Isolation of Mouse T-Cell Lymphoma Lines from Different Long-Term Interleukin 2-dependent Cultures

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ABSTRACT

A number of different biological properties have been ascribed to the hormone-like protein interleukin 2 (IL-2). However, the most salient feature of this lymphokine is its ability to sustain the long-term proliferation of T-cells from humans and mice. Reported herein are the results of studies demonstrating the isolation of growth factor-independent cell lines from the long-term IL-2-dependent murine T-cell line CTLL-2 that is used frequently as the source of target cells in IL-2 bioassays. Sustained log-phase growth of these T-cells in vitro has been achieved using Petri dishes of polymethylpentene; growth could not be sustained in similar dishes of glass, untreated polystyrene, polystyrene that had been treated for cell culture, or polycarbonate.

The IL-2-independent line grew as a T-cell lymphoma when injected i.p. into pristane-treated, but not untreated, syngeneic C57BL/6 mice. In contrast, cells from the IL-2 parental line CTLL-2 did not grow in vivo.

Characterization of the IL-2-independent lines propagated in vitro (denoted as line CEC) or in vivo (denoted as line CEP) demonstrated that they retained their dependency for 2-mercaptoethanol and expressed phenotypic profiles of their parental line CTLL-2 (Thy 1.2*, Lyt-1*; Lyt-2*). Isolation of an IL-2-independent T-cell lymphoma from a CTLL-2 line obtained from another investigator using a protocol that has proven reproducible under carefully controlled laboratory conditions and defined phenotypic traits of the syngeneic T-cell isolates provided evidence that the tumors were not a cross-culture contaminant arising as a result of a laboratory accident. Moreover, karyotypic analysis using a quinacrine:Hoechst banding technique revealed similar marker chromosomes in the IL-2-dependent and -independent lines.

IL-2-independent lines have also been established from the IL-2-dependent murine T-cell line CT-6.

Accordingly, the results of these studies suggest that, during prolonged cultivation that has included exposure to crude IL-2 preparations known to contain phorbol ester, possibly viruses, and other contaminants, the IL-2-independent lines have developed subpopulations that are thought to have undergone malignant transformation of unknown etiology to generate IL-2-independent murine T-cell lymphomas that can be passaged repetitively either in vitro or in vivo.

INTRODUCTION

IL-2 is a lymphokine produced by T-cells of the blood and lymphoid organs of humans and different animals following either specific (i.e., antigenic) or nonspecific (i.e., mitogenic) stimulation, and by lymphoblastoid T-cells of certain long-term human and murine leukemic-lymphoma lines (1-9). A number of biological properties have been ascribed to this hormone-like protein that has been purified to homogeneity from lymphocytes (10-12) and from prokaryotic cells in which the IL-2 gene had been expressed using recombinant DNA technology (13, 14); however, the most important feature of this immunoregulatory molecule is its T-cell growth-promoting activity (4, 5, 9, 15-17). IL-2-dependent monoclonal T-cells retaining functional and phenotypic properties characteristic of normal human and mouse lymphocytes have been described by many investigators (5, 8, 18-21). More recently, studies have demonstrated that the in vivo administration of IL-2 or IL-2-dependent immunocompetent lymphocytes may be of therapeutic benefit (22-28).

The absolute dependency of lines for this T-cell growth factor has provided the basis for bioassays used for the quantitative analysis of IL-2. First described in 1977 (2), the assay measures the growth of IL-2-dependent T-cells as defined by the incorporation of radiolabeled thymidine or by the enumeration of viable cells in microcultures containing a standard of defined IL-2 activity (e.g., positive control), an equal volume of diluent (e.g., negative control), or test material. The cells most frequently used for the IL-2 assay have been from the CTLL-2 (15), CT-6 (25), or HT-2 (21) lines, all of which have been cultured continuously for more than 6 yr. Thoroughly washed IL-2-dependent T-cells seeded at a density of 2 x 10⁶/ml in the presence of an optimal amount of IL-2 grow to a maximal cell density, usually recorded on culture Day 3 or 4, of 1.0-1.3 x 10⁶ cells/ml. Most cells grow as aggregates consisting of a few to several dozen cells. In the absence of IL-2, the cells die rapidly, and viabilities recorded for corresponding control cultures on Day 3 or 4 are very low (2, 4, 5, 9, 20), usually less than 1%. While conducting IL-2 assays, we noted a small number of atypical cells anchored to the bottom surface of polystyrene culture flasks. Examination of these adherent cells revealed that they were probably a viable subpopulation of slowly growing lymphocytes that were morphologically distinct from the rapidly proliferating free-floating IL-2-dependent T-cells. A method was developed that permitted the isolation and subsequent log-phase propagation of this lymphocyte subpopulation in the absence of IL-2. These cells grew as a highly invasive T-cell lymphoma when injected into pristane-treated syngeneic mice. Malignant T-cell lines have been successfully established from CTLL-2 lines obtained from different laboratories. Likewise, using carefully controlled cultivation procedures, leukemia T-cell lines have been isolated from T-cell line CT-6. The successful isolation of leukemic T-cell populations that express phenotypes and features characteristic of the parental line from which they were derived provides evidence that these IL-2-independent T-cell lines did not arise as a cross-contamination of cultures or other laboratory
accidents. We believe that these IL-2-independent leukemic T-cells arose as a result of malignant transformation during prolonged cultivation that frequently included repetitive exposure to crude IL-2 preparations obtained using different procedures, including those in which 4Phorbol-12-myristate-13-acetate, a known tumor promoter, is used as a means of activating mouse (e.g., EL-4; Ref. 1), human (e.g., Jurkat; Ref. 3) and nonhuman primate (e.g., MLA-144; Ref. 10) leukemic T-cell lines.

**MATERIALS AND METHODS**

**Cell lines.** The cell lines used in this study, references to publications describing their origin and characteristic features, and investigators from whom they were obtained included the following: (a) CTLL-2 (19), Dr. Steve Gillis, Immunex Corp., Seattle, WA; Dr. R. A. Miller, Boston University School of Medicine, Boston, MA; and Dr. H. Fuji, Roswell Park Memorial Institute, Buffalo, NY; (b) CT-6 (29), Dr. John Farrar, NIH, Bethesda, MD; and (c) HT-2 (21), Dr. James D. Watson, University of Auckland, Auckland, New Zealand.

Procedures used for the generation of the CTLL-2 T-cells have been described elsewhere (19). In brief, the CTLL-2 line was initiated from spleen cells of a C57BL/6 mouse (H-2b) which had been inoculated with allogeneic Friend leukemia virus-induced erythroleukemia cells, F-4-5 (H-2b). A secondary and tertiary in vitro mixed tumor-lymphocyte culture reaction was performed using mitomycin C-inactivated F-4-5 stimulator cells. The effector cells, defined as CTLL-2, were shown to mediate both allogeneic and syngeneic tumor-specific cytolyis (19), and monoclones of this line have been propagated in continuous culture with IL-2.

Cells of different lines were cultivated in 25-cm² polystyrene culture flasks (Coming Glass Works, Coming, NY) using Medium RPMI 1640 (Grand Island Biological Co., Grand Island, NY; Gibco) that contained FCS (10%; K. C. Biological, Inc., Lenexa, KS), previously heated (56°C, 45 min) and filtered (0.45 µm), antibiotics (penicillin, 100 units/ml; streptomycin, 50 µg/ml; Gibco), L-glutamine (2 mM; Gibco), 2-ME (2 x 10⁻⁵ M; Fisher Scientific, Pittsburgh, PA), and an optimal concentration of IL-2 (see below) for sustaining maximal cell proliferation. The loosely capped flasks with 12 ml of cell suspension were incubated in a stationary circular motion resulted in the formation of cell clusters consisting of 3-8 cells. During the next 2 wk, the size and number of these clusters increased. These were collected, deposited by centrifugation, resuspended in fresh medium, and cultivated in the absence of IL-2 at a density of about 10⁴-10⁵/ml.

**IL-2-independent derivatives of these lines were propagated using similar conditions and medium that did not contain the T-cell growth factor or other growth-promoting lymphokines. In addition, these cells were propagated in 100-mm Petri dishes of polymethylpentene (No. 5500-0010, Nalge Co., Rochester, NY; Ref. 30). Growth of these cells was also evaluated in comparative studies utilizing 100-mm Petri dishes of polycarbonate (No. 5502-0010, Nalge Co.); polystyrene, untreated (No. 1001; Falcon, Becton Dickinson Co., Oxnard, CA); polystyrene treated for tissue culture (No. 3003; Falcon); and glass (No. 3160-101; Coming Glass Works).

**Interleukin 2.** Murine T-cells provided by different investigators for these studies had been propagated using IL-2 generated using different procedures and included both crude and purified preparations. IL-2 that we used routinely was concentrated and partially purified using previously described procedures (31) and methods including cell lines EL-4 (1), MLA-144 (10), and Jurkat-FHCRC (5). For selected studies, we used hr-IL-2 (Ref. 13; kindly provided by Dr. Kirsten Koths, Cetus Corp., Emeryville, CA). The amount of hr-IL-2 required for achieving maximal cell proliferation varied with each line; however, in most instances, greatest growth was obtained with a hr-IL-2 concentration of 20-50 units/ml.

**Mice.** IL-2-independent T-cell lymphomas were passaged in 4- to 6-wk-old C57BL/6 mice that had received 0.2 ml of pristane (Aldrich Chemical Co., Milwaukee, WI) 1-2 days prior to injection of the cells (32). The tumors were transferred routinely by the i.p. injection of 0.2 ml of freshly collected ascites (~13 x 10⁶ cells; viability, >98%) or thoroughly washed cells (~25 x 10⁶; viability, >80%) isolated from an i.p. lymphoma that had been teased apart in 0.5 ml of PBS (pH 7.2; 0.14 M NaCl; 0.29 M KCl; 8.1 M NaHPO₄; 1.46 M KHP₂O₄).

**Phenotyping.** Phenotypic profiles of the mouse T-cells were established using previously described immunoperoxidase indirect procedures (33) and peroxidase-conjugated goat anti-rat immunoglobulin antibody (γ- and κ-chain specific; Accurate Chemical and Scientific Corp., Westbury, NY, ACS) and purified monoclonal antibodies Thy-1.2, Lyt-1, and Lyt-2 (Becton Dickinson Monoclonal Center, Inc., Mountain View, CA). Other reagents used included those for identifying surface immunoglobulins (peroxidase-conjugated rabbit anti-mouse immunoglobulins, adsorbed; ACSC) and macrophages (rat antibody IgG to mouse macrophage; ACSC).

**Chromosome Studies.** For cytogenetic analysis, cultures of exponentially growing cells were treated with Colcemid (0.1 µg/ml) for 2 h at 37°C. Cells collected by centrifugation were treated with 0.075 M KCI for 30 min at 37°C, then fixed in three changes of methanol:acetic acid (3:1), and spread on slides by air drying. Slides were stained with quinacrine and 33258 Hoechst (34) and examined under a fluorescence microscope. Suitable metaphases were photographed, and karyotypes were analyzed in accordance with the standard nomenclature (35).

**RESULTS**

**Isolation of IL-2-independent T-cells.** Mouse IL-2-dependent CTLL-2 T-cells had been propagated continuously with crude or partially purified IL-2 in our laboratory for more than 4 yr to provide a source of target cells for routine IL-2 bioassays. The IL-2-independent T-cell lines were isolated from cultures that had been initiated in a manner similar to that used for propagating the parental CTLL-2 T-cells; however, these "control" cultures were initiated so as to contain no IL-2. After 4 days of culture, a sample of the free-floating cell suspension revealed that more than 99% of the cells were dead as defined by the trypan blue dye exclusion assays. At this time, the flask was washed 4 times with PBS to remove all nonadherent cells. After adding 15 ml of PBS, the flask was chilled (4°C) for 2 h. Thereafter, the flask was shaken vigorously to dislodge the adherent cells. Total number of adherent cells was low, usually 1-10 x 10⁶; viability, however, was good (>90%). The cells, pooled from 3-8 flasks, were then deposited by centrifugation (200 x g, 12 min) and resuspended in FCS-supplemented medium without IL-2. Aliquots of 2-3 x 10⁵ cells were returned to the original polystyrene flask, a new flask, or to Petri dishes of polymethylpentene. Cells in the original or new polystyrene flasks quickly adhered to the surface. These cells remained viable for 2-3 wk but showed very little or no growth, and the addition of IL-2 was of no benefit. In contrast, cells plated in polymethylpentene Petri dishes showed little or no adherence, and agitation of the plate each day in a circular motion resulted in the formation of cell clusters consisting of 3-8 cells. During the next 2 wk, the size and number of clusters increased. These were collected, deposited by centrifugation, resuspended in fresh medium, and cultivated in the absence of IL-2 in new polymethylpentene dishes. Following another lag period, log-phase growth was achieved, and this subline, designated as CEC, has been cultivated for more than 10 mo. CEC cells injected i.p. into pristane-treated syngeneic C57BL/6 mice grew as a T-cell lymphoma that was designated as CEP to distinguish these in vivo-passaged cells from the CEC that have been maintained in vitro only.

To define whether the isolation of other IL-2-independent
populations could be established, additional studies were conducted using IL-2-dependent T-cell lines CT-6 and HT-2 as well as CTLL-2 lines provided by other investigators. Additional precautions were adopted in the laboratory to prevent cross-contamination of the cultures, and comparative phenotypic and karyotypic analyses of the IL-2-dependent and -independent populations were initiated.

**Growth of IL-2-Independent T-Cells in Different Cultureware.** Presented in Chart 1 are the results of a representative experiment comparing the growth of IL-2-independent CEC T-cells in 100-mm Petri dishes of glass and different plastics. Highest levels of cell growth were recorded for dishes of polymethylpentene, and almost all (>99%) of the cells grew as nonadherent free-floating clusters. Significantly lower levels of growth (P < 0.001) were observed in dishes of polycarbonate, polystyrene, and glass, and in these vessels, the absolute as well as the relative numbers of adherent cells were higher (P < 0.001) than in the dishes of polymethylpentene.

Experiments just described were performed using CEC T-cells derived from polymethylpentene dishes. Repetitive attempts have been made to passage serially the IL-2-independent CEC or CEP T-cells in dishes of polycarbonate or polystyrene. In all instances, a progressive decrease in cell growth occurred, and after 2–4 wk, there was no cell proliferation. Sustained log-phase growth could be initiated by returning the cells to dishes of polymethylpentene. Thus, these cells are unique in that they can be propagated only in polymethylpentene vessels, and they have retained this dependency for more than 10 mo.

**Growth i.p. of IL-2-Independent T-Cells.** Having demonstrated that the CEC cells could be cultivated in the absence of growth-promoting lymphokines, experiments were initiated to determine whether the cells could grow i.p. in syngeneic C57BL/6 mice. In these experiments, thoroughly washed CEC T-cells were injected in 0.5 ml of PBS into mice, and the animals were monitored for tumor growth for 8–10 wk, after which time they were sacrificed, and an autopsy was performed. Initial studies utilizing untreated mice failed to detect growth of the CEC T-cells. In subsequent studies, using mice that had previously been treated with pristane, tumor growth was observed repetitively. Using a protocol in which 25 × 10⁶ CEC T-cells were injected, each of 9 animals sacrificed on Day 14 showed tumors that had grown as both an ascites and as a lymphoma. Numerous tumor masses were located throughout the peritoneum, but metastatic lesions outside the peritoneal cavity were not observed. In subsequent studies in which the animals were not sacrificed, all mice died by Day 21. In contrast, no tumor developed in corresponding control animals that received pristane only or pristane and CTLL-2 T-cells.

No tumors developed in mice that were given s.c. injections of either ascites or isolated lymphoma CEP cells at concentrations that generated tumors when injected i.p. into pristane-treated animals.

The CEP cells, which have been passaged serially in vivo for more than 6 mo, were identified as Thy-1⁺ cells exhibiting a phenotypic profile and other features of the CEC cells. Fig. 1 presents a histological section of the diaphragm of a mouse in which the highly invasive nature of this T-cell lymphoma is illustrated by the penetration of the muscle fibers by the immature lymphoblastoid cells of this tumor.

**Phenotypic Characterization of the Cell Lines CEC and CEP.** Table 1 summarized the results of ongoing comparative studies that have been initiated to characterize the features of the IL-2-independent CEC and CEP T-cell lines in reference to the parental line CTLL-2.

The CTLL-2 T-cells required not only IL-2 but also 2-ME for growth. CTLL-2 cells have been cultivated in medium with IL-2, but without 2-ME, they showed little or no reduction in cell growth. CTLL-2 cells have been cultivated in medium with IL-2, but without 2-ME, they showed little or no reduction in cell growth.
proliferation during the first 3- to 4-day IL-2-dependent proliferation cycle. However, a marked reduction in proliferation was noted when the cells were subcultured under similar conditions but without 2-ME (data not presented). Likewise, both the CEC and CEP cells have retained this unique feature, and repetitive attempts to wean them from the 2-ME requirement have failed. In related studies, IL-2 was added to CEC and CEP cells that displayed log-phase growth for more than 4 mo in the absence of this or other growth-promoting lymphokines. In these experiments, CEC and CEP cells were washed thoroughly and seeded at a density of 2 x 10^5/ml of RPMI 1640 containing 2-ME and 10% FCS. Test cultures received highly purified hr-IL-2 (100 units/ml), and corresponding control cultures received an equal volume of the PBS diluent. In each of four experiments conducted during the third and fourth mo, hr-IL-2 was associated with a moderate (15-28%; P < 0.001) increase in CEC growth. However, when these experiments were repeated after 8 mo, hr-IL-2 did not augment CEC growth, and the proliferation and growth kinetic curves for these cells closely approximated that of the parental line and its growth factor-independent T-lymphoma derivative line CEC.

Studies were also undertaken to determine whether the CEC and CEP cells produce IL-2 spontaneously or would secrete this lymphokine following activation using conventional procedures including A, B, and C particles. Electron Microscopy. Examination of the CEC and CEP cells by transmission electron microscopy has failed to detect viruses, including A, B, and C particles.

Initiation of Other IL-2-independent T-Cell Lines. Realizing that the CEC and CEP cell lines may have arisen from a laboratory accident in which the parental CTLL-2 line had been cross-contaminated with other mouse cells, studies were undertaken to: (a) isolate IL-2-independent T-cells from CTLL-2 T-cell lines obtained from each of two laboratories and (b) define whether these new IL-2-independent isolates would grow as a malignant lymphoma.

IL-2-independent subpopulations were successfully isolated from CTLL-2 lines obtained from each of two laboratories and have been propagated for more than 4 mo in polymethylpentene dishes; these were designated as CEC and CPC, respectively. Attempts have been made to grow these two lines in vivo, and these efforts have generated malignant lymphoma populations designated as CEP and CPP.

Likewise, we have been successful in isolating IL-2-independent subpopulations from line CT-6, and these isolates have been designated as TPC and TPP.

Several attempts have been made to isolate IL-2-independent lymphoma populations designated as CEP and CPP.
been sustained for 10 wk; however, cell proliferation has been poor, and log phase has not been achieved.

DISCUSSION

Throughout these studies, we have been aware of the possibility that the IL-2-independent lines could have arisen as a result of a laboratory accident in which cultures of the parental line had been cross-contaminated by established mouse lymphoma lines. Evidence that has been obtained to indicate that this was not the case for IL-2-dependent lines CEC and CEP includes: (a) strict requirement for growth in culture vessels of polymethylpentene, a Teflon-like plastic that is used infrequently for cell cultivation; (b) dependency for 2-ME; (c) similar Thy-1, Lyt-1, and Lyt-2 phenotype as parental CTLL-2 T-cells; (d) marker chromosomes similar to those of the parental line; (e) growth in syngeneic C57BL/6 mice, and only in mice that have been treated previously with pristane; and (f) absence of viruses.

The strict dependency of the IL-2-independent T-cells for growth in vessels of polymethylpentene is unique. Variation in growth is noted for certain lymphoblastoid cells cultured in other plastics including polycarbonate, polypropylene, polystyrene, polysulfone, and other plastics; however, we know of no human or mouse cell lines that display an absolute requirement for a given plastic. We have noted previously that higher levels of cell growth and more cell adherence were observed consistently in polymethylpentene dishes than in similar vessels of polystyrene or glass (30), and it was based upon these observations that we selected this vessel for studies attempting to isolate the IL-2-independent populations.

Polymethylpentene is a crystalline polymer of the polyolefin family that offers a unique combination of transparency and resistance to heat and chemicals (polymethylpentene, TPX; Technical Information No. 1, Physical Properties of and Processing Technology for TPX; Mitsui Petrochemical Industries, Ltd., Tokyo, Japan). When compared to other plastics, particularly polystyrene that is utilized for most cultureware, polymethylpentene is thought to offer a number of advantages, including: (a) transparency to both visible and UV light; (b) stability to steam and dry heat (240°C); and (c) high gas permeability, particularly to O₂ and CO₂, thus providing the investigator with the ability to regulate optimal P CO₂ and P O₂ as well as a desirable pH for cells proliferating on the bottom surface of the vessel, even when the medium to atmosphere index is high. The Petri dishes used were fabricated from virgin polymethylpentene and contained no dyes, plasticizers, slip release agents, or other substances that are sometimes added in making different plastics. We have not been able to identify the reason for the dependency of the IL-2-independent cells for this particular plastic. Noteworthy was that new dishes had to be used for subculturing and that significantly lower levels of cell growth and more cell adherence were observed consistently in polymethylpentene dishes that we had attempted to recycle by cleaning with distilled water, detergents, acids, bases, enzymes, or a combination of these different methods. Moreover, relatively few vessels of polymethylpentene are of a geometric design suitable for cell culture, and multitwell microtiter plates are not available to permit cloning of these lines using conventional limiting dilution or micromanipulation procedures. Attempts to clone the lines using soft agar are in progress.

It could not be determined from these studies whether a small percentage of IL-2-independent cells propagate in IL-2-dependent cultures or whether IL-2-independent cells are being formed continuously from IL-2-dependent cells. In this respect, we know of no other reports describing the isolation of long-term growth factor-independent lines from IL-2-dependent parental cultures. Hass et al. (36) have described recently the transformation of radiation- or virus-induced neoplastic T-cells from an IL-2-inde-
ependent status to a growth factor-independent state following the intrathymic injection of the T-lymphoblastoma cells into C57BL/6 mice. Three unique cell lines have been established from patients with cutaneous T-cell lymphoma-leukemias (HUT-102, HUT-78, and CTCL-2); Refs. 6 and 7); of these lines, the latter two, initially dependent upon T-cell growth factor for proliferation, lost their requirement for exogenous IL-2 after several passages. All three lines proved to be a constitutive producer of IL-2, adsorbed IL-2, and displayed increased proliferation when exogenous IL-2 was added to the cultures. The conversion of these lines from an IL-2-dependent to IL-2-independent status is similar to that which we observed with the murine T-cell lines. The notable differences in these two studies, however, were that the mouse T-cell lines used in our studies were originally derived from healthy donors, conversion of IL-2-dependent status was detected after several yr of continuous cultivation, and the absence of IL-2 production.

Observations reported herein were those of experiments conducted with cell lines that had been cultivated for several yr and with cells that had been cloned and propagated using different procedures. Accordingly, procedures described herein are thought to have selected a subpopulation that had undergone malignant transformation; when this transformation occurred, however, is not known. Studies are currently in progress to determine whether growth factor-independent malignant T-cell populations can be isolated from human murine T-cell lines utilizing IL-2-propagated T-cells that had been cultivated for a much shorter time period before the selection process is initiated.

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REFERENCES


Fig. 1. Histological section of the diaphragm of a pristane-treated C57BL/6 mouse that had been sacrificed 16 days after receiving an i.p. injection of $25 \times 10^6$ IL-2-independent CEP T-cells. The separated smooth muscles of the diaphragm illustrate the invasive nature of this lymphoma. (H & E, $\times 100$). Inset, greater detail of the T-lymphoblasts of this same histological section. Cells thought to be dividing (metaphase figures) are identified with arrows. $\times 400$. 
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