, blood, nodes, liver</td>
</tr>
<tr>
<td><strong>Properties of fresh cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E Rosettes</td>
<td>68%-84%</td>
<td>72%</td>
<td>75%</td>
<td>49%</td>
</tr>
<tr>
<td>Nuclear morphology</td>
<td>Round</td>
<td>Convoluted</td>
<td>Blast-like</td>
<td>Convoluted</td>
</tr>
<tr>
<td>DNA Content</td>
<td>Diploid</td>
<td>Bimodal</td>
<td>Bimodal</td>
<td>Bimodal</td>
</tr>
<tr>
<td>Chromosome number</td>
<td>46</td>
<td>42-92</td>
<td>42-92</td>
<td>45-88</td>
</tr>
<tr>
<td>Tumorigenicity</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Properties of cultured cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Source</td>
<td>Blood</td>
<td>Blood</td>
<td>Node</td>
<td>Blood</td>
</tr>
<tr>
<td>Growth factor</td>
<td>LyCM</td>
<td>Con-A</td>
<td>LyCM</td>
<td>SPA</td>
</tr>
<tr>
<td>Proliferation time (wk)</td>
<td>&gt;4</td>
<td>&gt;52</td>
<td>&gt;52</td>
<td>16</td>
</tr>
<tr>
<td>Mitogen dependence</td>
<td>Continuous</td>
<td>Initial</td>
<td>Initial</td>
<td>Continuous</td>
</tr>
<tr>
<td>E Rosettes</td>
<td>&gt;70</td>
<td>15%-28% (initially)</td>
<td>58%-80%</td>
<td>82%</td>
</tr>
<tr>
<td>Slg, C' Receptors</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>EBNA</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Morphology</td>
<td>Blast-like</td>
<td>Convoluted</td>
<td>Blast-like</td>
<td>Convoluted</td>
</tr>
<tr>
<td>DNA Content</td>
<td>Diploid</td>
<td>Bimodal</td>
<td>Near-diploid</td>
<td>Bimodal</td>
</tr>
<tr>
<td>Chromosome number</td>
<td>46</td>
<td>47-85</td>
<td>45-92</td>
<td>ND*</td>
</tr>
<tr>
<td>Tumorigenicity</td>
<td>ND</td>
<td>Yes</td>
<td>No</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND, not done.*

The properties of 3 mitogen-initiated CTCL cultures are compared with those of the CTCL cells present in the patients from whom they were derived.

The properties of LyCM-stimulated cultures of 6 healthy donors are also presented.
mitogen requirements. The cultures failed to respond to any of the 5 mitogens in LTAs. Currently, after 15–18 mo in culture, their population doubling times are as follows: HUT 78, 26 hr; HUT 102, 38 hr.

DNA histograms indicated that HUT 102 cells had a near diploid DNA content (Fig. 3). However, its G0/G1 peak was broad and had a coefficient of variation (CV) of 8.7% (control PBMCs and B-lymphoblastoid cultures always had CVs of less than 5%). HUT 102 cells had a modal chromosome number of 46 (range 45–46), but 7% of the cells were tetraploid. Cytogenetic studies of patient 2’s skin and bone lesions were abnormal; most of the CTCL cells had a near diploid chromosome number (42–47) with occasional tetraploid cells. A DNA histogram of the cells in his bone lesion demonstrated a G0/G1 peak with a near diploid content, but with a widened CV (6.5%). DNA content and chromosome analyses of HUT 78 and
HUT 110 cells, and the CTCL cells present in the patients from whom they were derived, demonstrated bivalent aneuploid populations in all, with near diploid and subtetraploid populations (Figs. 2 and 3 and Table 2).

Nude mice injected i.c. with HUT 102 cells remained healthy and did not develop tumors during observation periods of up to 6 mo. All mice injected i.c. with HUT 78 cells developed neurologic signs or visible intracranial tumors with elevation of the frontal bones within 40–85 days. Histologic examination revealed tumors in the meninges, with invasion of the underlying brain tissue (Fig. 4). An intracranial tumor arising in a mouse injected i.c. with HUT 78 cells was reestablished in culture (HUT 78Nu). It grew vigorously from the onset and did not require or respond to mitogens. These cells, like the parent HUT 78 cells, lacked surface markers and EBNA. DNA histograms of HUT 78Nu cells revealed a single, subtetraploid peak (Fig. 3).

All mitogen-initiated and B-lymphoblastoid cultures were free of mycoplasma contamination.

**MIF Activity**

All three long-term CTCL cultures had MIF activity in the supernatant fluids (Table 3).

**Cytochemical Studies.**

The cytochemical staining reactions of the long-term CTCL cultures are presented in Table 4. All three cultures stained positive for ANAE, BG, and AP, and were negative (or +/−) for AP-T, B.EST, and ALP. The ANAE reaction was an ill defined punctate pattern, localized to the perinuclear area.

**DISCUSSION**

Multiple attempts by us to propagate CTCL cells in vitro without mitogen supplementation resulted in the

<table>
<thead>
<tr>
<th>Sample</th>
<th>% MIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>5.1%</td>
</tr>
<tr>
<td>Positive control</td>
<td>83.2%</td>
</tr>
<tr>
<td>HUT 78</td>
<td>72.9%</td>
</tr>
<tr>
<td>HUT 78Nu</td>
<td>52.4%</td>
</tr>
<tr>
<td>HUT 102</td>
<td>80.9%</td>
</tr>
</tbody>
</table>

Assay method and the controls are described in the text. Culture fluids were tested at dilutions of 1:5. Samples with % MIF greater than 20% are considered positive for MIF activity.
establishment of several B-lymphoblastoid cultures, but failed to replicate T cells. Presumably, these cultures represent in vitro transformation of circulating nonmalignant B cells by EB virus present in specimens from seropositive patients. The B-lymphoblastoid cultures differed from CTCL cells in many aspects—surface markers, morphology, DNA content, and chromosome number. While aneuploid CTCL cells were nontumorigenic in nude mice, the diploid B-lymphoblastoid cells induced invasive intracranial tumors. Some, but not all, B-lymphoblastoid and lymphoma cultures are tumorigenic in nude mice, especially by the intracranial route.\(^{15}\)

In vitro proliferation of T cells from normal donors in liquid and semisolid media requires stimulation with mitogens or antigens.\(^{10,24,25}\) T-cell mitogens, other than LyCM, are toxic after exposure for a few days. Long-term proliferation of normal T cells requires continuous exposure to LyCM or antigen. Crossen et al.\(^{26}\) demonstrated that PHA induces division in Sezary cells, a finding confirmed and extended by us. Our FMF studies unequivocally demonstrated that mitogens induce proliferation in aneuploid CTCL cells. Short- and long-term proliferation of CTCL cells required initial exposure to mitogens. Mitogens induced proliferation in 4 of 7 CTCL specimens, with the establishment of 2 long-term cultures. While Sezary cells have been reported to be unresponsive to some mitogens,\(^{27}\) selective hyperresponsiveness may occur.\(^{28}\) Our studies, using 5 mitogens, demonstrate that the responses of CTCL cells are highly variable, ranging from relatively unresponsive to normal or selectively hyperresponsive. Details of these studies will be presented elsewhere. Lymphocyte transformation assays may be used to screen mitogens for use as growth factors. While a correlation was not always present, CTCL cells from patient 7 were hyperresponsive to SPA, a stimulator of both T and B cells,\(^{29}\) and the cells replicated in the presence of this mitogen for several weeks. The establishment of long-term cultures was aided by the apparent resistance of CTCL cells to the toxic effects of sustained mitogen exposure. Cultures HUT 78 and 102 may represent the first reported long-term lymphoma cultures initiated with the use of mitogens as growth factors. We are currently investigating whether long-term cultures release their own growth factors. Nonlymphoid cells may also release factors that aid the growth of normal and malignant lymphocytes.\(^{24,30}\) Ishii et al. established a subtetraploid, non-E-rosetting culture from a patient with CTCL after attempted fusion and cocultivation with mouse fibroblasts.\(^{31}\)

In contrast to normal T lymphocytes, relatively few Sezary cultures replicated in the presence of LyCM. The supernatant fluids of lectin-stimulated lymphoid cells (LyCM) contain a T-cell growth factor and residual lectin. The former only acts on already proliferating lymphocytes, a function provided by the latter.\(^{25}\) The variable responses of CTCL cells to lectins may account for the failure of LyCM to induce proliferation in all CTCL cultures. We are currently exploring the effects of sequentially adding mitogens (selected from the results of LTAs) and LyCM on CTCL proliferation.

Unlike B-lymphoblastoid cultures, mitogen-initiated cultures expressed T-cell surface markers and were aneuploid, as were the CTCL cells from which they were presumably derived. Circulating CTCL cells rarely incorporate thymidine\(^{27}\) and are seldom observed in mitosis, perhaps accounting for the morphological differences between CTCL cells and the rapidly dividing cultures. These findings may also explain the inability of CTCL cells to replicate in vitro without mitogen stimulation. While CTCL cells frequently are hyperconvoluted and form E rosettes, they may lack one or both of these features.\(^{33,34}\) Cytophotometric and cytogenetic data indicate that the cells are often aneuploid, with a wide range of DNA content from near diploid to tetraploid.\(^{31,38,32}\) These variations in surface markers, morphology, and DNA content are reflected in the different properties of the mitogen-initiated cultures.

Fresh CTCL cells injected into nude mice by a variety of routes failed to induce tumors. However, one of the long-term cultures, HUT 78, induced invasive tumors after intracranial injection. The tumorigenic cells were subtetraploid, suggesting that they had a greater oncogenic potential than the near diploid subpopulation also present in the parent culture. Most of the fresh, nontumorogenic CTCL specimens contained large numbers of aneuploid cells. Surprisingly, diploid B-lymphoblastoid cultures established from the same patients were tumorigenic.
MIF activity has been demonstrated in the sera of five of six patients with SS and could be traced to the circulating CTCL cells. All three of our long-term CTCL cultures released a MIF-like substance into their supernatant fluids. Patients with CTCL cultures released a MIF-like substance into circulating CTCL cells. Thus, release of MIF by CTCL cells may be of considerable clinical significance. We are currently testing the cultures for release of other lymphokines, including LyCM. Initial CTCL proliferation was factor dependent, but eventually became independent, suggesting that the cultured cells release their own growth factors. Our preliminary unpublished data support this theory.

The cytochemical staining reactions of the long-term cultures were consistent with them having a T-cell origin. The cultures had varying degrees of positivity for ANAE, AP, and BG. While nonspecific acid esterase activity has been extensively utilized to indicate cells of monocytic origin, modification of the standard methodology to demonstrate ANAE activity delineates a distinctive ANAE-positive lymphocyte population in human peripheral blood and lymphoid tissues, which correlates with the T-cell population. Under these modified conditions, monocytes display diffuse cytoplasmic staining, while T cells have a more nodular or punctate reaction. The cultures stained positive in a granular pattern. While ANAE is a consistent finding in resting T cells, mitogens may differentially activate T-cell subpopulations that may retain or lack activity, thus, 100% of Con-A-stimulated blasts retain activity, while only 25% of PHA-stimulated blasts stain positive. HUT 78, and its tumorigenic subline HUT 78Nu, were initiated by Con-A stimulation, while HUT 102 was initiated with LyCM. Unfortunately, data on ANAE activity in LyCM-stimulated T cells are not available. ANAE activity is also present in lymphoid malignancies of T-cell origin, including CTCL. AP and BG are not useful in distinguishing normal T and B lymphocytes, but these enzymes are consistently present in T-cell malignancies, including CTCL. In contrast, these enzymes are rarely present in B-cell lymphomas. Thus, the cytochemical reactions of the cultured cells are similar to previous observations of fresh CTCL cells.

Freshly isolated CTCL cells may serve as effective helper cells for immunoglobulin synthesis by B cells, suggesting that they represent a specific T-cell subpopulation. However, CTCL cells demonstrate heterogeneity with regard to receptors for IgM and IgG. The variation in mitogen-initiated proliferation of CTCL cells suggests that they may arise from several subpopulations. Functional characterization of CTCL cultures may further our understanding of the biology of normal and malignant T cells.

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REFERENCES

Mitogen requirements for the in vitro propagation of cutaneous T-cell lymphomas

AF Gazdar, DN Carney, PA Bunn, EK Russell, ES Jaffe, GP Schechter and JG Guccion