Establishment of Continuous Cultures of T-Cell Acute Lymphoblastic Leukemia Cells at Diagnosis

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ABSTRACT

We have devised methods facilitating the establishment of continuous cultures of T-cell blasts from patients with acute lymphoblastic leukemia of T-cell type at diagnosis. The cultured cells closely resemble those of the patients at the time of diagnosis with respect to surface markers, karyotype, and T-cell receptor gene rearrangements. Cultured T-cell acute lymphoblastic leukemia (diagnosis) cells (a) are lymphocytes with a convoluted nucleus; (b) have doubling times of 24–48 h; (c) are dependent for growth on interleukin 2; (d) are reverse transcriptase negative; (e) do not form colonies in methylcellulose; and (f) are clonal with respect to T-cell receptor β chain rearrangements. Three T-cell acute lymphoblastic leukemia cultures had a normal diploid karyotype, and one had a 6q− deletion which was also present at the time of diagnosis.

INTRODUCTION

Childhood T-cell acute lymphoblastic leukemias comprise some 15% of the heterogeneous group of the acute lymphoblastic leukemias (1). At diagnosis the patients often have a peripheral WBC of greater than 10⁸/ml, extensive bone marrow involvement (80–90% blasts), and a rapidly dividing population of relatively immature, monoclonal or oligoclonal T-lymphoblasts. The patients have a poor prognosis and a short survival unless treated without delay. Even when treated, relapse is a common occurrence in T-ALL.

Little is known about the mechanism underlying the development of T-ALL. Progress in the cytogenetic characterization of the disease has been limited, in contrast to the case of the B-cell leukemias and lymphomas where many of the specific chromosomal changes associated with lymphoid malignancies have been identified. Recurrent chromosomal abnormalities involving the α or β chains of the T-cell receptor or deletions in the short arm of chromosome 6 are seen in only about one-third of T-ALL cases. However, a surprisingly large percentage of cases (30–50% depending on stage, Ref. 2; or 3 of 4 cases, Ref. 3) show apparently normal karyotype. Acquisition of autocrine growth properties or activation of specific oncogenes may play a role in the early phases of the disease (3–7). However, difficulties in maintaining cultures of leukemic cells from patients at diagnosis have impeded the study of the induction of T-ALL.

A number of leukemia or lymphoma cell lines of T-cell origin have been established from patients in relapse (3, 8–13). Success has, until recently, been limited to patient samples provided at the time of relapse; even in these cases only a small fraction of the samples gave rise to lines and only a small fraction of the cells grew out (8–10). Recently, Smith et al. (12) have reported conditions which have enabled them to grow out a cytogenetically normal, growth factor-independent population of leukemic cells from most patients with T-ALL at diagnosis or in relapse. The conditions used involved hypoxia and initial supplementation of insulin-like growth factor I.

Normal T-lymphocytes can be grown for extended periods of time in the presence of IL-2 (13–15). However, the length of time is variable (from about 2 weeks to >13 months), and it is not clear whether all samples are able to give rise to long-term cultures. One recent study reported that long-surviving T-cell cultures are rare and that most cultures of human T-lymphocytes possess a limited in vitro life span (16). It is probable that long-term cultures of normal T-lymphocytes remain polyclonal (17).

Here we report new conditions which allow the long-term culturing of virtually every T-ALL sample taken at diagnosis with outgrowth of nearly 50% of the cells plated as determined by trypan blue staining of cells before plating and at daily intervals after activation. These cultures should enable us to study the growth properties of T-ALL cells at diagnosis and possibly identify early events responsible for the development of the disease.

MATERIALS AND METHODS

Source of Leukemic Cells. The cells in this report were derived from patients (ages 6–16 years) with T-cell acute lymphoblastic leukemia at diagnosis. The protocol was approved by the Committee on Investigations Involving Human Subjects at the University of California, San Diego, and informed consent was obtained from the patients or their parents. Clinical and pathological data on each patient are presented in Table 1. Bone marrow or peripheral blood lymphocytes were collected for the purpose of routine clinical diagnosis, and cells that remained following the diagnostic procedures were cultured on the same day they were taken from the patients. Usually this was within 8 h of drawing.

Surface Markers on Cells from Patients and on Cultured Cells. Cells (10⁶) were washed twice in PBS plus 0.2% sodium azide and stained directly as follows. Monoclonal antibody (50 μl) (anti-T and anti-CALLA (Coulter), T101 (Hybritech), anti-Leu M5 (Becton Dickinson Co.) was added to each tube followed by a 30-min incubation on ice. Cells were washed twice with PBS plus 0.2% azide and 50 μl of diluted (1:20 in PBS) fluorescein isothiocyanate goat anti-mouse IgG (Antibodies, Inc.) were added. Following an additional 30 min incubation on ice, the cells were washed twice in PBS plus 0.2% azide, and fixed in 75 μl of 1% formalin. The percentage of fluorescent cells was counted by using a fluorescence microscope and a fluorescence-activated cell sorter.

Determination of Terminal Deoxynucleotide Transferase. Terminal deoxynucleotidyl transferase was determined by standard techniques (18).

Conditioned Medium from Activated Normal Peripheral Blood Lymphocytes. Peripheral blood lymphocytes (HPBLS) from normal donors were separated on Ficoll-Hypaque, washed, activated, and grown as described below for leukemic cells. For 2 to 3 weeks following activation these cultures proliferated and secreted factors. HPBLS CM was col-
**T-ALL CONTINUOUS CELL CULTURES**

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**Table 1 Clinical data**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Sample cultured</th>
<th>% of lymphoblasts in bone marrow</th>
<th>Culture designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. D.</td>
<td>12</td>
<td>M</td>
<td>BM + PBL</td>
<td>97</td>
<td>Andia</td>
</tr>
<tr>
<td>T. P.</td>
<td>8</td>
<td>M</td>
<td>BM + PBL</td>
<td>95</td>
<td>Timpani</td>
</tr>
<tr>
<td>G. M.</td>
<td>16</td>
<td>M</td>
<td>PBL</td>
<td>94</td>
<td>Gem</td>
</tr>
<tr>
<td>V. P.</td>
<td>15</td>
<td>F</td>
<td>BM</td>
<td>80</td>
<td>Vepa</td>
</tr>
<tr>
<td>R. M.</td>
<td>4</td>
<td>M</td>
<td>PBL</td>
<td>ND</td>
<td>Rym</td>
</tr>
</tbody>
</table>

* BM, bone marrow; PBL, peripheral blood lymphocytes; ND, not determined.
* 342,000 WBC/µl in peripheral blood, 88% blasts.
* 204,000 WBC/µl in peripheral blood, 83% blasts.
* Relapse patient.
* 400,000 WBC/µl in peripheral blood, 99% blasts.

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**RESULTS**

Continuous Cultures of T-ALL Cells. We have found conditions facilitating the long-term culture of T-cell blasts from the peripheral blood and from the bone marrow of T-ALL patients at diagnosis. Following a single exposure of Ficoll-Hypaque separated lymphoblasts to CI and PMA, most T-ALL samples (four of five) grown in medium D with IL-2 and HPBL CM gave rise to continuous cultures which could be maintained for essentially unlimited time (more than 6 months).

Sixty to 90% of T-ALL cells were estimated by visual inspection of the cultures to respond to treatment with CI plus PMA (within 1 to 2 h) by forming large aggregates of blasts. Following 8–10 h of activation some 50% of the cells were viable as estimated by trypan blue staining. The activated cells responded to IL-2 plus HPBL CM by dividing continuously (doubling time, ~48 h) and their viability remained high (80–90%). In long-term culture, the cells grew as partially aggregated suspension cells. When human AB serum was omitted from the medium, the cells appeared to differentiate and form adherent cell layers with fibroblastoid characteristics, but lacking T-cell markers (data not shown). T-ALL (diagnosis) culture supernatants were tested for the presence of reverse transcriptase activity and all were found to be negative, indicating lack of retrovirus production. In contrast to established lines of T-ALL (relapse) cells (e.g., Molt-4, CEM, HSB-2), cultured T-ALL (diagnosis) cells did not produce colonies in methyl cellulose or soft agar cultures, suggesting their “nonprogressed” (i.e., non-transplantable) state. Like the cultured T-ALL (diagnosis) cells, T-ALL cells taken directly from patients at diagnosis do not grow in methyl cellulose or soft agar cultures nor under the skin of nude mice (22), indicating that the cells have not yet undergone “progression” to full transplantability. As we have shown previously (23) there is a close, though not complete correlation between the cloning of blood cells in methyl cellu-lose cultures and their transplantation in vivo.

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**T-Cell Surface Markers.** Clinical data on four T-ALL patients at diagnosis and one patient in relapse from whom successful continuous cultures have been established are shown in Table 1. Immunophenotypic analysis to identify T-cell surface markers was performed on mononuclear cells separated from peripheral blood and bone marrow at diagnosis and again following propagation in culture (Table 2). Also shown in Table 2 are data on a culture grown from normal peripheral blood mononuclear cells that had been treated identically and propagated for 13 days. A panel of immunophenotypic markers was re- tained on T-ALL cells through long-term culture in the presence of IL-2 plus HPBL CM, including T11 (CD2, erythrocyte receptor), T3 (CD3, mitogenic pan-T), T4 (CD4, T-helper), T101 (CD5, pan-T-lymphocyte), Leu9-3A1 (CD7, pan-T-lymphocyte), T8 (CD8, T-cytotoxic), T9 (transferrin receptor), and T10 (thyrocyte). All cultured T-ALL cells were 100% positive for terminal nucleotidyl transferase (data not shown). Two markers that were tested for a percentage of the cells of some of the T-ALL patients at the time of diagnosis (CALLA or CD10, and OKT6 or CD1) was lost during long-term culturing of the cells. This suggests that during culturing, the T-ALL cells undergo a degree of differentiation, since the expression of CD11 is found on cortical thymocytes but not on medullary thymocytes or normal peripheral blood T-lymphocytes (24), and expression of CD3 [T-cell receptor (25)] is found on more mature thymocytes. Alternatively, it could be argued that a subpopulation of mature, polyclonal cells is overgrowing the population in culture. This possibility seems unlikely since analyses of karyotypes and T-cell receptor gene rearrangements support the argument that a clonal tumor cell population is growing out (see below). The induction of la seen in all cases is indicative of activation (26) and is to be expected following treatment with PMA and calcium ionophore. The absolute requirement for IL-2 for the proliferation of T-ALL (diagnosis) cells explains the low incidence of CD25 (Tac, IL-2 receptor) on the surface of the cultured cells. At the concentrations of...
Table 2 Comparison of phenotypes of leukemia cells or normal peripheral blood lymphocytes (PBL) before and after propagation in continuous culture

<table>
<thead>
<tr>
<th>Surface antigen</th>
<th>Monoclonal antibody</th>
<th>Patient A. D.</th>
<th>BM</th>
<th>PBL</th>
<th>Patient T. P.</th>
<th>Timpani</th>
<th>Normal PBL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD 1</td>
<td>OKT6</td>
<td>83</td>
<td>3</td>
<td>0</td>
<td>13</td>
<td>100</td>
<td>0</td>
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<tr>
<td>CD 2</td>
<td>T11</td>
<td>69</td>
<td>93</td>
<td>98</td>
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<td>100</td>
<td>86</td>
</tr>
<tr>
<td>CD 3</td>
<td>T3</td>
<td>3</td>
<td>83</td>
<td>86</td>
<td>85</td>
<td>100</td>
<td>73</td>
</tr>
<tr>
<td>CD 4</td>
<td>T4</td>
<td>27</td>
<td>14</td>
<td>25</td>
<td>20</td>
<td>20</td>
<td>32</td>
</tr>
<tr>
<td>CD 5</td>
<td>T101</td>
<td>94</td>
<td>79</td>
<td>83</td>
<td>90</td>
<td>98</td>
<td>77</td>
</tr>
<tr>
<td>CD 7</td>
<td>Leu9-3A1</td>
<td>97</td>
<td>87</td>
<td>89</td>
<td>88</td>
<td>72</td>
<td>84</td>
</tr>
<tr>
<td>CD 8</td>
<td>T8</td>
<td>90</td>
<td>71</td>
<td>68</td>
<td>21</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>CD 10</td>
<td>CALLA-J5</td>
<td>86</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>CD 25</td>
<td>TAC</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>4</td>
<td>4</td>
<td>17</td>
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<tr>
<td>T9</td>
<td>T9</td>
<td>33</td>
<td>35</td>
<td>28</td>
<td>35</td>
<td>40</td>
<td>29</td>
</tr>
<tr>
<td>T10</td>
<td>T10</td>
<td>83</td>
<td>57</td>
<td>66</td>
<td>82</td>
<td>90</td>
<td>16</td>
</tr>
<tr>
<td>1a</td>
<td>I2</td>
<td>3</td>
<td>71</td>
<td>83</td>
<td>4</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>CD11c</td>
<td>LeuM5</td>
<td>46</td>
<td>3</td>
<td>3</td>
<td>68</td>
<td>8</td>
<td>28</td>
</tr>
</tbody>
</table>

IL-2 that were used, most of the receptors for IL-2 can be expected to be blocked by the ligand and thus be inaccessible to the Tac antibody. Only growth with a limiting concentration of IL-2 might enable one to demonstrate the presence of the IL-2 receptor on the surface of a majority of the cells.

In the case of one patient (A. D.), where continuous cultures were established both from the bone marrow and from peripheral blood lymphocytes, we see similarity with respect to cell surface markers.

Cytogenetic Findings. Three of four T-ALL cultures which we examined possessed apparently normal karyotypes, as did the original patient samples from which they were derived. This result is not surprising since cytogenetics has often failed to reveal karyotypic abnormalities in T-ALL (2, 3); indeed, cytogenetics cannot, as a rule, detect mutations or deletions in single genes. One culture (Timpani) showed a 6q- deletion, which was also present in the original patient sample (not shown). This deletion has been correlated with high c-myc expression in hematopoietic malignancies (27). Since expression of c-myc is directly correlated with proliferation and inversely correlated with differentiation, it has been suggested that elevated expression of c-myc could contribute to the proliferation of leukemic cells.

The maintenance of the 6q- abnormality during culturing demonstrates that our culture conditions permit outgrowth of leukemic cells, and argues that phenotypic changes observed upon culturing (see above) represent clonal evolution rather than long-term growth of normal activated T-cells. It is highly unlikely that a chromosomal deletion such as 6q- would be present in the unaffected cells of the patient.

Monoclonal Rearrangements of T-Cell Receptor β Chain (Tβ) Locus in Cultured T-ALL (Diagnosis) Cultures. The available phenotypic markers of leukemic cells define specific blastogenic states and are not leukemia cell specific. Similarly, the results of cytogenetic analysis fail to provide consistent markers for leukemic cells. We have therefore relied on Southern blot analysis of T-cell receptor β chain gene rearrangements to demonstrate that our cultures represent the original leukemic clone. These rearrangements occur during T-cell ontogeny and are unique for each T-cell. They can therefore be used to identify clonal leukemic populations and to follow the evolution of that clone in culture.

Fig. 1 shows a Southern analysis of T-cell receptor β chain gene rearrangements in EcoRI-digested DNA isolated from original patient samples and from cultured T-ALL cells. As a control we have included human placental DNA which yields two bands at 12 kbp and 4.2 kbp, corresponding to the germ line configuration of the Cβ1 and Cβ2 alleles, respectively (Lane 2). In EcoRI-digested DNA from cultured normal peripheral blood lymphocytes (NPBL), placenta, patient sample (RM), 5-month continuous culture (Rym), patient sample (AD), 6-week continuous culture (Andia), and 7-week continuous culture (Vepa).
cell culture grown from it (Rym), and DNA from the original patient sample A. D. shares a common band with the DNA from the cell culture grown from it (Andia). This strongly suggests that the cultured cells derive from the original tumor cells. The loss or acquisition of bands in the cultured cells relative to the original patient samples could be due to the outgrowth of minor leukemic clones that are undetectable in the original sample, or to subsequent rearrangements arising during culturing, a phenomenon that has been previously documented (28).

Densitometric scanning of the Southern blot autoradiograms enabled us to estimate the fraction of clonal leukemic T-cells present in the original patient samples. Since rearrangements in the Cβ2 alleles are not revealed by digestion with EcoRI, the intensity of the rearranged Cβ1 band relative to the Cβ2 band reflects the proportion of the population representing that particular clonal rearrangement. The relative intensities on the same blot of the unrearranged Cβ1 and Cβ2 bands in placental cells. The loss or acquisition of bands in the cultured cells grown in culture to facilitate unlimited proliferation of cell culture grown from it (Rym), and DNA from the original sample, or to subsequent rearrangements arising during culturing, a phenomenon that has been previously documented (28).

The original patient samples are composed of clonal T-ALL cells and in part of polyclonal normal T-cells. It is unlikely that outgrowths of minor normal clones contribute to long-term clonal T-ALL (diagnosis) cultures for the following reasons. (a) Substantial percentages of the original patient samples are represented by the leukemic clones and these clones are maintained in culture; (b) normal polyclonal peripheral blood lymphocytes that are maintained in culture for up to 90 days with periodic restimulation remain polyclonal (17). These arguments suggest that the long-term cultures described here represent outgrowths of the original leukemic clones.

The absolute requirement of T-ALL (diagnosis) cells for exogenous IL-2 is surprising in light of the published reports (29, 30) that activation of pre-T-cells with CI plus PMA induces the secretion of IL-2, in addition to the induction of functional IL-2 receptors. Apparently the concentration of IL-2 that is secreted by the activated T-ALL (diagnosis) cells is insufficient to support long-term cell proliferation in culture.

While HPBL CM has been added routinely to all T-ALL cells grown in culture to facilitate unlimited proliferation of virtually all samples at any cell concentration, we have also seen that some T-ALL (diagnosis) cultures do not require HPBL CM and can be grown in medium D supplemented only with human recombinant IL-2 as long as the cell concentration is maintained at 4–6 × 10^6/ml or higher. This suggests that some samples of activated T-ALL (diagnosis) cells secrete an autocrine factor or factors required for their own proliferation, possibly by maintaining IL-2 receptor expression.

The nature of the additional factor(s) supplied in HPBL CM and possibly produced by some T-ALL (diagnosis) cells is not yet known. Preliminary experiments indicate that this activity cannot be substituted by recombinant interleukins (IL-1 through IL-6), cerebrospinal fluids, or interferon, nor by a combination of recombinant factors.

T-ALL (diagnosis) cultures are growth factor dependent (requiring IL-2 and an additional factor or factors) and have a minimum of apparent chromosomal aberrations. They therefore resemble normal peripheral blood lymphocytes in many respects. However, they differ in that they contain a dominant clone (or clones) from the beginning. That they do not grow autonomously, but require activation and exogenous growth factor, suggests that the in vivo environment must be playing an important role in T-ALL. The ability to maintain T-ALL (diagnosis) cells in culture should facilitate the study of these cells and their growth requirements. This in turn should lead to a better understanding of the early events in the disease.

The activated IL-2-dependent continuous cultures of T-ALL (diagnosis) cells that we describe here differ from the IL-2-independent lines recently established under hypoxic conditions from T-ALL (diagnosis) samples by Smith et al. (12). It is possible that patient samples contain clonal T-ALL cell blasts that are heterogeneous with respect to their differentiation state, as well as their responsiveness to activation. Thus the two methods might select for different subpopulations of T-ALL cells belonging to the same clone. Alternatively, different culture conditions may induce different changes in the cells.

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REFERENCES

8. Smith, S. D., Shatsky, M., Cohen, P. S., Warnke, R., Link, M. P., and Glader, B. E. Monoclonal antibody and enzymatic profiles of human malig-


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