Growth and Differentiation of a Human T-Cell Leukemia Cell Line, CCRF-CEM, Grafted in Mice

Peter J. Houghton, Joseph Mirro, Jr., Rakesh M. Goorha, Susana C. Raimondi, Arnold Fridland, and Janet A. Houghton

ABSTRACT

The growth of human CCRF-CEM T-cell lymphoblastic leukemia was studied in mice immune deprived by different techniques, and in CD-nu/nu athymic mice. Female CBA/CaJ mice were immune deprived by infant thymectomy, priming with 1-β-d-arabinofuranosylcytosine (200 mg/kg) 48 h prior to total body irradiation (925 cGy) designated ara-Cγ; or after thymectomy the mice received 925 cGy total body irradiation with marrow reconstitution (4 × 10⁶ nucleated cells), designated BMγ. Only in mice immune deprived by γ-radiation, subsequently given a single dose of cyclophosphamide (100 mg/kg) 18–24 h before transplantation of CCRF-CEM, was there progressive reproducible engraftment and tumor growth. For mice immune deprived in this manner the tumor engraftment rate was 100 and 80% of tumors achieved ≥1 cm³ within 46 days. In immune-deprived CBA/CaJ mice, but not CD-nu/nu athymic mice, tumor transplanted to the s.c. site metastasized to paraortic and axillary nodes. Metastatic spread to lymph nodes was confirmed by immunophenotyping and by karyotyping.

In contrast to the CCRF-CEM cells in culture, which expressed cytoplasmic CD3 (T3) but not surface CD3, both s.c. and metastatic CCRF-CEM cells xenografted in mice expressed surface CD3. The CCRF-CEM line was exposed to phorbol-12-myristate 13-acetate in vitro to mimic the apparent differentiation which occurred in the xenografted cells, and a similar expression of surface CD3 after treatment was seen. This surface expression of CD3 was accompanied by production of mRNA for the T-cell receptor α chain and surface expression of the T-cell receptor. Identical T-cell receptor β and γ chain gene rearrangements were found for the CCRF-CEM line in vitro and the xenografted cells in vivo, demonstrating that only one clone was present and that differences in immunophenotyping were not the result of clonal selection. These results suggest that host (mouse) hematopoietic factors could affect human leukemic cell differentiation.

INTRODUCTION

Despite successful transplantation of many tumor types, heterotransplantation of human hematopoietic malignancies has been difficult (1, 2). For acute myeloblastic leukemia, the lack of growth may be a result of terminal differentiation (3, 4). Clutterbuck et al. (4) reported that 17 of 19 fresh acute myeloblastic leukemia samples transplanted into mice resulted in formation of palpable tumors, but 16 of these tumors regressed. These authors suggested that tumor regression was a consequence of differentiation. Machado et al. (5) have demonstrated that the human myeloid cell lines KG-1, KG-1a, and HL60 may also undergo some changes in mice that are consistent with differentiation. The KG-1a cell line, however, could be propagated only in newborn mice (5), suggesting a possible influence of host immunity. Further immune deprivation of athymic mice enhances successful heterotransplantation of human leukemic cells (6), using established cell lines (1, 7). Transplantation to the intracranial site in athymic mice increases the ability to transplant human lymphomas (8). There are two reports of successful engraftment of human T-cell leukemia (1, 7), using the MOLT-4 line, and in both instances further immune deprivation of athymic mice was necessary. It has been postulated that T-cell leukemias may be sensitive to natural killer cells (9), and that the decrease in natural killer activity that occurs after irradiation (10) might explain the increased tumor growth (7).

Despite the increased rate of engraftment, few data are available concerning the uniformity of growth of individual tumors, which would allow such tumors to have utility as preclinical models. The T-cell leukemia, CCRF-CEM, has been used extensively for studies of drug metabolism and cytotoxicity in vitro, hence we were interested in developing a reproducible model which would allow extension of these experiments in vivo. CCRF-CEM cells have been grown as xenografts in athymic nude mice (11), although a systematic study of growth characteristics has not been reported. We have xenografted CCRF-CEM cells into immune-deprived mice and have documented that the resulting tumors are human by karyotyping, immunophenotyping, and genotyping (using T-cell receptor genes). We report the growth, metastatic characteristics, and the apparent ability of these human cells to differentiate in immune-deprived mice.

MATERIALS AND METHODS

CRF-CEM Cell Line. The human T-lymphoblastic leukemia CCRF-CEM was originally established by Foley et al. (12), and was obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in suspension culture in RPMI-1640 supplemented with glutamine and 10% fetal calf serum. For inoculation into mice, CCRF-CEM cells were suspended at 1 × 10⁷/ml in serum-free medium and 100 µl were inoculated s.c. into each flank. Cells were inoculated into mice 7–14 days after total body irradiation.

Immune Deprivation. Female CBA/CaJ mice, 3–4 weeks old, were thymectomized (13). Subsequently they received 925 cGy total body irradiation by using a 137Cs source. Mice either received priming with 1-β-d-arabinofuranosylcytosine, 48 h prior to radiation (designated ara-Cγ), or received 4 × 10⁶ nucleated bone marrow cells within 6 h of irradiation (designated BMγ). A third protocol was used where mice were immune deprived by γ-radiation and in addition received CPA (100 mg/kg) 18–24 h prior to tumor transplantation (designated BMγ + CPA). For serial transplantation, CCRF-CEM xenografts were excised aseptically, cut into pieces approximately 3 mm³, and transplanted to the s.c. space of recipient mice, within 2 weeks of irradiation. All procedures utilized a class B biological safety cabinet. Mortality associated with the increased rate of engraftment may be attributed to the increase in natural killer activity that occurs after irradiation (10) and might explain the increased tumor growth (7).

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associated with immune deprivation was <4% for each protocol. Six-week-old female CD-1 nu/nu mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). Mice were housed in filter top cages in humidity and temperature-controlled conditions. Experiments were in accordance with institutional guidelines, using protocols reviewed and approved by an internal review committee.

Xenograft Tumor. Tumor growth was determined by measurement of two perpendicular diameters at 7-day intervals (Mascal digital caliper interfaced with a microcomputer). Tumor volume was calculated from the formula \( V = \pi r^2 d \), where \( d \) is the mean diameter (13).

Xenograft s.c. tumors and grossly involved lymph nodes were removed by sterile technique after the mice had undergone cervical dislocation. The lymph nodes and the primary tumor were grossly excised from normal surrounding mouse tissue, placed in RPMI-1640 saline between steps, and minced by using scalpel and forceps. A single cell suspension was made by passing the cells through a 21-gauge needle. Viable cells were subsequently separated by using Ficoll-Hypaque (1.077 g/ml).

For controls, normal mouse lymphocytes were obtained after cervical dislocation from mice that were not inoculated with CCRF-CEM cells. Lymphocytes from peripheral blood were isolated by using Ficoll-Hypaque (1.077 g/ml), while normal lymphocytes from lymph nodes were isolated in exactly the manner described above for the metastatic tumor.

Morphological and Cytochemical Studies. Microscopic sections of xenografted tumor and metastatic lymph nodes were made by fixing tissue in 10% buffered formaldehyde and routine processing. Sections were stained with hematoxylin-eosin and examined by light microscopy. Cells in suspension were stained by using standard techniques, including Wright-Giemsa, periodic acid-Schiff reagent, myeloperoxidase, Sudan black B, and α-naphthyl butyrate esterase. The CCRF-CEM cell line, xenograft tumor, metastatic tumor, and normal mouse lymphocytes (from blood and lymph nodes) were examined by light microscopy after staining.

Surface Immunophenotyping. All cells were incubated in heat-inactivated pooled human AB serum to prevent nonspecific Fc binding. The expression of all cell surface antigens was detected by a direct immunofluorescence assay. Monoclonal antibodies from clusters of differentiation (CD) groups defined by the International Workshops on Leukocyte Differentiation Antigens were used for phenotyping (14). Antibodies for CD7 (Leu-9), CD2 (Leu-5b), CD8 (T8), CD3 (T3), CD4 (T4), and the T-cell receptor (WT-31) were used to assess the expression of T-cell surface antigens; CD10 (J5, CALLA), CD19 (B4), and CD22 (Leu-16) were used to detect B-cell-associated surface antigens. Myeloid-associated antigens were also assessed by using antibodies CD13 (MY7) and CD33 (MY9). All antibodies were directly conjugated to fluorochromes and were purchased commercially (Leu series from Becton Dickinson, Mountain View, CA, and all other MAbs from Coulter Immunology, Hialeah, FL). Antibody usage conformed to supplier's suggestions. In all experiments, mouse immunoglobulins were directly conjugated to the appropriate fluorochrome were used to control for nonspecific binding (negative control) and nonspecific reactivity was subtracted from the histogram.

After fluorescent staining, cells were analyzed with a Coulter EPICS 753 flow cytometer (Coulter Immunology). For MAbs conjugated to fluorescein, the fluorescence was detected with a 525-nm narrow band pass filter, with dead cells excluded from analysis, based on propidium iodide staining. Phycoerythrin fluorescence was detected with a 575-nm narrow band pass filter.

Cytoplasmic Immunophenotyping. Cryopreserved leukemic cells were tested for cytoplasmic antigens by an alkaline phosphatase staining method (15). Cyto centrifuge cells (2 × 10⁶/slide) were fixed in cold acetone (4°C for 3 min) and then were stained with Leu-4 (CD3) or isotype-matched myeloma immunoglobulin as the negative control (25°C for 45 min). Sequentially, with washing in phosphate-buffered saline between steps, an affinity-purified biotinylated horse antimouse IgG (Vector Laboratories, Burlingame, CA) was added (25°C for 30 min), followed by avidin DH:biotinylated alkaline phosphatase H complex (Vector Laboratories) (25°C for 45 min) and finally, an alkaline phosphatase substrate (Vector Laboratories) (25°C for 20 min).

TPA Induction of Differentiation in CCRF-CEM Cells. TPA (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO. Exponentially growing cell cultures were treated with TPA at a final concentration of 16 nm (10 ng/ml) for 5 or 7 days. CCRF-CEM cultures with an identical concentration of DMSO (0.1%), were used for controls in all immunophenotyping studies and in the analysis of T-cell receptor gene expression. DMSO at this concentration did not affect cell growth or differentiation.

Cytogenetic Analysis. The cytogenetic analysis was performed by the method of Williams et al. (16) that includes treatment with trypsin and staining with Wright stain to obtain G banding. The chromosomal abnormalities are described as recommended by the international System for Cytogenetic Nomenclature.

Molecular Genetic Analysis. High-molecular-weight DNA was extracted from the cell line and s.c. xenograft tumor by the method of Wigler et al. (17). DNA was quantified by using a fluorometric assay (Hoefer Scientific Instruments, San Francisco, CA). It was then digested with BamHI (Boehringer Mannheim, Indianapolis, IN), EcoRI (Bethesda Research Laboratory, Gaithersburg, MD) or HindIII (Bethesda Research Laboratory) restriction endonucleases (3 to 10 units/μg of DNA) electrophoresed through 1% agarose gels (FMC Bioproducts, Rockland, MD), and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH). Blots were then probed with oligolabeled DNA probes for the genes encoding the β and γ chains of the TCR. The probe for the β chain of the TCR was pB4000, which contains about 0.4 kilobase from the Cβ2 region and hybridizes equally well to the Cβ1 and Cβ2 (18). The γ chain probe P31 contains 0.7 kilobase from the joining region of the γ-TCR gene (19). After hybridization, the filters were washed (52°C) in 0.1 x standard saline citrate/0.1% sodium dodecyl sulfate and then exposed to Kodak XAR film (Kodak, Rochester, NY).

Cytoplasmic RNA (10–20 μg) was size-fractionated by electrophoresis through 1% agarose gel containing 2.2 M formaldehyde, followed by linkage to Nytran (Schleicher & Schuell) by UV light. The α chain gene of the complementary DNA clone containing an insert encoding the human T-cell receptor has been described (20). The insert was oligolabeled to specific activity of 1 to 2 x 10⁶ cpm/μg DNA (21). Total cellular RNA was prepared by a guanidium/cesium chloride procedure (22). DNA was denatured in 50% formamide/2.2 M formaldehyde, electrophoresed in 1% agarose, and linked to Nytran by UV light. After hybridization for 24 h, the membrane was washed twice in 0.1 M saline citrate solution at 50°C for 15 min. The membranes were exposed to Kodak XAR film (~70°C, 24–48 h) with an intensifying screen.

RESULTS

Preliminary experiments with CCRF-CEM showed that consistent growth of s.c. tumors was rare. This was further examined by using mice immune deprived by infant thymectomy followed by priming with aara-C5, bone marrow reconstitution alone (aara-BM), aara-BM conditioning with CPA (aara-BM + CPA) prior to CCRF-CEM transplant, or in CD athymic nude mice. Results, presented in Table 1, have been analyzed for frequency of palpable tumors, the proportion that attained 1 cm³, and the number of tumors that regressed having once become palpable.

For mice prepared by using aara-C5, 84% of implantations gave rise to palpable tumors, but only 47% reached 1 cm³ or greater. In this model 50% of tumors regressed. Growth patterns for tumors growing in opposite flanks of the same mice are presented in Fig. 1A. Clearly, growth was dependent upon the host, as bilateral tumors grew in a similar manner, i.e., progressive growth of each tumor, or regression of both. While such data did not eliminate the possibility of host-induced differentiation, it suggested that variable growth in the aara-C5 mice may be caused by host-mediated rejection. An alternative model in which mice were reconstituted with syngeneic bone marrow (aara-BM) was evaluated (Table 1). In these mice there was a higher frequency of palpable tumors, with 75% reaching...
GROWTH AND DIFFERENTIATION OF CCRF-CEM CELLS

Table 1 Heterotransplantation of CCRF-CEM lymphoblastic leukemia

<table>
<thead>
<tr>
<th>Passage</th>
<th>Host</th>
<th>No. of implant sites</th>
<th>No. of palpable tumors (%)</th>
<th>No. of tumors &gt;1 cm^3 (%)</th>
<th>No. of regressed tumors (%)</th>
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<tr>
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<td>4 (27)*</td>
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<tr>
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<td>8</td>
<td>4</td>
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<tr>
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<td>ara-C-y</td>
<td>12</td>
<td>5</td>
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</tr>
<tr>
<td>10</td>
<td>BM</td>
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<tr>
<td>11</td>
<td>BM</td>
<td>12</td>
<td>12 (94)</td>
<td>2</td>
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</tr>
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<td>14</td>
<td>10</td>
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<tr>
<td>14</td>
<td>BM + CPA</td>
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<td>28 (100*)</td>
<td>22 (79*)</td>
<td>6 (13)</td>
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<tr>
<td>15</td>
<td>BM + CPA</td>
<td>14</td>
<td>14</td>
<td>12</td>
<td>1</td>
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</table>

* Infant thymectomy, 1-β-D-arabinofuranosylcytosine (200 mg/kg), 925 cGy total body irradiation.
* Numbers in parentheses, mean for experiments using a given immune-deprivation technique.
* Infant thymectomy, 925 cGy total body irradiation, 4 x 10^6 nucleated bone marrow cells.
* As for (c), plus CPA (100 mg/kg) 1 day prior to transplant.
* Comparisons between ara-C-y and BM + CPA. P < 0.05.

Fig. 1. Growth of CCRF-CEM cells as xenografts in immune-deprived mice or athymic CD-na/nu mice. Tumor fragments were transplanted bilaterally into each flank of recipient mice. Growth of tumor was determined by measurement of two perpendicular diameters at 7-day intervals. Open and closed symbols represent growth of tumors in the same host. A, Passage 5 xenografts in CBA/CaJ mice prepared by ara-C-y; B, Passage 5 xenografts in CBA/CaJ mice prepared by ara-C-y; C, Passage 6 xenografts in athymic female CD-na/nu mice. Data are from representative experiments.

1 cm^3. However, many of these tumors (in two studies) regressed. Again, tumor growth was dependent upon the individual host, as determined by the similar growth pattern between bilateral tumors (Fig. 1B). Similar growth patterns were observed when CCRF-CEM tumors were transplanted in CD athymic nude mice (Fig. 1C). In these mice 75% of tumors grew to 1 cm^3 but approximately 37% of palpable tumors regressed.

As BM appeared to be superior to ara-C-y with respect to frequency of tumor takes (P = 0.05) and the proportion of tumors reaching 1 cm^3 or greater (P = 0.048), this model was chosen for further development. Mice immune-deprived BM were given a single nontoxic dose of CPA (100 mg/kg; 0.55 x 5% lethal dose level) 18 to 24 h prior to transplantation of CCRF-CEM. Results from 3 separate experiments are shown in Table 1. All implantations gave rise to palpable tumors with 7.5% reaching 1 cm^3 within 46 days of transplantation (Table 1). Growth of tumors in mice prepared by BM + CPA are shown in Fig. 2. The time for tumors to double in volume, during exponential growth was 1.1 ± 1.2, 9.2 ± 1.5, and 8.4 ± 1.2 (SE) in 3 separate studies.

Characteristics of CCRF-CEM Xenografts. Histological sections from both the s.c. tumor and metastatic lymph nodes revealed a monomorphic population of cells with a high nuclear:cytoplasmic ratio, prominent nucleoli, and a high mitotic index consistent with a malignant lymphoid tumor. Xenograft (s.c.) tumors were invasive both to the dermis and to underlying muscle (Fig. 3A). In mice immune deprived by ara-C-y, BM, and BM + CPA, there was gross evidence of metastatic spread to paraaortic and axillary nodes which achieved 5-7 mm diameter in some animals (Fig. 3B). No other organs appeared grossly involved. In approximately 10% of mice disseminated abdominal disease appeared secondary to involvement of paraaortic nodes. In these mice there was no obvious direct

* Infant thymectomy, 1-β-D-arabinofuranosylcytosine (200 mg/kg), 925 cGy total body irradiation.
* Numbers in parentheses, mean for experiments using a given immune-deprivation technique.
* Infant thymectomy, 925 cGy total body irradiation, 4 x 10^6 nucleated bone marrow cells.
* As for (c), plus CPA (100 mg/kg) 1 day prior to transplant.
* Comparisons between ara-C-y and BM + CPA. P < 0.05.

Fig. 2. Growth of CCRF-CEM xenografts in immune-deprived CBA/CaJ mice conditioned with CPA. Mice were immune deprived by BM, and in addition received CPA (100 mg/kg i.p.) 18-24 h prior to transplant of tumor. Each curve represents growth of an individual tumor, open and closed symbols being indicative of tumors growing in opposite flanks of the same host.

Fig. 3. Characteristics of CCRF-CEM xenografts growing in immune-deprived CBA/CaJ mice. A, photomicrograph of a s.c. tumor (x 60). Inset demonstrates invasion into underlying muscle (x 175). B, photomicrograph of paraaortic node metastasis (x 300).
extension of tumor from the s.c. site. Involvement of lymph nodes was not found in CD-\(nu/nu\) mice at autopsy.

The morphology and cytochemical characteristics of the CCRF-CEM cell line (from both early and late passage) and xenograft tumor were identical. Cultured and xenografted cells had the same lymphoid appearance with a large nuclear/cytoplasmic ratio, opened nuclear chromatin pattern with nucleoli. There were cytoplasmic vacuoles present in both the cell line and the xenograft tumor. The cytochemical staining pattern was identical (Table 2), except for a slight decrease in the intensity of the periodic acid-Schiff staining in the xenograft tumor cells.

Cytogenetic analysis confirmed that the cells in the s.c. tumor and the metastatic lymph nodes were of human origin. The karyotype of the CCRF-CEM human T-cell leukemia maintained in vitro had a near tetraploid modal number (Fig. 4A). The consistent abnormalities observed were: \(-X,-X\), extra copies of chromosome 20 and G-group size chromosome, \(der(1)t(1;?)p232;?)\), 2x del(8)(p11), 2x deleted and inverted 9, 2x der(9)t(9;?)p242;?), der(10)(p14). The modal chromosome number of the s.c. tumor and the metastatic lymph node samples were also near tetraploid and had the same markers described above. However, additional acquired changes in xenografted cells were as follows: del(3)(q25), der(5)t(5;?)p157;?), der(7)t(7;?)q367;?), marker (Fig. 4B).

The immunophenotypic data confirmed that the xenograft tumor from \(\theta yBM + CPA\) mice was human since cells from the xenograft expressed human CD7 and CD4 surface antigens like the original cell line (Table 3). The xenograft tumor and the in vitro CCRF-CEM cells were negative for CD2, CD8, CD19, CD10, CD20, and CD13. Both the CCRF-CEM cell line and the xenograft contained cytoplasmic CD3 antigen. There was no nonspecific binding of any MABs to mouse lymphocytes (Table 3).

There was a difference in the expression of 2 antigens between the cell line and the xenograft tumor. The xenograft tumor expressed surface CD3 while the original cell line did not. Alternatively, a few of the xenograft tumor cells weakly expressed surface CD33 (MY9) while cultured CCRF-CEM cells did express this antigen to a greater extent (Table 3). The xenograft tumor cells from \(\theta yBM + CPA\) mice appear to conform more closely to normal human T-lymphoid development by increasing CD3 expression and decreasing CD33 expression (MY9) on the cell surface all 3 times they were tested (Fig. 5). Furthermore, the CD4 surface expression is less intense in xenografted CCRF-CEM cells than in the CCRF-CEM cell line (Fig. 6). When CCRF-CEM xenograft tumor cells from \(\theta yBM + CPA\)-treated mice were reestablished in culture they continued to express CD3, less CD4, and very little CD33 (for at least 12 passages), a phenotype very similar to that when they were grown as xenografted tumor (data not shown).

The CCRF-CEM cell line was treated with TPA to induce differentiation as an in vitro model of the xenograft tumor.

### Table 2 Morphology and cytochemical reactivity

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cell line</th>
<th>Xenograft (s.c. tumor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology* (Wright-Giemsa)</td>
<td>Lymphoid</td>
<td>Lymphoid</td>
</tr>
<tr>
<td>Periodic acid-Schiff</td>
<td>Strong block positive</td>
<td>Block positive</td>
</tr>
<tr>
<td>(\alpha)-Naphthyl butyrate</td>
<td>Weakly positive</td>
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<tr>
<td>Myeloperoxidase</td>
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</tr>
<tr>
<td>Sudan black B</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

* For complete description see text.

TPA treatment resulted in a marked increased surface expression of CD3 which was accompanied by surface expression of the T-cell receptor identified by monoclonal antibody WT-31 (23) (Fig. 7). DMSO alone without TPA had no apparent effect on differentiation as measured by surface antigen expression.

Molecular genetic analysis was performed to confirm the human origin of the xenografted cells (Fig. 8) and to determine whether or not CCRF-CEM cells and xenografted tissue represented the same clone. It is evident that the s.c. tumor cells contained DNA sequences which hybridized to the human probes for the T-cell receptor \(\gamma\) and \(\beta\)-chain genes, while mouse lymphocytes or mouse cell lines do not (Fig. 8). The CCRF-CEM cell line and xenograft tumor cells demonstrated identical rearrangements in both \(\gamma\) and \(\beta\) chain genes of the T-cell receptor. This result confirms that the cell line grown in vitro and xenograft tumor cells growing in vivo are progeny of a single clone.

Expression of T-cell receptor genes \(\beta\) and \(\alpha\) was studied by Northern blot analysis (Fig. 9). Message for \(\beta\) chain gene was present in both the TPA-induced as well as uninduced CCRF-CEM cells, and as expected the message was also present in xenografted cells (Fig. 9B). By contrast, \(\alpha\) chain mRNA was present only in TPA-induced CEM cells and xenografted cells but not in uninduced CCRF-CEM cells (Fig. 9A). These results are consistent with the immunophenotyping results demonstrating the appearance of surface CD3 and T-cell receptor expression on TPA-induced CCRF-CEM cells and xenografted cells.

### DISCUSSION

Our objective in this study was to establish the CCRF-CEM T-cell leukemia as a xenograft, having sufficiently reproducible growth characteristics to allow this model to be used for evaluation of therapy in mice. To determine whether tumor growth was dependent upon host factors (i.e., induction of maturation, or immunity), tumors were implanted bilaterally into either flank (13). Thus inter- and intraanimal variation could be determined. The immune-deprivation model used first consisted of the "standard" preparation (\(\theta ara-C\gamma\)), in which priming with 1-\(\beta\)-n-arabinofuranosylcytosine is used prior to irradiation, and obviates the necessity for marrow reconstitution (24). As shown (Fig. 14; Table 1), most mice developed palpable tumors, but <50% reached 1 cm\(^3\), and 50% regressed completely. Of note is that growth of bilateral tumors was essentially similar, suggesting a significant host effect upon growth. We next examined growth of CCRF-CEM in CBA/CaJ mice immune deprived with marrow reconstitution (\(\theta BM\)), and in athymic CD-\(nu/nu\) mice. Results were quite similar to those obtained with \(\theta ara-C\gamma\) mice, in that a strong host effect was observed. Further, the frequency of successful grafts and regressions were similar in the athymic mice. Mice prepared by \(\theta BM\) had a higher frequency of palpable tumors, 75% of which achieved 1 cm\(^3\) or greater; however, there was a fairly high frequency of regression. Conditioning mice with a nontoxic dose of CPA, administered 18–24 h before tumor transplant increased the frequency of tumor development to 100%, with very few spontaneous regressions. Growth of individual tumors was consistent, with a doubling time of 9.1 ± 1.2, 9.2 ± 1.5, and 8.4 ± 1.2 days in three separate experiments. Thus, conditioning with CPA appeared to enhance tumor growth. As immune-deprived mice tolerate chemotherapeutic agents as well, or better than athymic mice, and may be housed under conventional conditions, CCRF-CEM xenografts in immune-
Fig. 4. A, karyotype of CCRF-CEM human T-cell leukemia maintained in vitro demonstrating that the cells were of human origin and had a near tetraploid modal number of chromosomes with the deleted and inverted chromosome 9. B, the karyotype of the CCRF-CEM heterografted cells were identical in the s.c. tumor and metastatic lymph node. The modal chromosomal number was also near tetraploid with the same markers as the cell line but with additional acquired changes (see text for details).
Table 3 Immunophenotype results

<table>
<thead>
<tr>
<th>Cluster group</th>
<th>CCRF-CEM line</th>
<th>Xenograft</th>
<th>Metastatic lymph node</th>
<th>Mouse controls</th>
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<td></td>
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* For all positive results given as percentage of positive cells, intensity of staining is given: +, weak; ++, moderate; ++++, intense; --, negative.
* ND, not done.

Fig. 5. Fluorescence histograms; (A and C) CCRF-CEM cells grown in vitro and used to establish xenografts. (B and D) CCRF-CEM cells from xenografts. Thin lines demonstrate results for control immunoglobulins, while heavy lines demonstrate results for test antibodies; A and B demonstrate increased CD3 expression by xenografted tumor, while C and D demonstrate the decreased expression of CD33 by xenografted tumor.

Fig. 6. Fluorescence histograms of CCRF-CEM cells grown in vitro and xenografted (A) compared to metastatic lymph node (B). Normal mouse lymphocytes do not express the human CD4 antigen (D).

Fig. 7. Fluorescence histograms of CCRF-CEM cells grown in vitro in the absence of TPA (DMSO control). A and C, or in the presence of TPA (16 nM): B and D. Thin lines demonstrate the results for control isotype matched immunoglobulins, while heavy lines demonstrate results of test antibodies. Treatment with TPA results in a marked increased expression of surface CD3 (A and B) and a marked increased expression of surface TCR (C and D).

As a xenograft growing s.c. in immune-deprived CBA/CaJ mice, CCRF-CEM was highly invasive, both to the dermis and to underlying muscle. In each immune-deprived model (athymic nude mouse), >90% of mice with tumor >1 cm³ had involvement of paraaortic and axillary nodes without direct extension of the s.c. tumor into the abdominal cavity. In approximately 10% of mice, massive abdominal disease appeared secondary to involvement of paraaortic nodes. The consistent and reproducible metastatic behavior of this tumor is thus of interest. Using the T-cell leukemia MOLT 4 or CCRF-CEM, neither Ziegler et al. (7), Ohsugi et al. (1), nor Weil-Hillman et al. (11) were able to detect metastatic spread in athymic nude mice. Of note is that we did not observe nodal metastases when CCRF-CEM was transplanted in CD-nu/nu athymic mice.

spread of tumor to both paraaortic and axillary lymph nodes was confirmed by karyotype analysis and by immunophenotyping, in addition to standard morphological techniques. Cytogenetic analysis demonstrated that the tumor cells observed in both tissues were derived from the original CCRF-CEM cell line (25).

Of note were differences in the immunophenotype between CCRF-CEM cells grown in mice (s.c. and metastases) compared to the original cell line, which suggests that differentiation in vivo of leukemic human T-cells may occur under the influence of mouse hematopoietic factors. Specifically, xenografted CCRF-CEM cells expressed CD3 antigen on the cell surface, whereas in culture only cytoplasmic CD3 was detected. Similarly, Northern blot analysis showed that xenografted but not cultured CCRF-CEM cells synthesized mRNA for α-chain gene.

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GROWTH AND DIFFERENTIATION OF CCRF-CEM CELLS

Fig. 8. Autoradiographs of Southern blots demonstrating identical rearrangements of the \( \beta \) and \( \gamma \) chain genes of the T-cell receptor in CCRF-CEM cells and the xenograft tumor. Samples (10 \( \mu \)g) of DNA were digested with restriction endonuclease, electrophoresed through a 0.8% agarose gel, and blotted to nitrocellulose filters. A, EcoRI digests probed with the \( \gamma \) chain joining region probe; B, HindIII digests probed with the \( \gamma \) chain probe; C, BamHI digests probed with the \( \beta \) chain constant region probe. In each figure, Lane 1 is DNA from KB cells (germ-line configuration); Lane 2 is DNA from HPB-ALL cells (rearranged genes; positive control); Lane 3 is DNA from the CCRF-CEM cell line grown in vitro; Lane 4 is DNA from xenografted tumor cells (s.c.); Lane 5 is DNA from mouse liver (negative control).

of TCR. These findings suggest that CCRF-CEM T-cells growing in mice were more closely following normal differentiation by expressing surface CD3-TCR complex along with surface CD4 (14, 15, 26). Parenthetically, it should be mentioned that the amount of \( \alpha \) chain mRNA in xenografted cells was much less (about 25-fold reduction) than in the TPA-induced CCRF-CEM cells. These are, at least, two reasons for the reduced amounts of \( \alpha \) chain mRNA: (a) internal control, using actin mRNA as an indicator of cellular RNA concentration, showed (Fig. 9C) that amount of total cellular RNA in xenograft is less (about 5-fold reduction). Similar conclusion was reached by the examination of \( \beta \)-chain mRNA (Fig. 9B) or RNA (data not shown) concentration in these cells; (b) it appears that induction of \( \alpha \) chain mRNA in xenografted cells is not as abundant as in TPA-induced CCRF-CEM cells. However, it should be pointed out that TPA is not a physiological inducer for T-cell differentiation and, therefore, there is no valid comparison in the quantitative aspects of \( \alpha \) chain mRNA induction.

Although the CD7+, CD4+, CD3- immunophenotype found in both the CCRF-CEM cell line and xenograft tumor is unusual for acute lymphocytic leukemia, it is reported for T-cell lymphomas (14). The clone of CCRF-CEM cells in our laboratory also express the myeloid-associated CD33 antigen. The expression of CD33, a myeloid-associated cell surface antigen on otherwise typical lymphoblasts is relatively common. In our prospective studies, 16% of children with otherwise typical acute lymphocytic leukemia will have lymphoblasts that express myeloid-associated cell surface antigens (27, 28). In fact, CD33 is one of the most commonly expressed antigens. We have called such cases “mixed-lineage leukemia” (27). These cases may result from transformation of an early stem cell capable of both myeloid and T-cell differentiation, or alternatively from aberrant gene regulation with expression of the CD33 antigen during lymphoid differentiation (27, 28). Since subclones of CCRF-CEM cells exist which do not express CD33 antigens, the simplest explanation is that this antigen is aberrantly expressed.

These immunophenotypic differences in the cell line and xenografted cells may represent either clonal selection during tumor growth or in vivo differentiation of human T-cells under the influence of mouse hematopoietic factors. However, both the CCRF-CEM cells grown in vitro and the xenograft tumor cells have identical patterns of rearranged \( \beta \) and \( \gamma \) genes of T-cell receptor. This finding demonstrates that cells in culture and from xenografted tumor are progeny from a single clone. Examination of two separate genes, namely \( \beta \) and \( \gamma \), and use of two different restriction endonucleases for each gene, discounted the possibility that the identical pattern of rearrangements is due to chance. Thus, the data indicate that in vivo differentiation of human T-cells occurs under the influence of mouse hematopoietic factors.

It has been shown that TPA can induce expression of CD3...
polypeptides with or without the T-cell receptor on the surface of T-cell lines (29, 30). Carrel et al. (29) reported that CCRF-CEM cells could express surface CD3 without surface TCR, and suggested that separate regulatory mechanisms may exist for CD3 surface expression and TCR. Their studies also suggested that expression of the TCR complex is not controlled by transcription of the TCR a chain, which may be increased after TPA treatment in CCRF-CEM subclones that do not express TCR on the cell surface. Some sublines of CCRF-CEM cells, however, do express CD3 polypeptides accompanied by the T-cell receptor when treated with TPA (30), which is consistent with our results. Our clone of CCRF-CEM cells demonstrated a marked increase in surface CD3 and TCR expression after treatment with TPA (Fig. 7). Furthermore, cells also demonstrated abundant appearance of mRNA for the a chain of the T-cell antigen receptor when treated with TPA (30), which is consistent with our (15) and other investigators (30) results which show that a chain transcription is usually accompanied by TCR surface expression.

In summary, we have established conditions for reproducible and metastatic growth of human T-cell leukemia in mice. Under these conditions, >90% of host animals develop distant lymph node metastases, and conditions for growth appear to stimulate maturation of those cells to give a more normally differentiated T-cell phenotype. The model described may be of value for study of T-cell differentiation, and as an in vivo model for preclinical pharmacological studies of T-cell leukemia.

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Growth and Differentiation of a Human T-Cell Leukemia Cell Line, CCRF-CEM, Grafted in Mice

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