

A lymphokine, provisionally designated interleukin T and produced by a human adult T-cell leukemia line, stimulates T-cell proliferation and the induction of lymphokine-activated killer cells

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ABSTRACT In early phases of human T-cell lymphotropic virus I-induced adult T-cell leukemia (ATL), the malignant cell proliferation is associated with an autocrine process involving coordinate expression of interleukin (IL) 2 and its receptor. However, during late-phase ATL, leukemic cells no longer produce IL-2 yet continue to express high-affinity IL-2 receptors. During studies to define pathogenic mechanisms that underlie this IL-2-independent proliferation, we demonstrated that the ATL cell line HuT-102 secretes a lymphokine, provisionally designated IL-T, that stimulates T-cell proliferation and the induction of lymphokine-activated killer cells. Conditioned medium from HuT-102, when added to the IL-2-dependent CTLL-2 line, yielded a stimulation index of 230. Since CTLL-2 was purported to be IL-2-specific, we performed a number of studies to exclude IL-2 production by HuT-102. Stimulation of CTLL-2 cells by HuT-102-conditioned medium was not meaningfully inhibited by addition of an antiserum to IL-2. Furthermore, uninduced HuT-102 cells did not express mRNA encoding IL-2 as assessed by Northern blot analysis. No biological activity on CTLL-2 cells was mediated by purified IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, or granulocyte/macrophage colony-stimulating factor, thus differentiating these factors from IL-T. Based on preliminary biochemical data, IL-T is a protein with a pI value of 4.5 and a molecular mass in SDS gels of 14 kDa. In addition to its action on CTLL-2 cells, 3200-fold-purified IL-T stimulated proliferation of the human cytokine-dependent T-cell line Kit-225. Furthermore, addition of IL-T enhanced cytotoxic activity of large granular lymphocytes (i.e., induced lymphokine-activated killer cells). Thus, IL-T is a lymphokine that plays a role in T-cell proliferation and induction of lymphokine-activated killer cells. Furthermore, IL-T may contribute to IL-2-independent proliferation of select ATL cells and lines.

T cells play both regulatory and effector functions in human immune responses that are often mediated by production of a set of polypeptides termed interleukins (ILs). Interleukins exhibit a high degree of redundancy and are also pleiotropic, controlling a wide range of functions. This redundancy is explained in part by sharing of common receptor subunits among members of the cytokine receptor superfamily. Specifically, receptors for IL-6, leukemia inhibitory factor, oncostatin-M, ciliary neurotrophic factor, and IL-11 share a gp130 signaling subunit, and IL-3, IL-5, and granulocyte/macrophage colony-stimulating factor utilize a common β_c receptor subunit (1, 2). There is a similar sharing of receptor elements within the IL-2 receptor (IL-2R) system, a system that involves α , β , and γ subunits, in that the IL-2R γ chain not only is a component of IL-2Rs but also is required for

actions of IL-4 and IL-7 (3-5). Abnormalities of IL/IL-receptor systems are observed in association with a broad array of human diseases. For example, in early phases of human T-cell lymphotropic virus I (HTLV-I)-induced adult T-cell leukemia (ATL), T-cell proliferation is associated with an autocrine process involving coordinate expression of IL-2 and its growth factor receptor (6). However, in most cases during the late phase of ATL, the leukemic cells and cell lines derived from them no longer produce IL-2 yet continue to express high-affinity IL-2Rs (7). As part of our effort to define pathogenic mechanisms that underlie this autonomous proliferation, we demonstrated that an IL-2-independent ATL cell line, HuT-102, secretes a lymphokine that stimulates T-cell proliferation and induces activation of large granular lymphocyte (LGL) cells. In the present report, we discuss the evidence supporting the existence of a non-IL-2 T-cell growth factor (provisionally designated IL-T) and results of preliminary purification and characterization studies of this IL molecule. In the companion report (8), we indicate that IL-2R β chain expression is required for IL-T action, thus expanding the spectrum of shared receptor subunits within the IL-2R system.

MATERIALS AND METHODS

Cells. The HuT-102-B2 cell line was obtained from A. Gazdar (National Cancer Institute). Kit-225-K6 is an HTLV-I-nonexpressing IL-2-dependent T-cell line derived from a patient with T-cell leukemia by T. Uchiyama (Kyoto University, Kyoto). The murine CTLL-2 cell line was obtained from Giovanna Tosato (Food and Drug Administration). LGLs depleted of T cells, B cells, and monocytes were prepared as described (9, 10).

Protocol for Generation of HuT-102 Conditioned Medium (CM) and for Purification of IL-T. The purification scheme used to obtain 3200-fold enrichment of IL-T activity is outlined in Table 1. HuT-102 cells were seeded at $1-1.5 \times 10^5$ cells per ml and cultured in roller bottles until a cell density of 1.0×10^6 cells per ml was reached. Cell-free supernatant was collected and was concentrated by ultrafiltration on a tangential flow device (Filtron Technology, Northborough, MA) with a 30-kDa cutoff. The bulk of the extraneous protein was removed from the retentate by precipitation with a 1/7 vol of Pro-Cipitate (Affinity Technology, New Brunswick, NJ). This mixture was centrifuged at $10,000 \times g$ for 30 min. In a stepwise manner, the supernatant was brought successively to 17.5% and 23.5% (wt/vol) PEG (M_r , 1500) followed by 6% PEG (M_r , 8000). Precipitated proteins were pelleted by

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Abbreviations: ATL, adult T-cell leukemia; CM, conditioned medium; HTLV-I, human T-cell lymphotropic virus I; IL, interleukin; LAK, lymphokine-activated killer; LGL, large granular lymphocyte; IL-2R, IL-2 receptor.

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Table 1. Purification of IL-T

Fraction	Total protein, mg	Total volume, ml	Total activity, units	Specific activity, units/mg of protein	Yield, %	Purification, fold
Conditioned medium	972	12,000	2.2×10^5	2.26×10^2	100	1
Ultrafiltered concentrate	989	255	2.2×10^5	2.22×10^2	100	1
Pro-Cip SN	526	280	2.2×10^5	4.18×10^2	100	1.9
PEG precipitate	33.4	31.5	7.2×10^4	2.15×10^3	32.7	9.5
Blue-Sepharose SN	8.4	3.7	5.6×10^4	6.67×10^3	25.5	29.5
Pro-Cip/CM-Blue-agarose, lectin SNA SN	2.27	0.52	2.6×10^4	1.15×10^4	11.8	50.6
Gel filtration	0.27	0.4	7.2×10^3	2.67×10^4	3.3	118.1
RP-HPLC	0.004	0.2	2.8×10^3	7.0×10^5	1.3	3200

RP, reversed phase; SNA, *Sambucus nigra* bark agglutinin; SN, supernatant; Pro-Cip, Pro-Cipitate. Units of activity were determined by the CTLL proliferation assay.

10,000 \times g centrifugation for 30 min at each of these PEG levels. Finally, an additional 17% PEG (M_r , 8000) plus 14.5% EtOH were added to the supernatant and centrifuged, and the resulting pellet was extracted once for 3 min with 95% EtOH to remove excess PEG. The IL-T in the pellet redissolved in 18 mM Mes (pH 5.7) was further purified by adsorption with Pharmacia fast Blue-Sepharose, followed by a repeat precipitation by addition of a 1/9 vol of Pro-Cipitate. The resulting supernatant was then adsorbed successively with CM-Blue-agarose (Bio-Rad) and the lectin *Sambucus nigra* bark agglutinin (SNA) on agarose (EY Laboratories). The unadsorbed solution was concentrated and brought to 0.3% SDS, heated at 56°C for 10 min, and injected onto a 1 \times 30 cm Superose 12 gel filtration column. The mobile phase for the run was 70 mM NH_4HCO_3 /15 mM triethylamine (TEA)/12 mM trifluoroacetic acid/100 mM hexafluoroisopropanol/25% (vol/vol) acetonitrile/12% (vol/vol) isopropanol (resulting pH \approx 8.2). Fractions were lyophilized and redissolved in 100 μ l of deionized water and those with peak bioactivity were brought to 3.8 M guanidine hydrochloride/120 mM Tris-HCl, pH 8.9/15 mM TEA/40 mM trifluoroacetic acid/70 mM diisopropylethylamine/7% acetonitrile, 0.2- μ m (pore size)-filtered, and injected onto a 4.6 \times 100 mm Poros R2/H reversed-phase column (Perseptive Biosystems, Cambridge, MA). A linear gradient from 0 to 100% eluant B was used. Eluant A was 10 mM diisopropylethylamine/30 mM TEA/15 mM formic acid/8% isopropanol, pH \approx 11. Eluant B was 65% acetonitrile/25% isopropanol/15% H_2O /5 mM TEA/3.5 mM formic acid, pH \approx 10.5. Fractions were lyophilized and redissolved in 100 μ l of H_2O and tested for bioactivity.

Cytokines. Recombinant human IL-2 was obtained from Hoffmann-La Roche. Recombinant IL-1 β was obtained from Biogen. IL-3, IL-4, IL-6, IL-7, IL-9, IL-10, IL-11, and IL-12 were obtained from R & D Systems; IL-13 was obtained from D. Caput Sanoti Elf (Bio Recherches, Labège, France), and ATL-derived factor was from J. Yodoi (Institute for Immunology, Kyoto University).

Analysis of IL-2 and IL-T Activity. To assess biological activity, the cytokine-dependent CTLL-2 cell line was washed three times in phosphate-buffered saline and dispersed in a 96-well microtiter plate at 15,000 cells per well. Standard IL-2 and IL-T preparations as well as the materials to be assessed for capacity to induce CTLL-2 proliferation were added to the wells and incubated for 24 hr, at which time they were pulse-labeled with 1 μ Ci of [^3H]thymidine (1 Ci = 37 GBq). Six hours later the cells were harvested, and radioactivity was measured. The response is expressed in units of biological activity where 1 unit of activity is defined as the quantity of standard cytokine required to yield one-half of the peak plateau of [^3H]thymidine uptake observed with the CTLL-2 assay cells utilized. Samples were also analyzed for immunoreactive IL-2 by using a commercial ELISA kit (CytImmune Sciences, College Park, MD).

Antibodies. A polyclonal antiserum to recombinant IL-2 raised in goats using recombinant *Escherichia coli*-derived IL-2 as the immunogen was obtained from Hoffmann-La Roche.

Analysis of IL-2 mRNA Expression. Equal amounts of RNA from HuT-102 and the IL-2-producing MLA 144 cell lines were obtained as discussed (7) and analyzed for IL-2 mRNA expression by Northern blot analysis using a ^{32}P -labeled PCR-amplified probe extending from bp 1 to bp 459 of the IL-2 gene coding region and a 1.9-kb BamHI cDNA clone for human β actin as a control (Clontech).

PCR Analysis of IL-2 Production. Oligonucleotide primers and probes were synthesized on an automated DNA synthesizer (Applied Biosystems) by the phosphoramidite method (11). For amplification of IL-2 mRNA, an IL-2/1 sense primer, 5'-TACAAGAATCCCAACTCACC-3', and an IL-2/2 antisense primer, 5'-ATGAATGTTGTTTCAGATCCC-3', were used. The predicted amplification product was the 217-bp IL-2 cDNA sequence from bp 151 to bp 368. With this exception, the procedures used, including primers and probes for actin, were as described (7).

Functional Assays. Cytotoxicity (^{51}Cr release) and proliferation (^3H]thymidine incorporation) assays were done as discussed (9, 10).

RESULTS

Identification of IL-T. During the late phase of ATL, the leukemic cells no longer produce IL-2. As a part of our effort to define pathogenic mechanisms that underlie the autonomous proliferation of ATL cells, we demonstrated that the IL-2-independent ATL cell line HuT-102 secretes a lymphokine that stimulates T-cell proliferation. Specifically addition of CM from HuT-102 to an IL-2-dependent CTLL-2 mouse indicator cell line at a dilution of 1:4 yielded a stimulation index of >230 (457,000 cpm per 15,000 cells per μ Ci of [^3H]thymidine as compared to 1970 cpm with unstimulated IL-2-deprived CTLL-2). The parallel addition of recombinant IL-2 (7.5 units/ml) to CTLL-2 cells in this assay yielded a stimulation index of 294 (580,000 cpm) after [^3H]thymidine addition. Since the CTLL-2 cell line used was purported to be IL-2-specific in its lymphokine-induced proliferation, we performed studies to exclude IL-2 as the predominant factor in HuT-102 CM-inducing T-cell proliferation. Stimulation of CTLL-2 cells induced by IL-2 throughout the dose range from 0.2 to 20 units/ml was completely inhibited by prior addition of a polyclonal goat antiserum to IL-2 (Fig. 1). In contrast, addition of the polyclonal anti-IL-2 antiserum to HuT-102 CM led to only a minimal inhibition in cytokine-induced stimulation of CTLL-2 cells. In addition, when assessed in an IL-2 ELISA assay, there was no reactive IL-2 demonstrable in unconcentrated HuT-102 CM. To exclude the possibility that HuT-102 produced an aberrant form of IL-2 that lacks the epitope identified by the anti-IL-2 antibodies,

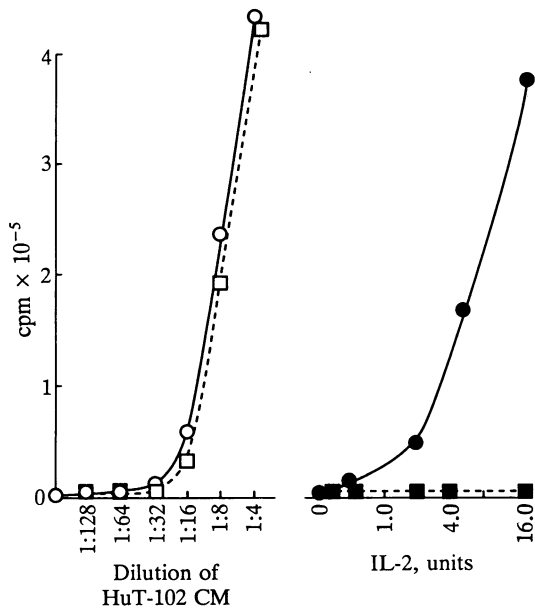


FIG. 1. Proliferative response of CTLL-2 cells to HuT-102 CM containing IL-T (○), HuT-102 CM plus anti-IL-2 antiserum (□), IL-2 (●), or IL-2 plus anti-IL-2 antiserum (■). The IL-T and IL-2 preparations were added to 15,000 washed CTLL-2 cells per well and incubated for 24 hr, at which time they were pulse-labeled with 1 μ Ci of [³H]thymidine. Six hours later, the cells were harvested and radioactivity incorporation was determined.

mRNA extracted from unstimulated HuT-102 cells was assessed for IL-2 mRNA. HuT-102 did not manifest IL-2 mRNA when assessed by Northern blot analysis. Furthermore, only minimal IL-2 mRNA could be identified by reverse transcription-PCR analysis, whereas high-level IL-2 mRNA expression was easily demonstrated in the simultaneously studied IL-2-producing MLA 144 cell line. On the basis of these studies, we conclude that the predominant T-cell stimulatory lymphokine in HuT-102 CM is distinct from IL-2.

To exclude the possibility that IL-T is identical to yet another defined cytokine, a series of purified or recombinant cytokines was assessed for biological activity on the CTLL-2

cell line. No activity was mediated by IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, granulocyte/macrophage colony-stimulating factor, oncostatin-M, transforming growth factor β 1, interferon γ , tumor necrosis factor α , leukemia inhibitory factor, stem cell factor I, or the HTLV-I-associated cytokine ATL-derived factor, thereby demonstrating that IL-T is distinct from these cytokines.

Chemical Characterization and Purification of IL-T. CTLL-2-stimulatory activity in HuT-102 CM was resistant to trypsin addition but was abolished by addition of endoproteases Lys-C and Asp-N and staphylococcal V8 protease and was reduced by >50% by addition of chymotrypsin, indicating that IL-T is a protein. On isoelectric focusing of HuT-102 CM, a major sharp peak of biological activity was observed at pH 4.5, in contrast to the pattern with IL-2, where an isoelectric point in the pH 6.5–8.0 range was defined.

The molecular mass of IL-T was estimated by nonreducing SDS/PAGE analysis using a 12% gel. A Ferguson plot of the gel was generated using prestained molecular mass markers. CTLL-2-stimulatory activity was eluted from the 12- to 16-kDa gel slice. A separate SDS/PAGE determination was performed with a continuous elution cylindrical gel device (model 491 Prep Cell, Bio-Rad). Bioactivity was eluted from the region between the 6.5-kDa and 17-kDa internal molecular mass markers.

IL-T was purified 3200-fold from HuT-102 CM by using the procedures outlined in Table 1. The fraction from the final reversed-phase purification step with the highest specific activity was employed for functional analyses. The final fraction contained 7.0×10^5 units of biological activity per mg of protein and represented a 3200-fold purification when expressed as biological activity per unit of protein as compared to the starting HuT-102 CM.

Biological Characterization. The 3200-fold-enriched IL-T stimulated proliferation of the CTLL-2 cell line. In addition, HuT-102 CM and the 3200-fold-enriched IL-T stimulated proliferation of the human IL-2-dependent Kit-225 cell line (Fig. 2). This stimulation was not inhibited by addition of an anti-IL-2 antiserum.

To assess the effect of IL-T on cytokine-dependent cytotoxicity, LGLs were purified from peripheral blood of normal individuals and were incubated for 36 hr with or without IL-T or IL-2 before a 4-hr coculture with ⁵¹Cr-labeled Daudi cells

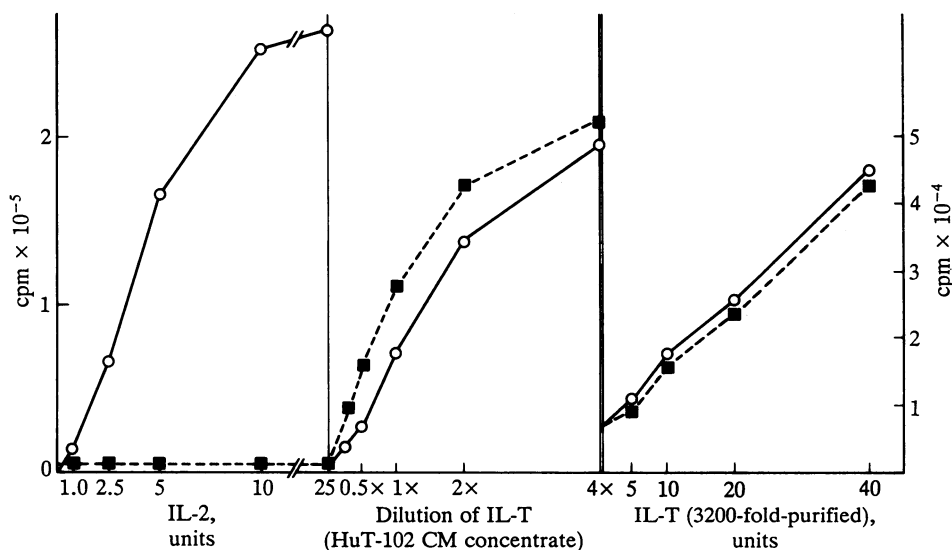


FIG. 2. Proliferative response of Kit-225 K6 cells to IL-T-containing HuT-102 CM, 3200-fold-purified IL-T, or IL-2, as indicated (○), or to the same cytokines in the presence of an antiserum to IL-2 (■). Kit-225 cells were washed free of IL-2 and dispersed in a 96-well microplate at 15,000 cells per well. The antiserum was added at 25 hr, the cytokines were added at 25.5 hr of culture, and the culture was incubated for an additional 48 hr. At this time cells were pulse-labeled with 1 μ Ci of [³H]thymidine, and then 6 hr later cells were harvested, and radioactivity incorporation was determined.

(9). Addition of 10^{-10} or 11^{-11} M IL-2 to LGLs led to activation of these cells resulting in 60% specific lyses of cocultured target Daudi cells. In simultaneous studies, addition of a 1:20 dilution of HuT-102 CM led to activation of LGL cytotoxicity against Daudi cells with 34% specific cell lysis observed. Addition of the polyclonal anti-IL-2 antiserum abrogated IL-2-induced lymphokine-activated killer (LAK) activity but had no effect on induction of cytotoxic activity mediated by IL-T-containing HuT-102 CM. Thus, IL-T not only stimulates proliferation of cytokine-dependent murine and human IL-2-dependent T-cell lines but also facilitates generation of LAK cells when added to LGLs.

DISCUSSION

Successful T-cell-mediated immune responses are initiated when an antigen interacts with the T-cell receptor for that specific antigen. After this encounter, T cells enter a program of activation leading to *de novo* synthesis and secretion of cytokines and to induction of expression of cytokine receptors. Interaction of the cytokine with its induced cellular receptor then triggers proliferation, culminating in the emergence of effector T lymphocytes. Initial studies focused on IL-2 and its receptor as the cytokine system involved in this process (12, 13). However, mice made deficient in IL-2 by homologous recombination developed normally immunologically during the first few weeks of life (14). Furthermore, a high degree of redundancy has been demonstrated in T-cell activation and function. Specifically, functioning alone or in concert, a series of cytokines have been defined that act on T lymphocytes.

Abnormalities of IL/IL-receptor systems are observed with a broad array of human diseases, including the forms of leukemia or autoimmune disease that are caused by HTLV-I (6, 15). HTLV-I is associated etiologically with ATL and a chronic progressive myelopathy, tropical spastic paraparesis (16, 17). We demonstrated that the *in vitro* spontaneous T-cell proliferation observed in association with tropical spastic paraparesis was profoundly inhibited by specific anti-IL-2R or anti-IL-2 blocking antibodies, indicating that HTLV dysregulation of an autocrine IL-2/IL-2R system contributed to this state of T-cell activation (18). Evidence for a similar autocrine process of T-cell proliferation has been presented for early-phase HTLV-I-associated ATL (6). However, in late-phase ATL, the leukemic cells no longer produce nor require IL-2 for their proliferation yet continue to express high-affinity IL-2Rs (7, 15). In the present study, we demonstrate a mechanism that may play a role in the IL-2-independent proliferation of select ATL cells. In particular, we have shown that HuT-102 cells secrete a lymphokine, provisionally designated IL-T, that stimulates T-cell proliferation and LAK cell activity. HuT-102 CM and 3200-fold-purified IL-T stimulated the proliferation of human and murine IL-2-dependent T-cell lines. Furthermore, this lymphokine induced LAK activity when added to LGLs. No CTLL-2 stimulatory activity was manifested by a series of cytokines, thus differentiating these factors from IL-T.

IL-2 and IL-T share a number of functional similarities. Both IL-2 and IL-T stimulate proliferation of cytokine-dependent IL-2R-expressing human and murine T-cell lines. Furthermore, both induce LAK cell activity when added to LGLs. In addition, the cytokines are of a similar size. On the other hand, the molecules have distinct biochemical properties. For example, the pI value defined by isoelectric focusing of IL-T is 4.5, in contrast to the complex pattern of peaks between pI values of 6.5 and 8.0 observed with IL-2 (19). Several laboratories studying HuT-102 (19, 20) and T cells derived from a thymic epithelial tumor (21) observed comparable differences between normal IL-2 and a T-cell growth factor they identified in the CM of these cell populations.

Although these T-cell growth factors were initially reported as biochemical variants of IL-2, these authors (19, 21) presented biochemical evidence that they are distinct molecules that may represent the product of a gene other than the one encoding IL-2. We now present data clearly distinguishing IL-T from IL-2. With most preparations, anti-IL-2 antisera that completely neutralized IL-2 action did not affect that of HuT-102 supernatants or partially purified IL-T (data not shown). With select polyclonal antisera, modest inhibition of stimulation mediated by HuT-102 CM was observed. This inhibition may be nonspecific in character, may indicate that HuT-102 produces small quantities of IL-2 and IL-T, or may reflect shared epitopes between IL-2 and IL-T. Of greater significance in differentiating IL-T from IL-2, the HuT-102 cell line did not express mRNA encoding IL-2, as defined by Northern blot analysis, and showed only minimal evidence for production of an IL-2 mRNA as assessed by reverse transcription-PCR. Thus, IL-T appears to be a lymphokine that is distinct from IL-2 yet shares many biological features with IL-2.

The demonstration of functional redundancy between IL-T and IL-2 may contribute to our understanding of the regulation of T-cell maturation in the thymus and of mature T-cell activation in the periphery. In particular, after demonstration of IL-2Rs in immature thymocytes, a key role was considered for IL-2 in T-cell development (22). However, demonstration of grossly normal thymic maturation and peripheral T-cell composition in mice made deficient of IL-2 by gene targeting indicated that IL-2 does not play an indispensable role in this process (14). The demonstration of sharing of the IL-2R γ subunit among receptors for IL-2, IL-4, and IL-7 suggested that these ILs might contribute to T-cell development (3-5). Our present observations and the demonstration that the IL-2R β chain is required for IL-T action (see ref. 8) suggest that IL-T should also be examined for a role in thymic maturation. Another implication of the present findings concerns the use of the murine CTLL-2 cell line to measure IL-2 concentrations. Since both IL-2 and IL-T elicit a response in this assay, one cannot utilize this procedure as a definitive method to quantitate IL-2 concentrations. Specifically, biological samples have been obtained that manifest apparent IL-2 activity when assessed by the CTLL-2 assay yet were negative when an ELISA assay that is immunologically specific for IL-2 was employed.

Finally, demonstration of the production of the T-cell growth factor IL-T by HTLV-I-associated ATL cells that no longer synthesize IL-2 yet continue to display IL-2Rs suggests that in select cases the generation of IL-T and its subsequent interaction with expressed IL-2Rs may stimulate malignant T-cell proliferation and thereby contribute to the IL-2-independent growth manifested by some HTLV-I-associated ATL cells and lines. Thus, the characterization of the lymphokine IL-T not only may provide another perspective concerning normal T-cell maturation and action but also may aid in the definition of mechanisms underlying disordered T-cell function and proliferation in disease.

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