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Common and Pre-B Acute Lymphoblastic Leukemia Cells Express Interleukin 2 Receptors, and Interleukin 2 Stimulates In Vitro Colony Formation

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The role of interleukin 2 (IL 2) as a possible regulator of in vitro proliferation and differentiation of non-T acute lymphoblastic leukemia (ALL) cells was investigated. For this purpose, leukemic cells from the blood or bone marrow of eight untreated patients with common or pre-B ALL were analyzed using the anti-Tac monoclonal antibody (reactive with the IL 2 receptor) in indirect immunofluorescence. The receptors for IL 2, which were initially absent from the cell surface, were induced on high percentages of the ALL cells after the in vitro exposure to the lectin phytohemagglutinin or the phorbol ester 12-0-tetradecanoylphorbol-13-acetate in six patients, suggesting that the cells had become sensitive to IL 2. In colony cultures to which feeder leukocytes and IL 2 had been added, colony growth was obtained in five of eight cases. Whereas the cells from one patient formed colonies in the absence of exogenous stimuli, the cells from others were dependent on the addition of feeder leukocytes plus IL 2. In the latter cases, feeder leukocytes alone, releasing some IL 2, stimulated growth suboptimally at different cell concentrations. Their stimulative effect was significantly enhanced when leukocyte-derived IL 2 or pure recombinant IL 2 was supplemented. Alone, IL 2 (up to 500 U/mL) did not support colony formation. Apparently, IL 2 and feeder leukocytes are both required for the induction of colonies in these cases of ALL. From cell sorting of fluorescent anti-common ALL antigen (CALLA) stained cells it appeared that colonies descended from cells with high as well as low or negative CALLA expression. Immunophenotyping demonstrated the presence of the original leukemia markers on colony cells, but was not indicative of maturation of ALL toward more differentiated B cells. We suggest that IL 2 can stimulate the in vitro proliferation of certain neoplastic B lymphocyte progenitors.

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INTERLEUKIN 2 (IL 2) is the soluble factor responsible for the in vitro proliferation of antigen or lectin-activated T lymphocytes.[1] Prior activation is required to induce receptors for IL 2 on the cell surface membrane. The monoclonal antibody anti-Tac specifically binds to these receptors (also called Tac antigens).[2,3]

Recently, activated human B cells have also been demonstrated to express IL 2 receptors, and results from ^{3}H-thymidine incorporation assays indicate that a subpopulation of these cells proliferates in the presence of immunopurified IL 2 without the addition of other factors.[4] In addition, it has been shown that in vitro colony formation by B chronic lymphocytic leukemia cells is induced by IL 2.[5]

Here we present our first attempts directed toward the characterization of factors required for the in vitro colony formation by neoplastic B cell progenitors, ie, common and pre-B acute lymphoblastic leukemia (ALL) cells. The findings suggest that, in certain cases, IL 2 may be involved in the proliferation of these leukemias. ALL cells initially lack but express IL 2 receptors following exposure to 12-0-tetradecanoylphorbol-13-acetate (TPA) or to phytohemagglutinin (PHA). The addition of IL 2 to colony cultures can stimulate ALL colony formation.

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MATERIALS AND METHODS

Patients and separation of leukemia cells. Eight untreated patients with ALL were studied. The leukemic cells were classified as common or pre-B ALL cells on the basis of immunologic phenotyping[6] (Table 1). Leukemic cells were isolated from peripheral blood or bone marrow following Ficoll-Isopaque (Nygaard, Oslo) separation.[7]

Contaminating E rosette-positive lymphocytes were then removed from the leukemic cell fractions by rosetting with 2-aminoethylisothiouronium bromide (AET)-treated sheep erythrocytes and Ficoll-Isopaque sedimentation according to Madsen et al.[8]

Immunofluorescence studies. Surface antigens on fresh and cultured cells were assayed by means of indirect immunofluorescence using murine monoclonal antibodies (MoAb) and goat anti-mouse immunoglobulin (Ig) coupled with fluorescein isothiocyanate (GAM/FITC, Nordic, Tilburg, The Netherlands). Details on the labeling procedures have been given elsewhere.[9] Cells were evaluated for specific fluorescence with either a Zeiss fluorescence microscope (200 cells per slide were scored) or a fluorescence-activated cell sorter (FACS 440, Becton Dickinson, Sunnyvale, Calif). Control incubations with GAM/FITC alone, included in each test to check for nonspecific binding of the conjugate, were negative in all experiments. Cell surface membrane-associated immunoglobulins (sIg) were assayed as described.[10] Cytoplasmic immunoglobulin M heavy chains (Cy μ) were stained in cytocentrifuged cells (Shandon, Cheshire, England) with 1:25 diluted goat anti-human Ig M/FITC (GAHuIgM/FITC, Nordic).[11] Two hundred cells per slide were examined with a fluorescence microscope. Control stainings with goat anti-human IgG/FITC and goat anti-human κ or λ light chain/FITC were negative.

Dual stainings: after the first MoAb (anti-Tac; IgG2a) treatment and labeling with 1:40 diluted goat anti-mouse IgG2a coupled to tetramethylrhodamine isothiocyanate (GAM/IgG2a/TRITC; Nordic), the cells were spun on a microscope slide and fixed for ten minutes at −20 °C in 5% vol/vol acetic acid in methanol. After three washings with phosphate-buffered saline (PBS), they were further treated with either 1:10 diluted MoAb VIL Al (IgM) and 1:40 diluted goat anti-mouse IgM FITC conjugate (GAM/IgM/FITC)

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Table 1. IL 2 Receptor Expression in Eight Cases of ALL

Patient			Leukemic Phenotype (Percentage of Positive Cells)†			IL 2 Receptor Expression (Percentage of Positive Cells)†		
No.	Age (yr)	Source*	CALLA	Cy μ	BA_2	Before Incubation	18 h PHA Incubation‡	18 h TPA Incubation‡
1	61	PB	60	50	82	0	53	43
2	2	PB	83	86	87	0	57	62
3	17	BM	76	45	70	0	38	51
4	7	BM	75	75	76	0	24	26
5	3	PB	60	0	95	0	63	64
6	16	BM	60	0	27	0	0	0
7	43	PB	53	0	74	0	62	65
8	38	BM	91	0	99	0	0	0

Blast cell phenotyping also included the T cell markers T3, T11, and WT1. These were negative in all cases. Patients No. 1 through 4, pre-B ALL; patients No. 5 through 8, common ALL.

*Leukemic cells wee isolated from peripheral blood (PB) or bone marrow (BM).

†Determined by fluorescence microscopy.

‡Details are given in Materials and Methods.

or GAHuIgM/FITC for the simultaneous visualization of IL 2 receptors and common ALL antigens or IL 2 receptors and cytoplasmic IgM heavy chains, respectively.

Control stainings (VIL Al [IgM] + GAM/IgG2a/TRITC, anti-Tac [IgG2a] + GAM/IgM/FITC, GAM conjugates without MoAb pretreatment) were all negative.

Monoclonal antibodies. The following MoAb were used in this study (in parentheses are the dilutions used in indirect immunofluorescence): (1) anti-Tac (1:1,000), anti-IL 2 surface membrane receptor[2] (Dr T. Uchiyama, Kyoto, Japan); (2) VIL Al (1:100), anti-common ALL antigen (CALLA)[12] (Dr W. Knapp, Vienna); (3) BA2 (1:250), anti-pre-B cell differentiation antigen[13] (Hybritech, San Diego); (4) WT1 (1:100), anti-pan-T cell antigen[14] (Dr W.J.M. Tax, Nijmegen, The Netherlands); (5) OKT11 (1:40), anti-sheep erythrocyte rosette receptor (Ortho Pharmaceutical Corp, Raritan, NJ); (6) OKT3 (1:40), anti-mature thymocyte and T lymphocyte antigen (Ortho Pharmaceutical Corp); and (7) S4-7 (1:500), anti-myeloblast → granulocyte and monocyte antigen[15] (Dr G. Rovera, Philadelphia).

Colony culture. Colony culture experiments were performed according to a previously described culture system with PHA and irradiated (2,500 rad) human peripheral blood leukocytes as stimulators.[15] This system supports the formation of normal T lymphocyte as well as myeloid leukemia colonies[16,17]; 2×10^5 cells were plated in each culture dish. On certain occasions, modifications were made to the system: (1) replacement of PHA (reagent grade; Wellcome, Dartford, England) by 100 ng of the phorbol ester TPA (Sigma Chemical Corp, St Louis); (2) addition of IL 2; and (3) omission of feeder leukocytes. Two sources of IL 2 were used: 15,000 mol wt fractions of gel-filtered (Ultrogel AcA 54; LKB, Bromma, Sweden) culture media from lectin-stimulated human leukocyte cultures and pure IL 2 obtained by recombinant DNA techniques (rIL 2; Biogen SA, Geneva).

Suspension culture. Suspension cultures were performed in 6-mL tubes; 2×10^6 cells were cultured in 1 mL of alpha medium (with 10% vol/vol fetal calf serum) with either 100 ng/mL TPA or 1% vol/vol PHA for 18 hours in a fully humidified atmosphere of 5% CO_2 at 37 °C. They were washed three times with PBS and prepared for indirect immunofluorescence.

Cell sorting. In one series of experiments, cells were inoculated into culture following MoAb VIL Al (anti-CALLA) and GAM/FITC incubations and fluorescence-activated cell sorting (by FACS 440).

RESULTS

Induction of IL 2 membrane receptors on pre-B ALL cells. Fresh ALL cells did not express IL 2 receptors on their membranes as assessed with the MoAb anti-Tac. On

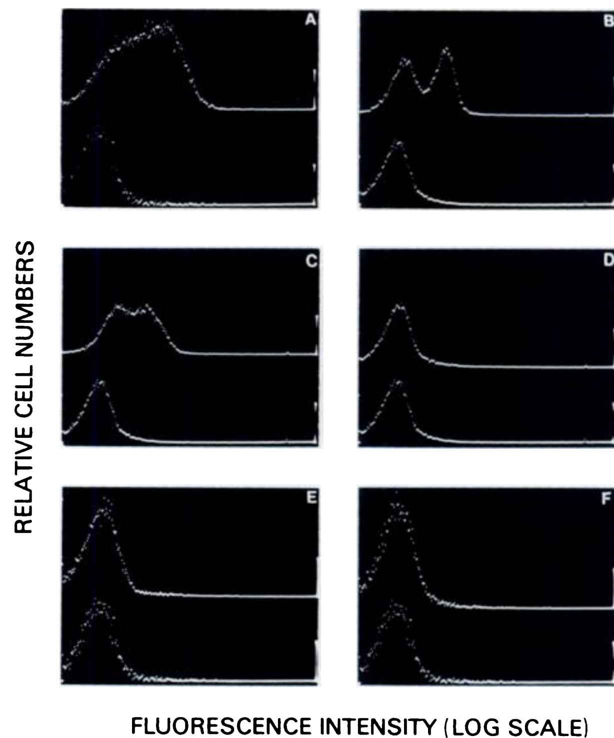

Fig 1. Flow cytofluorometric analysis of membrane antigens expressed by pre-B ALL cells of patient No. 1 following incubation with TPA. In each panel, the histograms of the log fluorescence intensity (horizontal axis) *v* relative cell numbers (vertical axis) are shown. The upper graphs represent stainings with MoAb and goat anti-mouse Ig coupled to fluorescein isothiocyanate (GAM/FITC); the lower graphs represent control stainings with GAM/FITC alone. (A) anti-Tac, (B) VIL Al, (C) BA2, (D) WT1, (E) OKT11, and (F) OKT3.

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the other hand, IL 2 receptors were found in high percentages of cells following incubation in suspension with TPA or PHA in each of four cases of pre-B ALL and in two of three cases of common ALL (Table 1). Less than 1% of the incubated cells formed E rosettes, indicating that the anti-Tac binding was not due to contaminating E-positive T lymphocytes. Evidence for the presence of IL 2 receptors on pre-B cells following TPA exposure was obtained when the cells from patient No. 1 were phenotyped in more detail. Cytofluorometric analysis (Fig 1) demonstrated the binding of anti-Tac (57% positive), anti-CALLA (54% positive), and anti–pre-B cell antigen (BA2) (66% positive) antibodies on the incubated cells, whereas the T cell markers T3, T11, and WT1 all remained negative. A similar binding pattern (not shown here) was found after incubation with PHA. In addition, two-color fluorescence labeling methods revealed the simultaneous expression of the IL 2 receptors with CALLA and with Cy μ on individual cells; 80% of the CALLA-positive cells expressed the IL 2 receptors (200 CALLA-positive cells counted) (Fig 2A1 and A2), and such receptors were found on 47% of the Cy μ-positive cells (Fig 2B1 and B2). As expected, IL 2 receptors could be induced on normal peripheral blood T lymphocytes using these methods (24% to 55% anti-Tac–positive cells after PHA and TPA incubation, respectively).

ALL colony formation. Colony data from PHA-supplemented cultures are summarized in Table 2. In four cases (patients No. 1, 4, 7, and 8) colony formation was obtained (in a range of 47 to 63 colonies per 10^5 plated cells) in the presence of irradiated feeder leukocytes (in the culture underlayer) and IL 2.

The stimulative effect of IL 2 is evident from the comparison with cultures with leukocytes but without the addition of IL 2. Colony formation by cells from patients No. 1, 4, and 7 was enhanced by the extra IL 2. We hypothesized that suboptimal concentrations of IL 2 had been produced by the feeder leukocytes.

In these cases, PHA and IL 2 alone, ie, without leukocytes, were not active (Table 2) even when IL 2 was present in concentrations up to 100 U/mL (not shown). Apparently feeder leukocyte-derived factor(s) other than IL 2 were also essential for these ALL cells to form colonies.

The additional role of leukocyte stimulation is illustrated in cultures reconstituted with increasing numbers of feeder cells in the presence of 25 units of IL 2 (Fig 3). Colony numbers increased progressively when titrated numbers of feeder leukocytes were added to the cultures. In addition, the stimulative effect of IL 2 is evident from these experiments: colony numbers were significantly higher in the cultures supplied with exogenous IL 2 than in those without extra IL 2. These results suggest that IL 2, in combination with leukocyte-derived factors, was required for in vitro colony formation by the pre-B ALL cells.

To exclude the possibility that contaminating molecules in the IL 2 preparation had been responsible for the stimulation of colony growth, the cells of patient No. 1 were cultured with increasing concentrations (25 to 500 U/mL) of pure rIL 2 in the presence of PHA and 2×10^6 feeder leukocytes. The addition of rIL 2 enhanced colony growth in a dose-dependent fashion. Colony numbers in cultures with 500 rIL 2 were about three times higher than those in cultures without rIL 2. Similarly to IL 2, rIL 2 (500 U/mL) could not induce colony formation in the absence of additional feeder leukocyte stimulation.

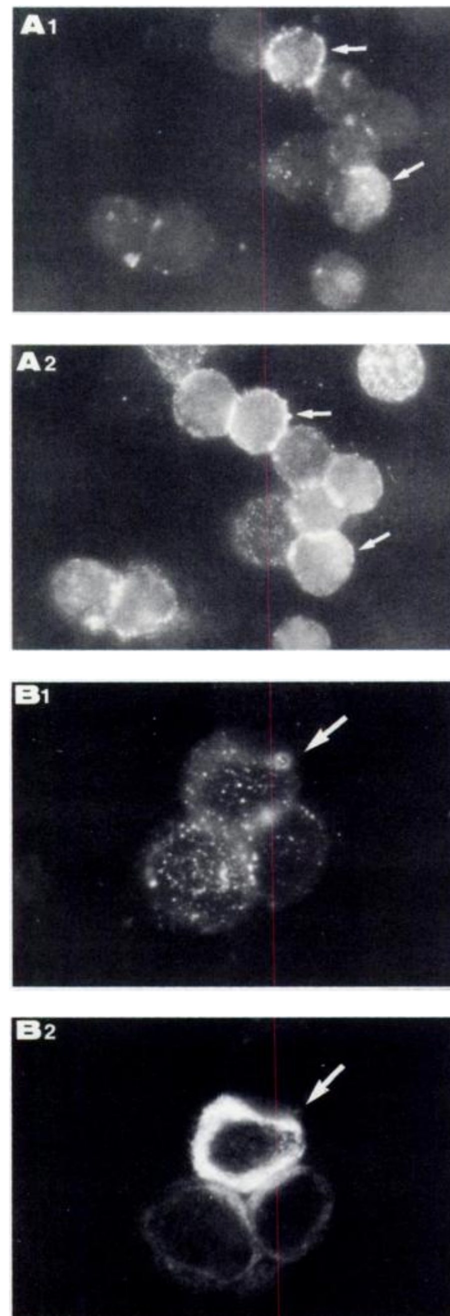

Fig 2. Two-color immunofluorescence microscopy of pre-B ALL cells of patient No. 1 after incubation with TPA for 18 hours. The arrows indicate double fluorescent cells. (A) anti-Tac (A1) and VIL Al (anti-CALLA) (A2); (B) anti-Tac (B1) and anti-Cy μ (B2).

Cultures from the cells of patients No. 2, 3, and 5 did not give rise to colonies under any of the conditions tested. On the other hand, cells from patient No. 6 produced colonies in PHA containing culture medium without further additives,

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Table 2. ALL Colony Formation in PHA-Supplemented Cultures

Patient No.	Growth Stimulation	Colonies per 10^5 Plated Cells: 2×10^6 Irradiated Leukocytes + 25 U IL 2	2×10^6 Irradiated Leukocytes	25 U IL 2	None	Immunologic Markers on Colony Cells* (Percentage Positive): CALLA	BA2	Cy μ	sIg	E	WT1
1		49	31	0	0	53	69	29	0	0	0
2		0	0	0	0	—	—	—	—	—	—
3		0	0	0	0	—	—	—	—	—	—
4		63	0	0	0	10	58	ND	0	12	8
5		0	0	0	0	—	—	—	—	—	—
6		25	22	80	80	49	76	0	0	22	ND
7		47	36	5	0	57	77	0	0	0	10
8		54	49	2	0	22	67	0	0	0	11

All experiments included control cultures without the addition of PHA; no colonies were formed in these cultures. ND, not determined.

*Pooled colonies were harvested from the plates with a Pasteur pipette, suspended to a single-cell suspension, and prepared for immunofluorescence microscopy as described in Materials and Methods.

indicating that proliferation occurred independent of exogenous growth stimulators.

Phenotyping of ALL colony cells. Colony cells were examined morphologically and immunologically. Their microscopic appearance was consistent with that of lymphoblasts. Immunologic phenotyping (Table 2) of the colony cells confirmed the presence of the preculture ALL markers (CALLA, BA2, Cy μ), although the percent expressions before and after culture differed in some instances. Indications for the in vitro maturation of ALL cells were not obtained. A loss of CALLA expression was noted following culture in two of eight cases, but this was not accompanied by the acquisition of cytoplasmic IgM heavy chains or the appearance of sIg (Table 2).

The cultured cells were checked for the presence of T lymphocytes with markers E and WT1 inasmuch as the applied culture conditions are known to be highly permissive for T cell colony formation (Table 2). Significant contaminating proliferation of T lymphocyte colony-forming cells did not occur in each of these patients. The presence of myelomonocytic cells was excluded using marker MoAb S4-7.

Expression of CALLA on ALL colony-forming cells. Using a FACS 440 cell sorter, VIL AI- and GAM/FITC- stained cells from patients No. 1 and 8 were sorted in two fractions: (1) cells with a high CALLA density and (2) cells without CALLA or with a low CALLA density. The results listed in Table 3 show that colony-forming cells were recovered from both fractions.

DISCUSSION

IL 2, previously referred to as T cell growth factor,[1] has been identified as the essential stimulatory component for in vitro T lymphocyte proliferation. However, recent studies have suggested that IL 2 can also stimulate the proliferation of human B lymphocytes.[4] Very little is known about factors involved in the growth of precursor cells of the human B lymphocyte lineage. The present experiments provide indications that IL 2 can act on leukemic B cell progenitors, ie, common and pre-B ALL colony-forming cells. Cells obtained from eight patients at diagnosis were studied. In six cases, the ALL cells could be induced to express membrane receptors for IL 2 (which they initially lacked) by the in vitro exposure to a lectin (PHA) or a phorbol ester (TPA), similar to normal peripheral blood T lymphocytes. These findings raised the question of whether ALL cells may respond to IL 2 and proliferate in culture. To investigate this possibility, we plated the cells in a colony culture system containing PHA or TPA and leukocyte feeder cells either with or without the addition of human IL 2. Colony growth was obtained with the cells of five patients.

The addition of leukocyte-derived IL 2 to the culture medium significantly enhanced colony formation by the leukemic cells of patient No. 1, whereas those from patient

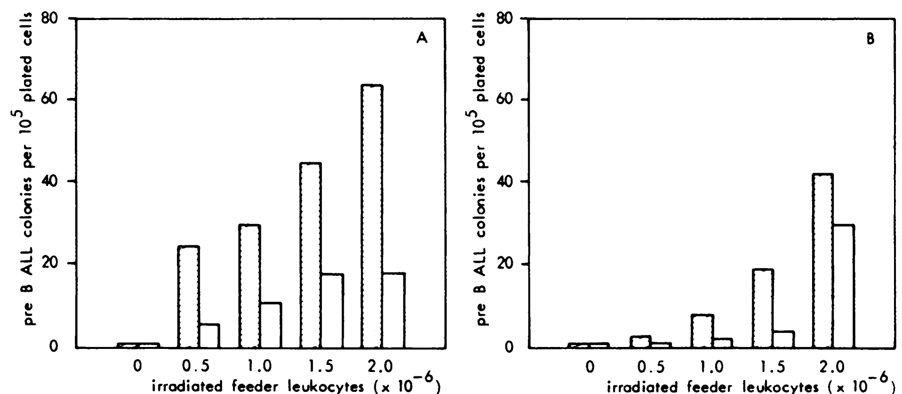

Fig 3. Pre-B ALL colony formation in patient No. 1 with (▨) or without (□) 25 units of IL 2 added to the upper layers of the cultures. Colonies (50 cells or more) were counted on day 7 of the culture. The effect of IL 2 on colony growth was assessed in relationship with varying numbers of irradiated peripheral blood leukocytes in the underlayer. Sections A and B indicate the experiments in which cultures were supplemented with TPA (100 ng per dish) and PHA (0.01 mL per dish), respectively.

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Table 3. CALLA on ALL Colony-Forming Cells

Sorted Fractions*	Cell Recovery (%)	Colony-Forming Cells per 10^5
Patient No. 1		
Unfractionated	100	47
CALLA-negative or weakly positive‡	35.4	40†
CALLA-positive	64.6	46
Patient No. 8		
Unfractionated	100	24
CALLA-negative or weakly positive‡	25.4	6
CALLA-positive	74.6	27

Colonies were grown in the PHA leukocyte feeder system supplemented with 25 units of IL 2. It was not possible in these cases to separate the CALLA-negative cells from the cells weakly expressing the antigen since histogram analysis of the CALLA fluorescence distribution did not reveal distinct CALLA-positive and CALLA-negative populations.

*Cells were stained with MoAb VIL A1 and GAM/FITC and sorted on the basis of fluorescence intensity.

†Colony cells were harvested and analyzed for CALLA expression. Forty-three percent of the cells showed bright fluorescence after VIL A1 and GAM/FITC staining.

‡Fluorescence intensity did not exceed the maximal fluorescence of cells stained with GAM/FITC alone.

No. 4 were absolutely dependent on the supply of IL 2. Pure (recombinant) IL 2 exerted a similar effect. These results support the idea that the in vitro proliferation of ALL cells can be stimulated by IL 2. The findings also suggest that IL 2 was not the only growth factor involved in these cases: omission of feeder leukocytes from IL 2-containing cultures abrogated colony growth, and a positive relationship between feeder cell numbers and ALL colony response was established (Fig 3). In this respect, the stimulation of the ALL colony-forming cells clearly differs from that of clonogenic T lymphocytes. Normal E rosette-positive peripheral blood T cells produce colonies in PHA and IL 2-containing cultures and do not need additional feeder cell stimulation (data not shown).

A second difference relates to the optimal IL 2 concentration needed for colony growth: T colony-forming cells are stimulated maximally by 10 to 25 U/mL of IL 2, whereas ALL colony numbers (patient No. 1) do not reach plateau values in the presence of 500 U/mL of IL 2 (data not shown).

In view of the IL 2 receptor expression on high numbers of the cells following stimulation with PHA or TPA, a role for IL 2 in ALL colony formation through a direct interaction between IL 2 and IL 2 receptors on the clonogenic ALL cells is strongly suggested. Nevertheless, the alternative explanation for these findings, ie, that the effect of IL 2 had been indirect (on the feeder cells), has to be considered. Unfortunately, we have not yet been able to produce a cell-free source of stimulation that can efficiently replace the leukocytes, which is a prerequisite to address this problem.

Although we have, so far, only studied a small group of patients, a marked diversity in growth requirements was noted. In discrepancy with the essential culture conditions described above for patients No. 1 and 4, the cells from patient No. 6 produced colonies independently of exogenous growth factors. In addition, the leukemic cells of patients No. 2, 3, and 5 did not give rise to colonies, although they were capable of IL 2 receptor expression. This inability to proliferate may reflect different requirements for growth factors other than IL 2. For example, this could indicate that in certain cases the applied leukocyte feeder does not provide sufficient concentrations of these factors for proliferation.

Recently, the murine growth factor, IL 3, has been shown to support the growth and maturation of mouse pre-B cell clones.[17] It is possible that an analogous regulator produced by the feeder leukocytes is involved in pre-B ALL cell proliferation in humans.

ALL colony-forming cells were analyzed for CALLA expression by fluorescence-activated cell sorting (Table 3). In the two cases investigated, colony-forming cells were recovered from cell fractions with bright as well as dull or negative CALLA fluorescence. These findings are consistent with the CALLA positivity of ALL colony-forming cells, but they do not exclude the possibility of a coexistent CALLA-negative precursor subset. Further investigations along this line appear to be of extreme importance in view of the immunotherapeutic use of anti-CALLA monoclonal antibodies.

We have not obtained indications for the in vitro differentiation of ALL cells to a more mature cell type, neither in PHA- nor in TPA-supplemented colony cultures. The very limited capacity of ALL to differentiate in vitro has been noted by others,[19,20] and so far, only one case of ALL has been reported in which the leukemic cells matured to sIg-bearing B cells.[21] Recently, Ralph and co-workers demonstrated that high concentrations (ie, 10^3 to 10^4 U/mL) of IL 2 can induce the maturation of normal human B lymphocytes.[22] The question of whether high IL 2 concentrations are also effective in inducing differentiation of ALL cells cannot yet be answered.

We conclude that the induction of IL 2 receptors and the subsequent proliferative response in the presence of IL 2 in vitro found in ALL cells raises the possibility of a direct regulatory role of IL 2 in early stages of human B cell differentiation.

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