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Genes for Interleukin 7 Are Transcribed in Leukemic Cell Subsets of Individuals with Chronic Lymphocytic Leukemia

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Summary

Regulation of expression of interleukin 7 (IL-7) mRNA is aberrant in the leukemic subset of cells of chronic lymphocytic leukemia (CLL) patients. The entire coding sequence for IL-7 as well as an alternatively spliced IL-7 mRNA are transcribed in these leukemic cells. No IL-7 mRNA expression is detected in fresh peripheral blood mononuclear cells from normal individuals. Furthermore, the "normal" nonleukemic subsets of cells isolated from the same CLL patients also do not express IL-7 mRNA. The only subset of cells in which IL-7 mRNA is detected is the one that contains the leukemic cells themselves. The polymerase chain reaction was used to examine cytokine expression, and flow cytometry was used to purify the various subsets of peripheral blood mononuclear cells examined in these studies, as well as to examine IL-7 receptor expression. A proportion of the cells from the CLL patients express receptors that are capable of binding IL-7, whereas T cell–depleted normal cell preparations do not express receptors for IL-7 that are detectable with IL-7 fluorokines. The IL-7 receptor–bearing cells in CLL patients include a portion of leukemic cells and a fraction of the T cells, as well as some non-T, non-B cells. These findings suggest that IL-7 and IL-7 receptor expression in CLL may be relevant not only to growth regulation of the leukemic cells but to the immunological abnormalities that occur in the disease as well, possibly via the induction of inappropriate immune activity of IL-7 receptor–bearing cells.

Chronic lymphocytic leukemia (CLL)[1] is a relatively stable, common form of leukemia, with >90% of B cell CLL cases resulting from the leukemic transformation and clonal expansion of a single B lymphocyte within the $CD5^+$ subset (1–3). $CD5^+$ B cells account for 5–25% of circulating and splenic B lymphocytes in normal adults, and may serve as the first line of immunological defense against microorganisms in infants, as these cells frequently produce IgM antibodies that are polyspecific (4–11). CLL cells themselves are relatively mature B cells but usually do not actively secrete Igs. They exist in a quasi-quiescent state, with only ~3% of the population in cell division. Patients with CLL often experience a suppression of immune responses and, in contradistinction, autoimmunity. An increased incidence of colon and skin cancers may be a reflection of depressed T cell immunity (12–16). The abnormal immune functions observed in CLL may be mediated by products of the B cell malignancy and, furthermore, may be a determining factor in the chronic nature of the disease.

A number of studies have suggested that growth factors and/or their receptors have a role in CLL. High concentrations of soluble IL-2 receptors, for example, have been detected in the serum of CLL patients, and IL-1 production by CLL cells has been documented (17–23). Other studies have suggested a role for TNF in supporting the proliferation of CLL cells (24, 25). Another potential growth regulatory factor for CLL would be IL-7 and/or IL-7 receptors. IL-7 is a stromal cell–derived growth factor for early lymphoid stem cells, including progenitor B and Pre-B cells, as well as for pre-T and cytotoxic cells (26–31). Our laboratory became interested in cytokine growth signals associated with leukemia cells when we found an IL-1 receptor–associated marker on CLL cells (32–34). We postulated that a cytokine(s) and its receptor(s) might contribute significantly both to leukemic transformation and to the immune anomalies associated with CLL. Cytokines or cytokine receptors could also effect the chronic state of the leukemia by inducing nonleukemic immune cells

[1] *Abbreviation used in this paper:* CLL, chronic lymphocytic leukemia.

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to function in an abnormal manner. The question we first asked was whether endogenously produced cytokines and/or cytokine receptors might serve a role in the leukemic transformation of B cells. We approached the analysis of cytokine and cytokine receptor expression via the PCR (35) and included an examination of the expression of a variety of cytokines/cytokine receptors associated with B cell growth and differentiation. Flow cytometry was used to isolate mononuclear cell subsets, to examine expression of receptors for IL-7, and then to phenotype IL-7 receptor–bearing cells. A portion of the data obtained is presented here and demonstrates that PBMC from CLL patients express IL-7 mRNA while cells from normal blood donors do not.

Materials and Methods

Cell Sources. Peripheral venous blood from leukemic patients was obtained through the Hematology Service at Rush Presbyterian-St. Luke's Medical Center (Chicago, IL). Normal donor cells were from laboratory personnel or were purchased as buffy coats from units of same-day donations through Lifesource (Chicago, IL). Mononuclear cells were immediately isolated after a 1:1 dilution of the blood in 10 mM PBS, pH 7.2, by centrifugation on 5 ml per tube of a Ficoll-Na diatrizoate solution (Organon Teknika Corp., Durham, NC). The cells were removed from the interface, washed, counted, and frozen in aliquots of ~1–3 × 10^7 each. The cells recovered upon thawing were generally from 75 to 95% viable. Samples of the leukemic patients' cells were also phenotyped for cell surface markers by flow cytometry. A summary of relevant clinical information and surface phenotype expression on the individual patients reported here is presented in Table 1.

Cell Surface Markers. Directly labeled mAbs to the cell surface markers, CD3, CD4, CD5, CD8, CD19, CD20, CD13, CD45, HLA-DR, as well as for κ or λ light chains, were purchased from Becton Dickinson & Co. (Mountain View, CA). The mAbs were directly conjugated with either FITC, PE, or peridinal chlorophyll protein (PerCP). OKT3, used in T cell depletion experiments, was purchased from Ortho Diagnostics (Raritan, NJ). The Fluorokines™, IL-7 conjugated with either biotin or with PE (IL-7PE), were purchased from R & D Systems (Minneapolis, MN).

Isolation of Cell Subsets. Leukemic cells were incubated on ice for 30 min with PE-labeled anti-CD5 (Leu-1) and with FITC-labeled anti-CD19 or CD20 mAbs, then washed thrice. The cells were analyzed in a FACStar Plus® (Becton Dickinson & Co.) flow cytometer, and the double-labeled $CD5^+$,$CD19^+$ or $CD5^+$,$CD20^+$ cells, or, in some cases, the single-labeled $CD19^+$,$CD20^+$ or $CD5^+$ cells, or the double-negative non-T, non-B, $CD5^-$,$CD19^-/20^-$ cells, were isolated by sorting. The sorted cells were again analyzed by flow cytometry to confirm the degree of purity, then washed and solubilized in a 4.0-M solution of guanidinium isothiocyanate. Normal cells, which contain a substantially smaller proportion of CD5,CD20 double-positive cells, were enriched for B cells initially by removing the majority population of T cells with mAbs directed against CD3. Briefly, 3–10 × 10^7 normal donor cells were treated with 100 μl of OKT3 for 30 min on ice, washed, then incubated with goat anti–mouse IgG antibodies conjugated to iron filings (Collaborative Research Inc., Bedford, MA). The iron filings plus adhering T cells were removed by three cycles of incubation on ice with a magnet. The T cell–depleted, B cell–enriched preparations were then treated with the fluorochrome-conjugated antibodies as above, analyzed, and sorted by a FACStar Plus® flow cytometer into $CD5^+$,$CD20^+$ B cell suspensions or $CD5^+$,$CD20^-$ non-T, non-B cells, or, alternatively, $CD20^+$,$CD5^-$ B cells, and the $CD5^+$ cells that include the $CD5^+$,$CD20^+$ B cells. We discovered that time and temperature were the two most important variables that would determine mRNA "stability" and, thereby, detection by PCR. The cells, thus, were kept cool throughout the fractionation procedure, and RNA was isolated immediately after cell sorting.

Isolation of RNA, cDNA Synthesis, and PCR. Cell pellets were resuspended and dissolved in 4.0 M guanidinium isothiocyanate containing 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl, and 1.0% 2-ME, and layered onto a 5.7 M cushion of CsCl (36). The RNA was pelleted in a rotor of an Optima ultracentrifuge (TL100.2; Beckman Instrs. Inc., Fullerton, CA) for 2 h at 175,600 *g*. The supernatant was carefully removed, the RNA pellet dissolved in water, and the RNA precipitated overnight at −20°C in 0.3 M sodium acetate and 2.5 vol of cold 100% ethanol. The RNA was pelleted during 30 min in a centrifuge (Eppendorf North America, Inc., Madison, WI), washed in 70% cold ethanol, and dried under vacuum. The RNA was then dissolved in 30 μl sterile water, and 10 μl was used for cDNA synthesis. Random priming of mRNA was with oligo$(dT)_{15}$ (Promega Corp., Madison, WI). AMV reverse transcriptase was used to synthesize the cDNA at 37°C for 2 h in a final volume of 25 μl. In a later refinement of the cDNA

Table 1. *Cell Surface Phenotypes of CLL Patients*

CLL patient no.	Sex	Age	WBC	Stage	Percent λ	Percent κ	CD5	CD20	CD3
		yr							
2	M	42	29	2	2	97	99	77	2
3	M	42	14	4	1	76	98	87	9
4	M	41	59	2	95	6	97	94	4
5	F	59	53	1	4	97	95	92	4
10	F	80	13	0	10	67	84	60	24
11	F	67	22	1	1	84	18	41	11
16	F	52					14	75	6
25	F	67	4	2	23	11	69	36	51

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synthesis technique, the incubation time was reduced to 15 min, with full ability to detect the respective PCR products. 1 μl of cDNA was generally used as a template for amplification in the PCR. Higher concentrations of cDNA (5, 10, or 20 μl) were used to confirm negative PCR reactions. Primers for selected cytokines and cytokine receptors used in these studies, the expected size of the PCR products for each, and respective restriction enzymes that would yield discernible-sized fragments are listed (Table 2). All primers reported here were purchased from Clontech Laboratories Inc. (Palo Alto, CA). Standard GeneAmp™ PCR was performed with Taq DNA polymerase, 200 μM dNTPs, 0.4 μM forward and reverse primers, 3 mM $MgCl_2$, and H_2O to a final volume of 50 μl in siliconized tubes. After 35 or 40 cycles of PCR, 7 μl of each sample was subjected to separation by agarose gel electrophoresis, stained with ethidium bromide, analyzed, and photographed. Proof of identity of the PCR product of the appropriate size was determined by restriction enzyme digestion into fragments of known sizes (Table 2). The data presented in this manuscript were obtained largely with IL-7 primers and include others only as necessary for controls to demonstrate that an experiment was a technical success. A more inclusive report with these as well as other primers will be published elsewhere. The primer set for the IL-2 receptors are for the p55, TAC, α chain.

Southern Hybridizations. Clonal rearrangement of leukemic Ig genes was examined in Southern hybridizations with the J_h probe, C76R51 (37). DNA was isolated from total PBMC and from subsets sorted by flow cytometry, then cut with the restriction enzyme EcoRI. The DNA samples were separated overnight in a 0.7% TAE-buffered agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labeled C76R51 (38).

Results

IL-7 mRNA Expression Was Detected by PCR in PBMC from CLL Patients but Not from Normal Donor Cells. Cytokine expression was evaluated to determine whether a particular cytokine was consistently expressed in CLL patients' PBMC that was not expressed in normal individuals. Our data revealed that IL-7 was expressed in cells from 20 of 20 CLL patients tested (Table 3). The IL-7 PCR product migrated in agarose gel electrophoresis to the approximate size predicted for an mRNA-derived template (681 bp; Fig. 1, lane *2*). Digestion of the IL-7 PCR product with the restriction enzyme PVUII revealed two fragments as expected (379 and 302 bp; Fig. 1, lane *3*). To rule out contaminating DNA as the source of template, PCR analyses were also performed with an equivalent amount of the RNA preparation. No IL-7 product was evident in the absence of a cDNA template (Fig. 1, lane *4*). The reverse and forward primers for IL-7 span five introns of genomic sequence, therefore, a PCR product derived from genomic DNA would be substantially larger (i.e., several kilobases). The data from these experiments revealed that mRNA for IL-7 indeed was expressed in cells from CLL patients.

Table 3. *IL-1β, IL-2 Receptor, and IL-7 mRNA Expression in PBMC Isolated from CLL Patients and Normal Donors*

	CLL donors	Normal donors
IL-1β	14/18	7/7
IL-7	20/20	0/7
IL-2R	16/18	5/7

No IL-7 mRNA was detected in cells obtained from normal individuals (0/7) (Table 3). Positive PCR products, either IL-1β, IL-2R, or both, demonstrated that the experiments were technical successes. One concern, however, was whether the difference in the ability to detect IL-7 mRNA was a reflection of the large numbers of $CD5^+$ leukemic B cells present in CLL patients or was undetected because of the substantially smaller number of $CD5^+$ B cells in normal individuals. Two approaches were used to resolve this issue. First, the minimal number of CLL cells required to detect IL-7 mRNA was determined. While a faint band for IL-7 was detected at low numbers of CLL cells (5×10^5 and $1 \times$

Table 2. *Primer Sets for PCR*

Cytokine	Sequence* forward/reverse	PCR product	Restriction enzyme	Fragment size
		bp		*bp*
IL-1α	5'ATGGCCAAAGTTCCAGACATGTTTG 3'	816	HindIII	617, 190
	5'GGTTTTCCAGTATCTGAAAGTCAGT 3'			
IL-1β	5'ATGGCAGAAGTACCTAAGCTCGC 3'	802	PVUII	591, 210
	5'ACACAAATTGCATGGTGAAGTCAGTT 3'			
IL-2R	5'GAATTTATCATTTCGTGGTGGGGCA 3'	398	NCOI	274, 123
	5'TCTTCTACTCTTCCTCTGTCTCCG 3'			
IL-6R	5'CATTGCCATTGTTCTGAGGTT 3'	251	HAEIII	142, 109
	5'AGTAGTCTGTATTGCTGATGTC 3'			
IL-7	5'ATGTTCCATGTTTCTTTTAGGTATATCT 3'	681	PVUII	379, 302
	5'TGCATTTCTCAAATGCCCTAATCCG 3'			

* From Clontech Laboratories, Inc. (Palo Alto, CA).

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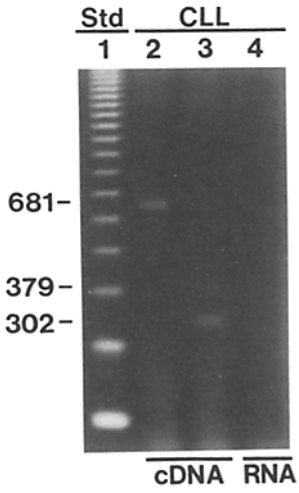

Figure 1. IL-7 PCR product from CLL patients' cells before and after restriction enzyme digestion with PVUII. Lane *1*, 123-bp ladder: lane *2*, IL-7 PCR product of cDNA template synthesized off RNA isolated from CLL patient's total peripheral mononuclear cells; lane *3*, after PVUII digestion; lane *4*, lack of a PCR product from the RNA preparation as template and the IL-7 set of primers. The expected size of the IL-7 PCR product was 681 bp, while the PVUII fragments were 379 and 302 bp. No product was observed with the RNA as template.

10^6) (Fig. 2, lanes *4* and 7), no IL-7 product was detected with high numbers of normal donor cells (10^7) (Fig. 2, lane *11*). "$CD5^+$,$CD20^+$ cell equivalents" were calculated based on dual marker studies of these donors. Whereas an IL-7 PCR product was readily detected with ~1,300 "$CD5^+$,$CD20^+$ CLL cell equivalents," and actually could be observed at 650 "cell equivalents" (Fig. 2, lane 7), no IL-7 PCR product was evident at ~2,050 "$CD5^+$,$CD20^+$ normal cell equivalents" (Fig. 2, lane *11*). IL-1β and IL-2R PCR products were readily

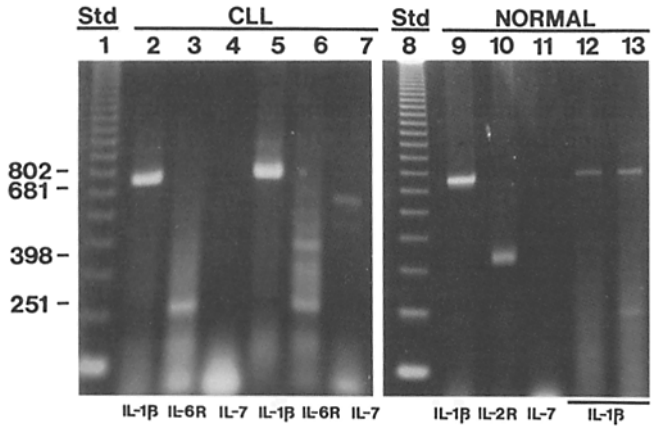

Figure 2. Normal donor cells do not yield an IL-7 PCR product. RNA was prepared from increasing numbers of CLL no. 10 and normal donor (LS no. 8) peripheral mononuclear cells. An IL-7 product was observed with the lowest number of CLL cells used in this experiment, 0.5×10^6 (lanes *2–4*). More intense bands were observed with higher numbers of CLL cells. Results from 10^6 cells are reported here (lanes *5–7*). Cell equivalents were calculated to determine the maximum number of cells that contributed template for the 7 μl of PCR product loaded into the gel based upon a theoretical yield of 100%. This number was used to calculate the minimum number of normal donor cells required to start with an equivalent number of $CD5^+$ B cells. Normal donor cells at 10×10^6 represented greater than twice the minimum number of cell equivalents required (lanes *9–11*). Lanes *1* and *8*, 123-bp ladder; lanes *2–4*, 0.5×10^6 CLL cells, PCR products of primers for IL-1β, IL-6 receptor, and IL-7, respectively; lanes *5–7*, 10^6 CLL cells, PCR products of primers for IL-1β, IL-6 receptor, and IL-7, respectively; lanes *9–11*, 10×10^6 normal donor cells, PCR products of primers for IL-1β, IL-2 receptor, and IL-7, respectively; lanes *12* and *13*, IL-1β PCR products from 10^6 and 0.5×10^6, respectively.

detected with 10^7 normal cells, while IL-1β was also readily detected with 10^6 as well as 5×10^5 normal cells (Fig. 2, lanes *9*, *10*, *12*, and *13*, respectively). The IL-1β as well as IL-6R primers also served as positive controls for the CLL RNA and cDNA preparations (Fig. 2, lanes *1* and *5*, as well as lanes *3* and *6*, respectively). It was apparent from these studies that IL-7 mRNA was expressed in mononuclear cells isolated from CLL patients but not in an equivalent number of cells isolated from normal individuals. In the second approach to investigate IL-7 mRNA expression in normal cells, flow cytometry was used to enrich for the normal $CD5^+$, $CD20^+$ B cells. No detectable expression of IL-7 mRNA (data reported below, see Fig. 5) was observed in the sorted normal B cells.

The Subsets of B Cells That Contain the Leukemic Cells Expressed mRNA for IL-7 while the Nonleukemic Subsets of the CLL Patients Did Not Express Detectable IL-7 mRNA. We sought to determine whether the leukemic cells themselves expressed IL-7 mRNA. Dual-color fluorescence and cell sorting were used to isolate cell subsets with antibodies directed against either of the pan-B cell markers, CD19 or CD20, and the B cell subset marker, CD5. mAbs to CD20 were used with normal cells and the initial three CLL patients (nos. 1–3), while mAbs to CD19 were used more recently with the CLL cell preparations. CD5 also serves as a pan-T cell marker. Examination of T-depleted, B-enriched mononuclear cells from a normal donor revealed a density map profile of fluorescence expected from these two markers (Fig. 3 *A*). The majority subset now consisted of single-positive $CD20^+$ cells as seen in the upper left quadrant, while the second most prominent subpopulation was the double-negative, non-T, non-B cells observed in the lower left quadrant, and, a third, less prevalent subpopulation of residual $CD5^+$ T cells was observed in the lower right of the contour map (Fig. 3 *A*). The double-positive $CD5^+$,$CD20^+$ cells were hardly discernible as a distinct subpopulation in the upper right quadrant. In contrast, most CLL patients had a majority subpopulation of $CD5^+$,$CD19^+/20^+$ cells, as observed in the upper right quadrant (Fig. 3 *B*). The proportion of T cells, $CD5^-$ B cells, and non-T, non-B cells observed varied with each patient. The double-positive $CD5^+$, $CD19^+/20^+$ population in five of the CLL patients ranged from 25 to 93% with a median of 71%. The contrast between normal donor and the $CD5^+$ leukemic patients as observed in Fig. 3, *A* and *B*, reflects the overwhelming contribution of the monoclonal leukemic population. 2 of 11 patients examined by dual-color analyses, however, had relatively few double-positive cells, but instead had a $CD5^-$,$CD19^+$ majority B cell subpopulation and evidently had the more rare form of $CD5^-$ CLL (Fig. 3 *C*). Parameters were then set to isolate the normal $CD5^+$,$CD20^+$ cells, the CLL patients' $CD5^+$,$CD19^+$ or $CD5^-$,$CD19^+$ cells, as well as other selected subpopulations. Contour maps of the dual-labeled mononuclear cells from the three respective types of individuals before and after cell sorting are presented: normal (Fig. 4 *A*), $CD5^+$ CLL (Fig. 4 *B*), and $CD5^-$ CLL (Fig. 4 *C*). Each isolated subset was reexamined by flow cytometry and found to consist of >96%

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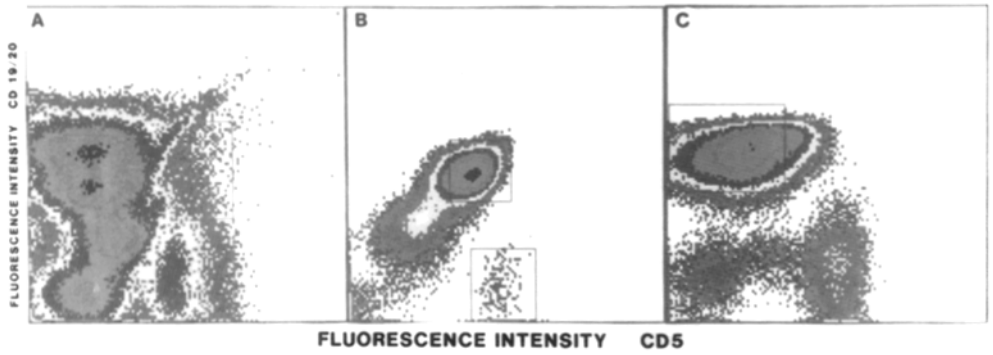

Figure 3. Flow cytometry analysis of peripheral mononuclear cell preparations from T cell–depleted normal donor, from a $CD5^+$ CLL patient, and from a $CD5^-$ CLL patient. Live acquisition data of 10^6 events from analyses on a FACStar Plus®. Cell preparations were stained with a fluorescein-conjugated anti-CD5 and a PE-conjugated anti-CD19 or CD20. (*A*) T cell–depleted normal donor cells, LS no. 1. (*B*) $CD5^+$ CLL no. 4 cells. (*C*) $CD5^-$ CLL no. 16 cells.

purified cells (Fig. 4, *right*). The cells were then washed and processed for the isolation of RNA. Complementary DNA was synthesized, and PCR assays were performed. The PCR data demonstrated that the B cell subsets that contained the respective leukemic population expressed IL-7 mRNA (Table 4 and Fig. 5, lanes *2* and *3*, respectively). IL-7 mRNA was expressed in $CD5^+$,$CD19^+/20^+$ cells of the $CD5^+$ patient, whereas IL-7 mRNA was detected in $CD5^-$,$CD19^+/20^+$ cells of the $CD5^-$ patient. Neither the normal donor $CD5^+$,$CD20^+$ nor the normal $CD5^-$,$CD20^+$ B cell subsets expressed detectable levels of IL-7 mRNA (Table 4 and Fig. 5, lanes *4* and *7*, respectively). Normal $CD5^+$ B cells, however, did express IL-1β, thereby demonstrating: (*a*) that the experiments were technically successful, and (*b*) that the expression of IL-1β is a normal function of $CD5^+$ B cells (Fig. 5, lane *5*). The predominant $CD5^-$,$CD20^+$ normal B cells also express IL-1β but to a lesser degree (Fig. 5, lane *7*). Equivalent levels of IL-6R PCR products were detected in both subsets of B cells (Fig. 5, lanes *6* and *9*). IL-2R expression was evident in both B cell subpopulations (38a). These data demonstrate, therefore, that the synthesis and secretion of IL-1β by CLL cells most likely is unrelated to their leukemic transformation. Expression of IL-7, however, is unique to cells from the CLL patients.

Other cell subsets from the CLL patients were also examined for expression of IL-7 mRNA: T cells, as defined by the surface expression of CD5 but the absence of CD19/20; non-T, non-B cells, as defined by the lack of surface expression of both, CD5 and CD19/20; conventional B cells, as defined by the expression of CD19 but the absence of CD5; and the total pool of $CD5^-$ cells, which contained a mixture of conventional B cells and non-B, non-T cells. The only

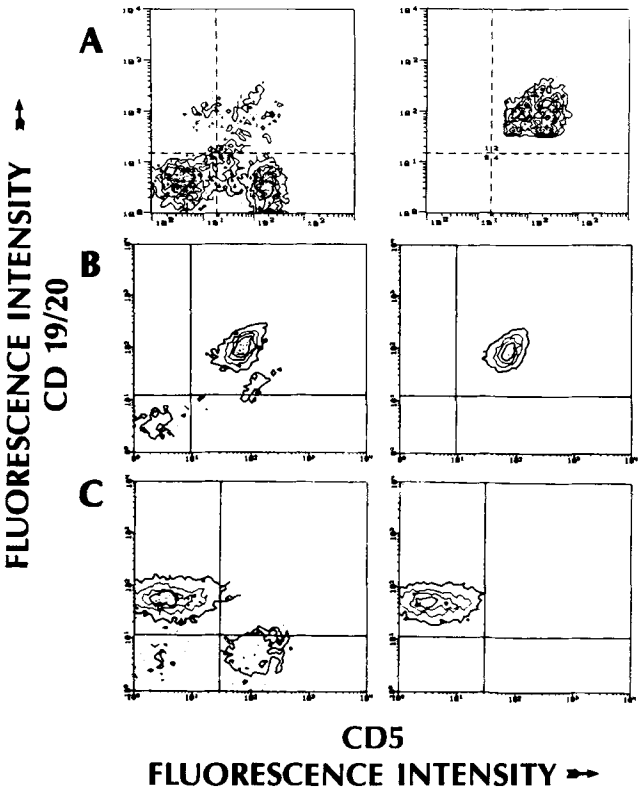

Figure 4. Fractionation of cell subsets from a T cell–depleted normal donor, a $CD5^+$ CLL patient, and a $CD5^-$ CLL patient. Contour maps were derived from an analysis of 10,000 cells before (*left*) and after (*right*) cell sorting. (*A*) T cell–depleted normal donor cells, LS no. 2. (*B*) $CD5^+$ CLL no. 10 cells. (*C*) $CD5^-$ CLL no. 11 cells.

Table 4. *Cytokine and Cytokine Receptor Expression in Leukemic Cell Subsets*

Patients	IL-1α	IL-1β	IL-2R	IL-7
$CD5^+$,$CD20^+/19^+$				
CLL 1	−	+	+	+
CLL 2			+	+
CLL 3			−	+
CLL 10	−	+	+	+
CLL 4	−	+	+	+
$CD5^-$,$CD20^+/19^+$				
CLL 16			−	+
CLL 11		+	+	+

CLL nos. 1–3 were fractionated by flow cytometry sorting using the mAb Leu-16 directed against the B cell marker CD20. CLL nos. 10, 4, 11, and 16 were fractionated similarly using the mAb Leu-12 directed against the B cell marker CD19.

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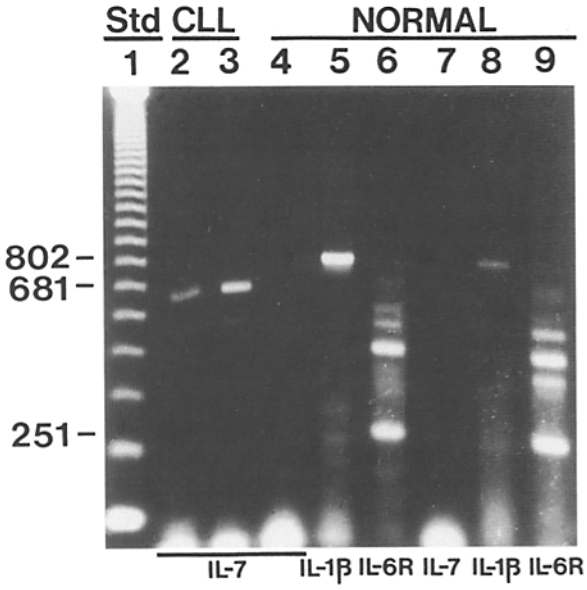

Figure 5. PCR products of mRNA obtained from purified B cell subsets. Lane *1*, 123-bp ladder; lane *2*, $CD5^+$,$CD19^+$ CLL no. 4 cells with IL-7 primers; lane *3*, $CD5^-$,$CD19^+$ CLL no. 11 cells with IL-7 primers; lanes *4–6*, $CD5^+$,$CD20^+$ normal LS no. 8 B cells with primers for IL-7 (lane *4*), IL-1β (lane *5*), and IL-6 receptor (lane *6*); lanes *7–9*, $CD5^-CD20^+$ normal LS no. 8 B cells with primers for IL-7 (lane 7), IL-1β (lane *8*), and IL-6 receptor (lane *9*).

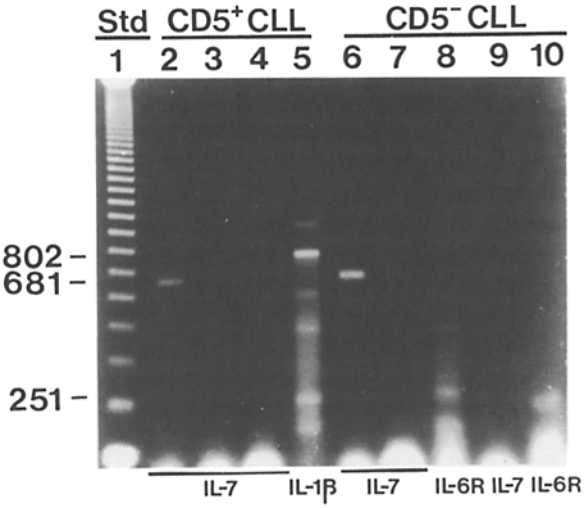

Figure 6. Only the leukemic subsets of B cells have mRNA for IL-7. Peripheral mononuclear CLL patients' cells were tagged with antibodies to the markers of CD5 and CD19 and fractionated into T and B cell subsets as well as non-T, non-B cells. Lane *1*, 123-bp ladder; lanes *2–5*, PCR products of flow cytometry–sorted cells from $CD5^+$ patient, CLL no. 4. Lanes *2–4*, IL-7 PCR primers with $CD5^+$ B cells (lane *2*), with $CD5^+$,$CD19^-$ T cells (lane *3*), and with all $CD5^-$ cells (lane *4*). The latter sort should include the $CD5^-$ majority class of B cells as well as non-T non-B cells. Primers for IL-1β served as their positive control (lane *5*). Lanes *6–10*, PCR products of flow cytometry sorted cells from $CD5^-$ patient, CLL no. 11; lane *6*, cDNA-derived IL-7 PCR products from $CD19^+$,$CD5^-$ cells; lane 7, IL-7 primers with $CD5^+$,$CD19^-$-sorted T cells, with IL-6 receptor primers serving as the positive control (lane *8*); lanes *9* and *10*, IL-7 and IL-6 receptor primers, respectively with $CD5^-$,$CD19^-$ non-T, non-B cells.

cell fractions that yielded mRNA-derived IL-7 PCR products were the leukemic subsets from the CLL donors (Fig. 6, lanes *2* and *6*). Neither the nonleukemic $CD5^-$,$CD19^+$ B cells isolated from the same $CD5^+$ leukemic individual (lane *4*) nor their $CD5^+$,$CD19^-$ T cells (lane *3*) yielded an IL-7 PCR product. Positive control primers for IL-1β with the $CD5^-$-sorted fraction yielded a major band at 802 (lane *5*), demonstrating that sufficient cDNA was available. Likewise, in the $CD5^-$ CLL patient, neither the $CD5^+$,$CD19^-$ T cells (lane 7) nor the collective $CD5^-$,$CD19^-$ non-T, non-B cells yielded an IL-7 PCR product (lane *9*), but each expressed IL-6R PCR products (Fig. 6, lanes *8* and *10*). The yield of PCR products obtained with positive control primers tested in the $IL\text{-}7^-$-sorted fractions, of either the CLL patients or the normal donors, were of levels comparable to those obtained with these primers in the $IL\text{-}7^+$-sorted fractions. Furthermore, the $IL\text{-}7^-$ cell fractions remained negative after increasing the volume of template in the PCR mixture by as much as 20-fold. The absence of detectable IL-7 PCR product, therefore, was most likely due to the absence of or substantially diminished levels of IL-7 mRNA.

Proof of Clonality of CLL Patients' Cells Using an Ig Heavy Chain Marker, J_h Probe. Southern hybridizations revealed that leukemic cells purified by flow cytometry cell sorting exhibited clonality in that no germline genes and only rearranged genes were detected in the sorted fractions (Fig. 7, lane 7 [unsorted]; lane *8* [sorted]). Unrearranged germline genes were strongly detected at 18 kb with normal donor cells (Fig. 7, lane *2*), and only moderately detected in unsorted cells of five different leukemic patients (Fig. 7, lanes *3–7*). In fact, in patient CLL no. 2's total PBMC sample, few normal cells were detected as evidenced by the weakness of the germline band and the preponderance of the clonally rearranged band (Fig. 7, lane *4*). Analysis of flow cytometry–sorted

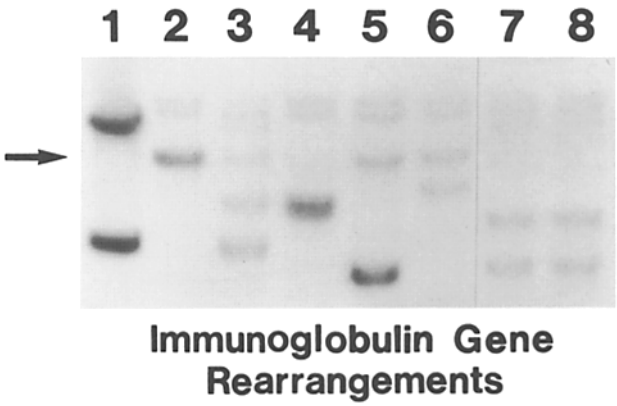

Figure 7. Southern hybridizations demonstrate clonality of CLL cells. EcoRI-digested DNA was subjected to electrophoresis and probed with ^{32}P-labeled J^h probe. DNA was isolated from total PBMC of normal donor LS no. 7 (lane *2*) and of patients CLL no. 23 (lane *3*), CLL no. 2 (lane *4*), CLL no. 10 (lane *5*), CLL no. 11 (lane *6*), and CLL no. 4 (lane 7), and from flow cytometry–sorted double-positive CD5,CD19 B cells from CLL no. 4 (lane *8*). HindIII-digested λ markers at 23.1 and 9.42 kb are visible in lane *1*.

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cells revealed only clonally rearranged bands and no germline gene band in the $CD5^+$,$CD19^+$ leukemic subset (Fig. 7, lane *8*). These data are consistent with previous studies demonstrating clonal Ig gene rearrangements in neoplastic B cells (39–41).

IL-7 Receptors Are Expressed on Cells from CLL Patients. If IL-7 serves a function in CLL, then one would expect that CLL patients' cells would express receptors for IL-7. Fluorescent dye–tagged cytokines were used with flow cytometry to evaluate IL-7 binding and to characterize the cells that expressed IL-7 receptors. Positive binding of IL-7 was observed with cells from four of four CLL patients examined. The percentage of total cells that stained specifically for IL-7 receptors varied between the patients from 1.1 to 53.4%. Each patient also had a variable percentage of the leukemic subset of B cells ranging from 20 to 96%. The percentage of specific IL-7 receptor–bearing cells in a given patient, however, was reproducible with an average deviation from the mean in CLL no. 2 of 2.67% in three replicate binding studies. The binding of biotinylated IL-7 to cells from two patients (CLL nos. 2 and 5) but not to T-depleted normal donor cells (normal control) is depicted in Fig. 8. CLL cells incubated with the Streptavidin-PE (Avidin-PE) reagent without biotinylated IL-7 served as the control for nonspecific reagent binding, and their fluorescence curve was superimposable over the normal T-depleted donor curve, demonstrating no evidence of binding. The specificity of IL-7 binding was evaluated with recombinant, unlabeled IL-7 incubated at a 100-fold greater molar concentration than IL-7-PE. An inhibition of IL-7-PE binding demonstrated the specificity of IL-7 binding to its receptors (Fig. 9 *B*). Approximately 53% of this CLL patient's cells specifically bound IL-7-PE (Fig. 9 *C*). IL-7 receptor–positive cells were surface phenotyped using anti-CD5 conjugated with FITC and anti-CD20 conjugated with PerCP. Three distinct subpopulations of IL-7 receptor–bearing cells were identified. The majority of the IL-7 receptor–positive cells were $CD5^-$,$CD20^-$ non-T, non-B cells. The $CD5^+$,$CD20^+$ leukemic subset comprised approximately half of the IL-7 receptor–positive cells, while $CD5^+$ T cells accounted for the remainder. Virtually no conventional $CD5^-$,$CD20^+$ B cells were IL-7 receptor positive. These studies revealed, therefore, that while a proportion of the leukemic $CD5^+$,$CD20^+$ subset of B cells expressed detectable IL-7 receptors, other subsets in the patients' peripheral blood, including T cells and non-T, non-B cells, also expressed IL-7 receptors.

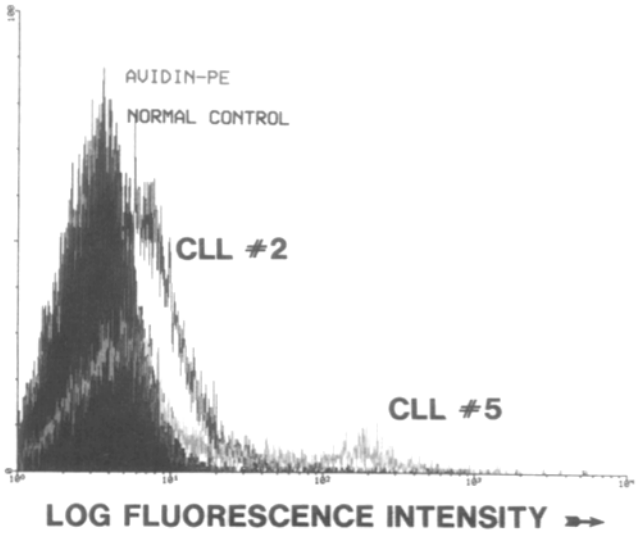

Figure 8. Flow cytometry analysis of PE-streptavidin-biotin-conjugated IL-7 bound to cells from two CLL patients, CLL no. 2 (*black lines*) and CLL no. 5 (*gray lines*), but not to normal T-depleted PBL (*filled area*). CLL cells incubated with PE-streptavidin but without biotinylated IL-7 are indicated as the hatched lines over the solid peak.

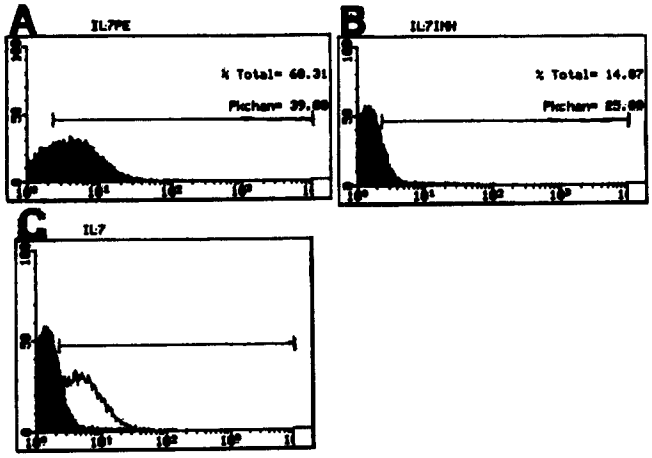

Figure 9. Specificity of IL-7 binding to CLL cells. (*A*) CLL no. 2 cells were labeled with PE-conjugated IL-7. (*B*) A second sample of cells was preincubated with 100-fold molar excess of unlabeled purified human rIL-7 before addition of the PE-conjugated IL-7. (*C*) *A* and *B* were overlaid and the specific IL-7 receptor–bearing cells in the white area under the curve were further analyzed for expression of CD5 and CD20.

Discussion

We have demonstrated that mRNA for the cytokine IL-7 is expressed in cells from the leukemic subset of PBMC of patients with CLL. Normal B cells do not make detectable quantities of IL-7 mRNA. IL-7 serves as a progenitor lymphoid cell growth factor normally produced by stromal cells; thus, one possible role of IL-7 in CLL may be to serve as an autocrine or paracrine growth signal for the proliferating subset of B-CLL cells. Recent studies have suggested that IL-7 can trigger proliferation in a variety of leukemic cells,

Table 5. *Cytokine and Cytokine Receptor Expression in $CD5^+$,$CD20^+$ Normal Cells*

Normals	IL-1α	IL-1β	IL-2R	IL-7
LS 2	–	+	+	–
LS 7	–	+	+	–
LS 8		+	+	–

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including CLL (42–45). Most CLL cells, however, are not proliferating, but accumulating, as rather mature B cells. IL-7, apparently, does not trigger proliferation in such mature B cells (46). Another possible role for IL-7 in CLL may be to serve as as a differentiation signal to push the leukemic cells into this mature stage of nonproliferating B cells. A third, alternative hypothesis for the function of IL-7 in CLL is suggested by the fact that IL-7 binding was detected in a variety of cells from CLL patients, including T cells and non-T, non-B cells, as well as a portion of the leukemic B cells. A recent publication suggested that four of six CLL patients' cells specifically bound radiolabeled IL-7, however, these studies did not reveal the proportion of CLL cells or other subsets that may also express IL-7 receptors (42). It has also been shown that IL-7 serves as a signal for the induction of cytotoxic T and NK cells (47–50). These studies suggest that IL-7 can serve as a potent inducer of antitumor cytotoxic cells. Tumor cell–derived IL-7 in CLL could be triggering IL-7 receptors on nonleukemic cells and inducing such cell-mediated antitumor immunity, thereby resulting in the diminution of the disease with a prolongation of the leukemic course.

The fact that IL-7 expression was only detected in cells obtained from leukemic patients and not from normal donors suggests that IL-7 mRNA expression may be related to the leukemic transformation of these B cells (Table 3). Enriched normal $CD5^+$,$CD20^+$ B cells gave no evidence for IL-7 expression, but, IL-2 receptor expression was readily detected (Table 5). The normal majority B cell subset, $CD5^-$,$CD20^+$, also expressed IL-2 receptor but did not express IL-7 (Fig. 5, lanes *2* and *3*). It had been suggested that IL-1β expression by CLL cells was related to their leukemic transformation (18–20). Our PCR data with normal B cells refute that hypothesis and indicate that IL-1β expression occurs as a natural event in B cells (Fig. 5, lane *5*). The most compelling evidence, however, that IL-7 mRNA expression is related to leukemic transformation stems from our studies with fractionated cells from $CD5^+$ vs. $CD5^-$ CLL patients (Fig. 6). In the $CD5^-$ CLL patient, an IL-7 PCR product was readily detected in the leukemic cell subset, $CD5^-$,$CD19^+$ (Fig. 6, lane *6*). Yet, in the $CD5^+$ patient, an IL-7 PCR product was not detected in the same nonleukemic "normal subset," $CD5^-$,$CD19^+$ (Fig. 6, lane *4*), but, again, was detected in the leukemic cell subset, $CD5^+$,$CD19^+$ (Fig. 6, lane *2*). These data strongly support the suggestion that the expression of IL-7 may be associated with the leukemic transformation of B cells.

Our findings with IL-7 differ from murine lymphoma studies in that all human patients tested to date yield positive IL-7 PCR products with reverse-transcribed total RNA prepared from their cells (Table 3), while no IL-7 PCR product was detected in the mouse cells (51). The reasons for these differences are unclear. The primer sets used in each study were quite different. The IL-7 primer sets used in our studies spanned six exons, with the 5′ end of the PCR product beginning at the ATG initiation codon for the first amino acid of the signal sequence and ending subsequent to the coding sequence in the 3′ noncoding region (52). The 681-bp IL-7 PCR product, therefore, contained the entire coding sequence for the IL-7 precursor protein. The primer set for IL-7 used in the murine lymphoma study began and ended in the 3′ noncoding region of the cDNA sequence. Perhaps the differences in the primer sets used in the two studies could account for the dissimilar results observed, or perhaps stability problems for the IL-7 mRNAs occurred with the murine lymphoma cells.

In the IL-7 PCR products from CLL cells, a second, smaller, minor band was generally also evident at ~550 bp, as observed in Figs. 1, 2, 5, and 6. This second band represents a PCR product derived from an alternatively spliced cDNA. The isolation of the original cDNA clones for human IL-7 revealed a deletion in one of the cDNA clones that was found to be due to alternative splicing (27). The alternative transcript lacked the entire exon 4, which encodes 44 amino acids, thereby reducing the transcript size by 132 nucleotides. This size reduction corresponds precisely with the size of the second IL-7 PCR product band observed in our gels. The PVUII restriction site of this alternatively spliced IL-7 transcript remains intact (27, 52). Upon PVUII digestion of our IL-7 PCR products, both the major and minor bands virtually disappeared and only the two expected digestion product bands were obtained, as observed in Fig. 1. One of the restriction products of the alternatively spliced, minor band is identical with the 302-bp band of the major IL-7 band's restriction product and should merge with it, thereby appearing as a more dense band than that of the larger 379-bp band, as observed in Fig. 1, lane *3*. The second PVUII digestion product of the minor IL-7 PCR product should be ~247-bp and does not appear as a distinct third band in the figures presented here, but a diffuse band at the second marker band (246 bp) has been observed in gels where the undigested sample demonstrates a more dense alternate spliced product. The significance of the alternatively spliced transcript remains unknown. Our ability to detect both the entire intact coding sequence for IL-7 and an alternative spliced transcript that had been defined through molecular cloning lends further credence to our conclusions that CLL cells transcribe IL-7 mRNA. The regulation of expression of IL-7 in CLL as well as a determination of its function in the disease state remain as questions that need to be answered and are major areas of experimentation in our laboratory.

Our data suggest that within a given patient only a proportion of the CLL cells express IL-7 receptors and that other peripheral mononuclear cells, including some T cells and some non-T, non-B cells, also express IL-7 receptors. As has been the case for many of the cytokines, IL-7 has now been shown to have effects on a broader variety of cells than initially described, hence, IL-7 could also be effecting the T cell defects observed in CLL patients. Besides the possible role of IL-7 in autocrine signaling of CLL growth and/or differentiation, it is also possible that IL-7 may directly trigger the disease-associated immunological aberrations in CLL.

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