USE OF A NOVEL COLONY ASSAY TO EVALUATE THE CYTOTOXICITY OF AN IMMUNOTOXIN CONTAINING POKEWEED ANTIVIRAL PROTEIN AGAINST BLAST PROGENITOR CELLS FRESHLY OBTAINED FROM PATIENTS WITH COMMON B-LINEAGE ACUTE LYMPHOBLASTIC LEUKEMIA

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Autologous bone marrow transplantation (BMT)[1] is being evaluated at several medical centers as an alternative approach to allogeneic BMT for the treatment of acute lymphoblastic leukemia (ALL) patients who do not have matched sibling donors (1, 2). A major limitation of autologous BMT is the likely presence of residual leukemia in the remission marrow (2). This difficulty makes *ex vivo* purging of autologous stem cell grafts before transplantation essential for therapeutic efficacy (3). Current *ex vivo* purging strategies involve the treatment of autologous marrow with cyclophosphamide congeners (3–5), mAb plus complement (6–9), and immunotoxins (IT) (10–18). Our laboratory is committed to the evaluation of the clinical potential of IT in autologous BMT for ALL. We have begun a phase I trial in T-ALL using intact ricin IT as purgative reagents to eliminate occult leukemic T-lineage blasts from autologous marrow grafts prior to reinfusion (18). In common B-lineage ALL, our Bone Marrow Transplant Team at the University of Minnesota has emphasized the use of mAb plus complement for *ex vivo* bone marrow treatment (19). Despite such *ex vivo* manipulation of autologous stem cell grafts, recurrent leukemia in autotransplanted patients remains the major cause for treatment failure in ALL (19–21).

Available methods do not determine whether relapses occur because of incom-

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[1] *Abbreviations used in this paper:* ALL, acute lymphoblastic leukemia; BMT, bone marrow transplantation; IT, immunotoxin; LCM, lymphocyte-conditioned medium; M/E, myeloid/erythroid; NSE, nonspecific esterase; PAP, pokeweed antiviral protein; PAS, periodic acid–Schiff; sIg, surface Ig.

plete purging of marrow or incomplete eradication of leukemia in patients. Present clinical trials usually rely on preclinical studies in which the efficacy of purgative reagents is evaluated against in vitro established tumor cell lines (7, 9). However, there is a marked heterogeneity in surface antigen expression among leukemia cell populations (22, 23) and neither the phenotype nor drug or toxin sensitivity of leukemic progenitor cells is as yet known. Therefore, model systems such as clonogenic assays of homogeneous leukemic cell lines, while potentially useful for quantitating the relative effectiveness of various purgative reagents and optimizing treatment conditions, cannot be extrapolated for clinical ALL with any certainty. We (14–17, 24, 25) and others (7, 9, 10, 26) have discussed the problems inherent in using established cell lines in vitro as a model system for evaluating the antileukemic efficacy of a purging reagent. These problems have received little attention because of the difficulty in growing freshly obtained ALL cells in vitro (26, 27). To our knowledge, none of the reagents currently under clinical evaluation have been tested directly against clonogenic blasts from ALL patients, and therefore there is no evidence that they can effectively eliminate leukemic progenitor cells. In the absence of such data, it is difficult to determine the value of *ex vivo* marrow purging. Clearly, the critical evaluation of purging methods at the level of leukemic progenitor cells will be most important to determining whether the therapeutic outcome of autologous BMT in ALL can be improved by modifications in the current clinical purging protocols.

Herein, we report the results of a preclinical study examining the efficacy of B43-PAP, a human B cell–directed IT containing pokeweed antiviral protein (PAP), against leukemic progenitor cells from patients with common B-lineage ALL. We show that B43-PAP selectively eliminated blast progenitor cells in all 10 patients, with minimal toxicity to normal progenitor cells. These studies are of special interest because, to our knowledge, B43-PAP is the first mAb-toxin conjugate to prove effective against common B-lineage ALL blasts and their progenitors.

The leukemic progenitor cell assay described in this paper is devised to provide a basis for future comparative studies of different purging strategies, with the aim of defining an optimal and standardized protocol for removing residual clonogenic leukemia cells from autologous marrow grafts in common B-lineage ALL. Our investigation represents the first application of this novel colony assay to a major unresolved problem in human BMT for ALL.

Materials and Methods

Patient Material. 12 patients with ALL were included in this study. Clinical and diagnostic data are summarized in Table I. Bone marrow aspirate samples were procured by conventional methods. Morphological classification was performed on Wright-Giemsa–stained slides using a modification of the original FAB classification (28). Blast cells were isolated by a single centrifugation on Ficoll-Hypaque (1.077 g/cm^3). Marker analyses by indirect immunofluorescence and flow cytometry were performed using a panel of mAb that define B-lineage (i.e., B43, BA-1, BA-2, BA-3) and T-lineage (i.e., 35.1, T101, 3A-1) leukocyte differentiation antigens. FITC-labeled goat F(ab')$_2$ anti–mouse IgG (Cappel Laboratories, Cochranville, NC) served as secondary antibody. Surface immunoglobulin (sIg) was assayed by direct immunofluorescence using FITC-conjugated goat F(ab')$_2$ anti–human total Ig (Kallestad, Austin, TX). Background fluorescence was determined by

TABLE I
Clinical and Diagnostic Data on Patients with ALL

Patient	Sex	Age (yr)	Diagnosis (FAB)	New case or relapse	Bone marrow (% blasts)
1	M	18	L1	New	97
2	M	3	L2	New	99
3	F	5	L1	Relapse	94
4	M	9	L1	Relapse	91
5	F	1	L1	New	91
6	F	12	L1	Relapse	91
7	M	2	L1	Relapse	100
8	F	2	L1	New	100
9	F	2	L2	New	79
10	F	2	L1	New	78
11	F	4	L1	New	100
12	M	5	L1	New	95

incubating cells with an IgG2a murine myeloma protein (UPC 10; Litton Bionetics, Charleston, SC). Cells were analyzed for immunofluorescence using a Spectrum III Cytofluorograph (Ortho Diagnostics, Raritan, NJ). We attempted to examine only the lymphoblasts within each specimen by appropriate gating. Samples were scored as positive when >20% of cells bound the antibody used.

Colony Assay for Common B-lineage ALL Progenitor Cells. Freshly obtained bone marrow blasts were suspended in α-MEM supplemented with 0.9% methylcellulose, 15% FCS, 15% platelet-rich fresh human plasma, 10% PHA-LCM (PHA-stimulated lymphocyte-conditioned medium), 1% MEM vitamin solution, 0.5% MEM amino acids solution, 0.5% MEM nonessential amino acid solution, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-ME, 4 μg/ml D-L-serine, and 4 μg/ml asparagine. PHA-LCM was prepared by culturing 10^7 peripheral blood mononuclear cells per milliliter from a patient with polycythemia vera for 4 d at 37°C in 5% CO_2 in α-MEM containing 1% (vol/vol) PHA (HA15; Wellcome Diagnostics, Dartford, United Kingdom) and 1% human albumin (10 GM; Sigma Chemical Co., St. Louis, MO). All batches of PHA-LCM, FCS, and human plasma were titrated to determine the optimal concentrations, and kept frozen at −20°C until use. Blast colony formation in our assay system required the presence of PHA-LCM. A single batch of PHA-LCM was used throughout this study, since data on different patients can be compared only under standardized assay conditions. This particular batch of PHA-LCM was prepared by stimulating peripheral blood mononuclear cells of a polycythemia vera patient with PHA. However, other batches of PHA-LCM, prepared by using peripheral blood lymphocytes of healthy volunteer donors as well as the supernatants of activated helper T cell clones derived from normal individuals are also able to promote in vitro B-lineage blast colony formation. We strongly believe that pluripotent colony-stimulating factor (CSF) and/or IL-3 in these conditioned media support colony formation by leukemic progenitor cells in common B-lineage ALL. Three of five PHA-LCM batches obtained from different donors promoted blast colony formation, whereas only 3 of 17 plasma batches and two of three FCS batches yielded successful cultures (data not shown). Hence, the reproducibility of blast colony formation requires a careful and thorough screening of the PHA-LCM, plasma, and FCS batches. The ill-defined stimulatory activity present in the plasma of some individuals appears to be essential for the development of leukemic B-lineage blast colonies. Duplicate 1 ml samples were cultured in 35 mm Petri dishes for 7 d at 37°C in a humidified 5% CO_2 atmosphere. On day 7, colonies containing >20 cells were counted using an inverted phase microscope with high optical resolution. Subsequently, all colonies were pooled for further cytochemical and immunological analysis of colony blasts. In the experiments described in this paper, we started cultures with 10^5 bone marrow mononuclear cells (BMMNC)/ml. In some experiments, 10^4 cells

from pooled colonies were replated under the initial culture conditions to measure the self-renewal ability of blast progenitors.

Cytochemical and Immunological Analysis of Colony Blasts. Cells from pooled colonies were deposited on slides by cytocentrifugation. The morphology of lymphoblasts was studied in Wright-Giemsa–stained cytospin preparations. In cytochemical studies, lymphoblasts were evaluated for staining by periodic acid–Schiff (PAS) by the McManus method (29), and for nonspecific esterase (NSE) activity using α-naphthyl acetate as substrate. Sudan Black B and myeloperoxidase stains were also used to detect contaminating normal myelomonocytic cells. For surface marker analyses by indirect immunofluorescence, slides were fixed in acetone (10 min), dried, and 100 μl mAb was added directly on top of cells. mAb were used in antibody excess. After 30 min incubation at room temperature in a humidity chamber, cells were washed twice in PBS to remove unbound antibody. FITC-conjugated goat $F(ab')_2$ anti–mouse IgG (Cappel Laboratories) was used as second antibody (incubation for 30 min, then washed three times in PBS). For background fluorescence, an IgG2a murine myeloma protein (UPC 10; Litton Bionetics) was substituted for mAb. The percentage of cells expressing each marker was determined using a Zeiss fluorescent microscope equipped with Ploem epi-illumination. Quantitative data were obtained from examination of 100–200 cells. The marker profiles were analyzed using a panel of murine mAb that define lymphoid and myeloid/erythroid (M/E) differentiation antigens. Specifically, we used the following mAb. T cell panel: 35.1, anti-CD2(T,p50) (30); T101, anti-CD5(T,p67) (31); 3A1, anti-CD7(T,p41) (32). B cell panel: BA-2, anti-CD9(nT-nB,p24) (33); BA-3, anti-CD10(nT-nB,p100) (34); B43, pan-B (14); BA-1, anti-CD24(B,G,p45,55,65) (35). M/E panel: anti-MY8 (36); 2 RIO, anti–glycophorin A (37). Serological cluster designations were assigned at the First (Paris, 1982) and Second (Boston, 1984) International Workshops on Human Leukocyte Differentiation Antigens.

Immunotoxin Synthesis. PAP is a single-chain polypeptide toxin (M_r 29,000) that catalytically inactivates the 60 S subunit of eukaryotic ribosomes, and was isolated from spring leaves of pokeweed (*Phytolacca americana*) as previously described (38). mAb B43 (IgG1, pan-B) (14), 3A1 (IgG1, pan-T, anti-CD7) (32), and T101 (IgG2a, pan-T, anti-CD5) (31) were linked to PAP by a disulfide bond using *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), as previously described (39). Pan-T IT were purified on a Sephacryl S-200 column (1 × 150 cm). Pan-B IT B43-PAP was purified by adsorbtion on protein A–Sepharose as described earlier (14). No free PAP was detected on PAGE under nonreducing conditions. Free antibody contamination was estimated to be <20% by gel electrophoresis. The molar PAP/IgG ratio in the conjugates was estimated by a specific homologous RIA using radioiodinated PAP (39, 40). By competitive inhibition of ^{125}I-PAP binding to rabbit anti-PAP antibodies by PAP and PAP containing IT, this ratio was found to be 2:1 in B43-PAP, 1:1 in 3A1-PAP, and 1:1 in T101-PAP. IT were evaluated for their ability to inactivate protein synthesis in a cell-free translation system using rat liver ribosomes and polyuridylic acid as message, according to the method previously described (39, 41). All three IT were found to be equally potent inhibitors of polyuridylic acid translation at equimolar concentrations (data not shown). We have used an average molecular weight of 180,000 (3A1-PAP, T101-PAP) or 210,000 (B43-PAP) for IT to calculate the molar concentrations applied in this study.

B43 mAb reacts with most of B cells isolated from peripheral blood and lymphoid organs, EBV-transformed B lymphoblastoid, Burkitt's lymphoma, B-ALL and pre-B-ALL cell lines. The majority of patients with B cell lymphoma, chronic lymphocytic leukemia (CLL), B-ALL, common B-lineage ALL, hairy cell leukemia express the surface determinant recognized by B43 mAb. In contrast, patients with AML, T-cell ALL, or T cell–derived non-Hodgkin's lymphoma show no reactivity with B43. This mAb does not bind to committed myelomonocytic (CFU-GM; colony-forming units/granulocytes and macrophages), erythroid (CFU-E), megakaryocytoid (CFU-MK) precursors, granulocytes, erythrocytes, platelets, T-lymphocytes, or plasma cells. The detailed information about the reactivity pattern of B43 will be published in a separate report. (Uckun, manuscript in preparation).

IT Binding. The binding of PAP IT to leukemic blasts was determined by a double

sandwich method in which we incubated the cells in a sequential fashion with (*a*) IT (4×10^{-8} M, 30 min, 4°C), (*b*) affinity-purified rabbit anti-PAP antiserum (50 μg/ml, 15 min, 4°C), and (*c*) FITC-conjugated goat anti–rabbit IgG (Miles Laboratories, Elkhardt, IN) (1:50 dilution, 15 min, 4°C) as previously described (14). Cells were analyzed by cytofluorometry using a FACS IV (Becton Dickinson Immunocytochemistry Systems, Mountain View, CA). Fluorescence profiles were plotted with the aid of an Apple II computer as histograms of relative intensity over 256 channels vs. number of cells in each channel (17). Background fluorescence staining was obtained by incubating cells with an equimolar mixture of free mAb (B43, 3A1, T101; 4×10^{-8} M each) and unconjugated PAP (8×10^{-8} M) instead of PAP-IT.

Treatment of Cells with IT. 10^7 cells/ml were treated with IT for 8 h (standard protocol) or 4 h (short incubation protocol) at 37°C in 5% CO_2 in RPMI 1640 plus 20% (vol/vol) FCS. After treatment, cells were washed twice in RPMI 1640 plus 5% (vol/vol) FCS to remove unbound IT.

Evaluation of B43-PAP-induced Inhibition of Protein Synthesis in Common B-lineage ALL Cells. The extent of protein synthesis inactivation was determined as percent inhibition of protein synthesis = 100 − 100 × ([^{3}H]leucine incorporation [cpm] per 10^5 treated cells)/([^{3}H]leucine incorporation [cpm] per 10^5 untreated cells). The incorporation of [^{3}H]leucine into cellular protein was determined as previously described (14). Controls were (*a*) untreated samples, (*b*) samples treated with two control IT directed against T-lineage associated surface determinants (i.e., 3A1-PAP and T101-PAP), (*c*) samples treated with a mixture of free B43 mAb and unconjugated PAP, and (*d*) samples of two different nontarget leukemic T cell lines that are sensitive to PAP (i.e., HSB-2 and MOLT 4).

Evaluation of Cytotoxicity of B43-PAP Against Common B-lineage ALL Progenitor Cells. Following B43-PAP treatment, the survival of leukemic progenitor cells was measured using the colony assay described above. The extent of cytotoxicity was expressed as percent inhibition of leukemic progenitor cells and was calculated as 100 − 100 × (mean number of blast colonies per 10^5 treated cells)/(mean number of blast colonies per 10^5 untreated cells). In these experiments, we included the following controls: (*a*) untreated samples, (*b*) samples treated with two control IT directed against T-lineage–associated surface determinants (i.e., 3A1-PAP and T101-PAP), and (*c*) samples treated with a mixture of free B43 mAb and unconjugated PAP. The nonspecific cytotoxicity of B43-PAP against normal bone marrow progenitor cells was evaluated in stem cell colony assays.

Stem Cell Colony Assays. Toxicity of B43-PAP treatment (8 h at 37°C) against normal monopotent (CFU-GM, CFU-E, CFU-MK) and pluripotent (CFU-GEMM) bone marrow progenitor cells was evaluated by colony assays. The detailed methodology of this colony assay system has been described elsewhere (25, 42). Data were expressed as percent control colony formation as 100 × (mean number of colonies per 10^5 treated cells)/(mean number of colonies per 10^5 untreated cells).

Results

Immunological Marker Profiles of Common B-lineage ALL Cells. 12 patients with ALL were studied. The immunological profiles of the patients' BMMNC are shown in Table II. No patient expressed the T-lineage associated markers CD2(T,p50), CD5(T,p67), or CD7(T,p41). In contrast, the cells from all the patients reacted with BA-3, an mAb directed against CD10 (nT-nB,p100)/CALLA surface determinant. There was a marked variability in the number of BA-3$^+$ bone marrow blasts among the patient population ranging from 33% (patient 5) to 98% (patient 11) (median, 80.5%). All patients expressed the B-lineage–associated antigens CD9 and CD24 but lacked sIg. Importantly, the marrow blasts from all the patients expressed the B-lineage–associated surface determinant recognized by the B43 mAb. B43 was conjugated to the plant

TABLE II

Immunological Marker Profiles of Patients' Bone Marrow Blasts

Patient	Cells (%) reactive with T-lineage markers			Cells (%) reactive with B-lineage markers				
	CD2 35.1	CD5 T101	CD7 3A1	CD9 BA-2	CD10 BA-3	B43	CD24 BA-1	sIg
1	4	4	3	92	59	87	88	4
2	13	9	5	83	94	77	88	11
3	5	4	3	85	79	87	42	3
4	1	1	0	72	87	91	95	1
5	3	3	3	75	33	76	91	7
6	7	3	3	94	94	83	95	5
7	1	1	1	96	61	94	90	3
8	2	2	2	90	73	87	41	5
9	11	12	10	75	78	74	85	14
10	5	4	4	72	95	85	96	2
11	2	2	2	34	98	79	98	4
12	8	5	10	39	82	83	86	7

Numbers represent percentage of cells reactive with the given antibodies directed against T- and B-lineage surface determinants. Marker analyses by immunofluorescence and flow cytometry were performed as described in Materials and Methods. sIg, surface Ig.

TABLE III

Immunotoxin Binding and Immunotoxin-induced Inhibition of Protein Synthesis in Common B-lineage ALL Cells

Patient	Cells stained* for PAP (%)			Percent inhibition of protein synthesis‡				
				B43-PAP		3A1-PAP	T101-PAP	B43-mAb + PAP
	B43-PAP	3A1-PAP	T101-PAP	8-h treatment	4-h treatment	8-h treatment	8-h treatment	8-h treatment
1	89.5	6.2	NT	99.5	98.2	6.5	NT	0.0
2	77.8	1.3	NT	91.5	89.7	0.0	NT	0.0
3	85.8	11.1	NT	99.6	95.6	9.2	NT	0.0
4	91.6	3.9	5.9	97.3	NT	2.5	NT	0.0
5	77.1	5.4	5.3	95.5	NT	0.0	28.9	NT
6	83.4	NT	8.6	95.4	NT	NT	0.0	2.4
7	96.0	2.7	3.6	98.7	NT	NT	32.4	0.0
8	89.4	NT	10.3	97.7	NT	NT	0.0	0.0
9	83.8	NT	7.7	NT	89.3	NT	27.9	0.0
10	90.2	NT	9.4	NT	98.8	NT	16.6	4.7
11	79.5	NT	4.6	NT	99.4	NT	4.9	0.0
12	86.8	NT	NT	NT	98.4	NT	NT	0.0

* The binding of PAP IT to leukemic blasts was determined by a double sandwich method as described in Materials and Methods. Cells were analyzed by cytofluorometry using a FACS IV. Results were expressed as the percentage of cells stained for PAP. NT, not tested.

‡ 10^7 cells/ml were incubated with IT (4×10^{-8} M) or unconjugated antibody (4×10^{-8} M) and PAP (8×10^{-8} M) mixtures at 37°C for the indicated treatment periods. After treatment, cells were washed to remove unbound IT and cultured at 37°C in 5% CO_2 atmosphere. The incorporation of [^{3}H]leucine into cellular protein was determined at 24 h posttreatment as described in Materials and Methods. Results were expressed as percent inhibition of protein synthesis = 100 − 100 × ([^{3}H]leucine incorporation [cpm]/10^5 treated cells)/([^{3}H]leucine incorporation [cpm]/10^5 untreated cells).

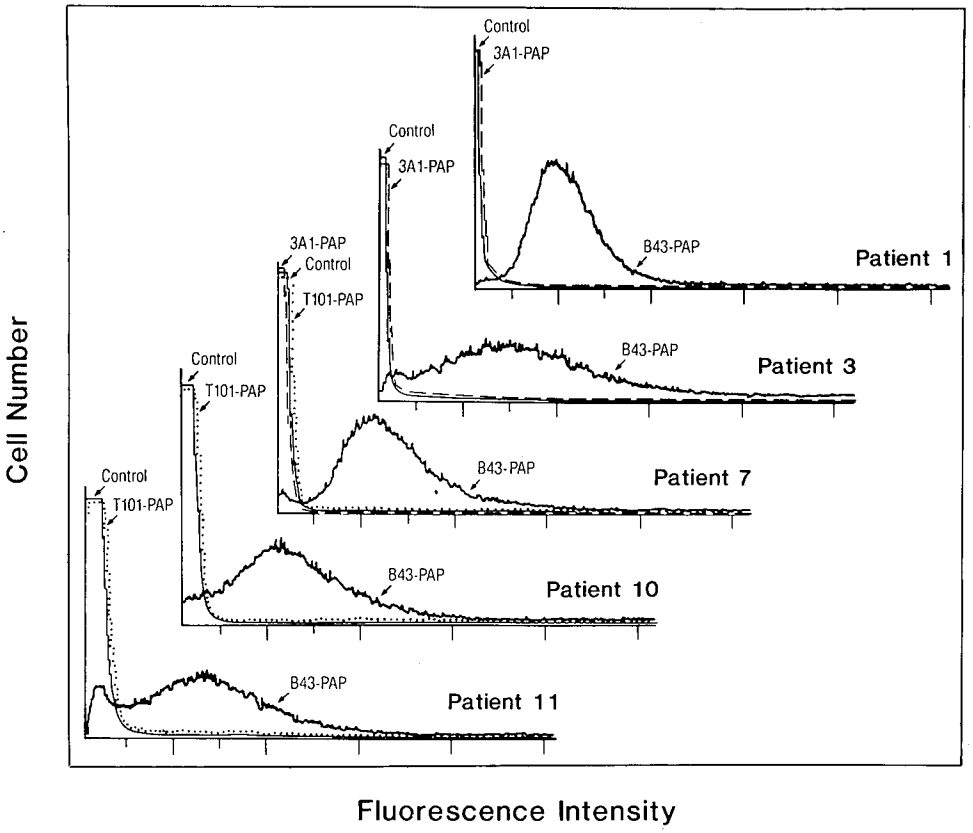


FIGURE 1. Binding of B43-PAP to common B-lineage ALL cells. Leukemic blasts were analyzed by indirect immunofluorescence and cytofluorometry using a FACS IV. Fluorescence profiles were plotted as histograms of relative fluorescence intensity over 256 channels vs. cell number in each channel. Background fluorescence (Control) was obtained using a mixture of free mAb and unconjugated PAP. T101-PAP and 3A1-PAP were used as control IT.

hemitoxin PAP in the present studies to generate an IT reactive with common B-lineage ALL cells.

Binding of B43-PAP IT to Common B-lineage ALL Cells. Bone marrow blasts from patients were examined for the presence of bound IT after treatment with 4×10^{-8} M PAP IT. As shown in Table III, common B-lineage ALL cells reacted with the B43-PAP IT in all 12 cases studied. The number of B43-PAP-labeled cells ranged from 77.1% (patient 5) to 96.0% (patient 7) (median, 86.3%). In contrast, these blasts did not bind control IT 3A1-PAP and T101-PAP, which are directed against T-lineage–associated surface determinants. Representative FACS histograms from five patients are shown in Fig. 1.

B43-PAP-induced Inhibition of Protein Synthesis in Common B-lineage ALL Cells. The cell type–specific cytotoxicity of B43-PAP was evaluated by inhibition of protein synthesis in target common B-lineage ALL cells. Bone marrow blasts (10^7 cells/ml) from eight patients were treated with 4×10^{-8} M B43-PAP for 8 h at 37°C. This treatment protocol produced 91.5% (in patient 2) to 99.6% (in patient 3) inhibition of protein synthesis in target B-lineage leukemic cells (Table

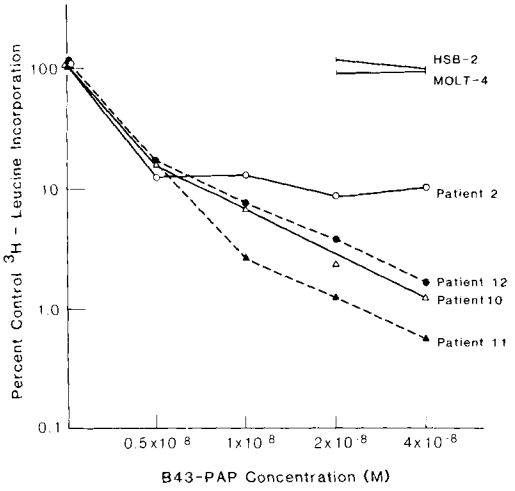


FIGURE 2. Inhibition of protein synthesis in common B-lineage ALL cells by B43-PAP. Leukemic blasts were incubated in triplicate for 4 h at 37°C in the presence of various concentrations of B43-PAP. Percent control [^{3}H]leucine incorporation in treated cells as an index of residual ribosomal activity was determined as described in Materials and Methods. HSB-2 and MOLT 4 are PAP-sensitive leukemic T cells that were used as nontarget controls.

III). In contrast, 3A1-PAP (anti-CD7) elicited <10% inhibition of protein synthesis in common B-lineage ALL cells at this IT concentration. By comparison, T101-PAP (anti-CD5) inhibited a mean of 26.5% of protein synthesis in four of seven cases (patients 5, 7, 9, and 10). The mean percentage of cells stained for bound T101-PAP by indirect immunofluorescence was only 6.5 in these four patients (Table III). In additional controls, a mixture of 4×10^{-8} M free B43 mAb and 8×10^{-8} M unconjugated PAP did not inhibit the [^{3}H]leucine incorporation by these B-lineage leukemic cells.

We also examined the efficacy of 4 h treatment with B43-PAP. Significant inhibition was observed at 0.5×10^{-8} M B43-PAP, and at 4×10^{-8} M, >90% of [^{3}H]leucine incorporation was inactivated in common B-lineage ALL blasts (Fig. 2). As shown in Table III, this short incubation protocol with 4×10^{-8} M B43-PAP induced substantial inhibition of protein synthesis in all 7 common B-lineage ALL cases. The median inhibition of cellular protein synthesis was 98.2%. At this conjugate concentration, B43$^-$ nontarget blasts from neoplastic T cell lines (i.e., HSB-2 and MOLT-4) were not inhibited. We have previously reported that these T cell lines are PAP sensitive and were inhibited at very low concentrations of T cell directed PAP-IT (13, 43). These findings provide further evidence that the high antileukemic activity of B43-PAP is cell type–specific and occurs via antibody binding.

Colony-forming and Self-renewing Abilities of Common B-lineage ALL Progenitor Cells. Freshly obtained bone marrow blasts from 12 common B-lineage ALL patients were assayed for primary colony formation in vitro. Successful cultures were obtained in 10 of 12 cases. Paired daughter cells appeared in 8 of these 10 cases within 24 h. In two cases (i.e., patients 2 and 4), there was a lag period of ~48 h until the first paired blasts were observed. After 72 h of incubation,

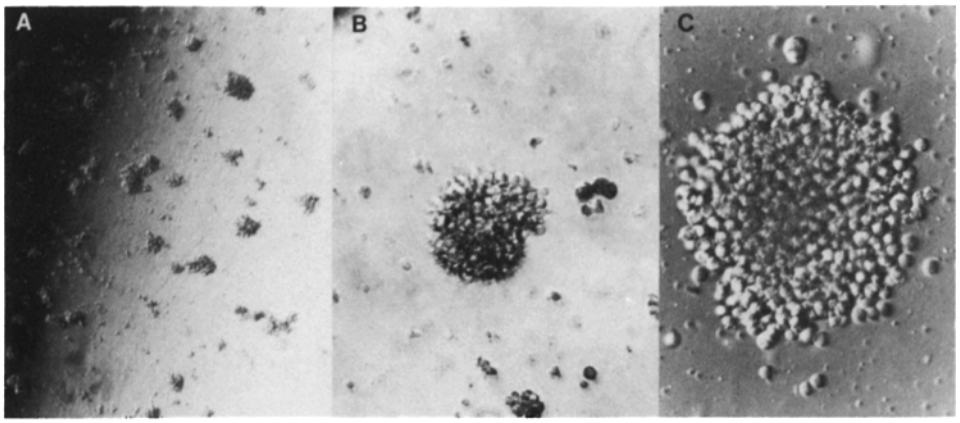


FIGURE 3. (*A*) Picture of part of a Petri dish containing compact clusters and colonies of B-lineage lymphoblasts cultured for 5 d (patient 3). Note the differences in size. (× 5). (*B*) Photomicrograph of a day 7 blast colony (patient 10). (× 19). (*C*) Photomicrograph of a day 7 blast colony (patient 5). (× 38).

spherical clusters of 10–20 tightly associated cells were discernible in all successful cultures (i.e., 10 of 12 cases) (Fig. 3*A*). Colonies containing >20 blasts were counted on day 7 (Fig. 3*B*). The blasts in these colonies were round and had a translucent cytoplasm with a distinct cell border of high refractility (Fig. 3, *B* and *C*). Colonies were compact and usually contained <200 cells. Therefore, we could easily and accurately count >2,000 distinct colonies in a single 35-mm Petri dish.

Blast colony formation was linear with respect to numbers of cultured cells between 10^3 and 10^5 leukemic blasts per Petri dish (data not shown). The apparent cloning frequency of blast progenitor cells in cultures started with 10^5 leukemic blasts varied from 0.09% (i.e., 90 colonies per 10^5 BM blasts) to 2.63% (i.e., 2,630 colonies per 10^5 BM blasts). In the 10 cases with successful cultures, day 7 blast colonies displayed a marked variability in cell number, ranging from ~20 cells to >200 cells per colony (Fig. 3, *B* and *C*).

The self-renewal capability of blast progenitor cells was evaluated in replating experiments. Table IV compares the secondary plating efficiency of blasts pooled from primary colonies in three cases with the primary plating efficiency of bone marrow blasts. Too few surplus blasts were recovered from the primary colonies of other patients to test for their self-renewal ability. In all three cases, secondary colonies appeared earlier than the primary colonies (within 72 h). Colonies contained >50 cells and started to disperse on day 5. Petri dishes were harvested on day 7 for immunological marker analyses. Secondary plating efficiencies were higher than the primary plating efficiencies in all cases but did not exceed 7.12% (patient 7).

Both primary and secondary blast colony formation was dependent on the copresence of FCS, human plasma, and PHA-LCM in this assay system. No colony formation was obtained in control cultures that lacked one or more of these three supplements (data not shown).

Morphological, Cytochemical, and Immunological Characteristics of Cultured

TABLE IV

Primary and Secondary B-lineage Blast Colonies in Culture

Patient	Primary colony formation*			Secondary colony formation‡		
	Colonies/10^4 BMMNC	Plating efficiency (%)	Surface phenotype (%)§	Colonies/10^4 blasts	Plating efficiency (%)	Surface phenotype (%)§
1	197 (193,201)	1.97	97 BA-2	250 (241,259)	2.50	90 BA-2
			0 BA-3			0 BA-3
			98 B43			83 B43
			25 BA-1			38 BA-1
3	133 (112,154)	1.33	90 BA-2	563 (540,586)	5.63	85 BA-2
			40 BA-3			55 BA-3
			90 B43			82 B43
			35 BA-1			50 BA-1
7	250 (248,252)	2.50	99 BA-2	712 (699,725)	7.12	79 BA-2
			58 BA-3			50 BA-3
			85 B43			96 B43
			91 BA-1			83 BA-1

* Duplicate samples of 10^4 freshly obtained bone marrow mononuclear cells were assayed for blast colony formation in methylcellulose cultures as described in Materials and Methods. On day 7, colonies containing >20 blasts were counted using an inverted phase microscope. Results were expressed as the mean number of colonies per 10^4 BMMNC, and as the plating efficiency. Numbers in parentheses represent the actual number of colonies from individual dishes.

‡ Duplicate samples of 10^4 blasts pooled from day 7 colonies were assayed for secondary colony formation in methylcellulose cultures as described in Materials and Methods. Cultures were evaluated for colony formation as above. Results were expressed as the mean number of secondary colonies per 10^4 blasts, and as the plating efficiency. Numbers in parentheses represent the actual number of colonies from individual dishes.

§ Surface phenotype analyses of colony blasts, determined by indirect immunofluorescence, were performed on acetone-fixed cytospin preparations as described in Materials and Methods. Numbers represent the percentage of colony blasts reactive with the mAb.

Blasts. The cells from pooled colonies showed >95% viability, as estimated by trypan blue exclusion. They had blast morphology with a basophilic cytoplasm, prominent vacuolation, irregular nuclear membrane, and prominent nucleoli (Fig. 4). The colony blasts from different patients displayed marked differences with respect to size; nuclear/cytoplasmic ratio; basophilia of cytoplasm; nuclear membrane contour; nucleolar size, number, and prominence; and amount and prominence of vacuolation (Fig. 4). Cytochemical evaluation of colony blasts showed that they are Sudan black–negative, myeloperoxidase-negative, NSE^-, and PAS^+. The PAS^+ cells showed a strong staining intensity. The stain was irregularly distributed within the cytoplasm and occurred in clumps or coarse and fine granules (Fig. 4). In summary, colony cells had morphologic and cytochemical features consistent with ALL.

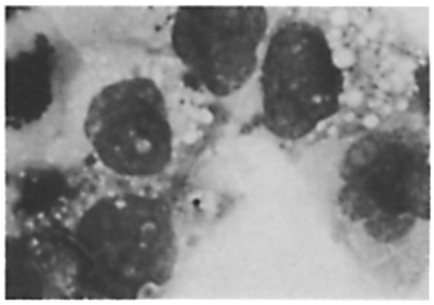
Blasts from pooled day 7 colonies displayed a marked heterogeneity in expres-

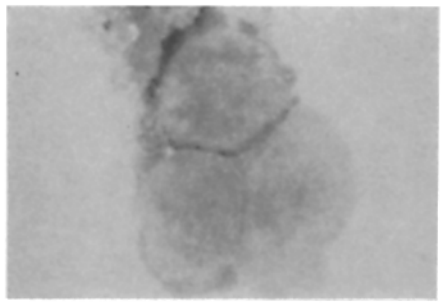
FIGURE 4. (*A*) Wright-Giemsa–stained cells pooled from day 7 blast colonies. Note the blast morphology with a basophilic cytoplasm, irregular nuclear membrane, prominent nucleoli and vacuolation. (*B*) Colony blasts were evaluated for staining by PAS. Note the strong staining intensity.

Patient 1

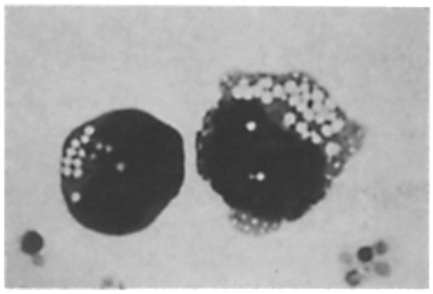
A

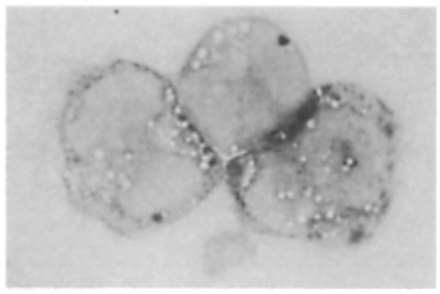
B



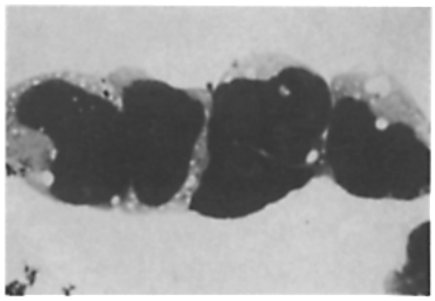


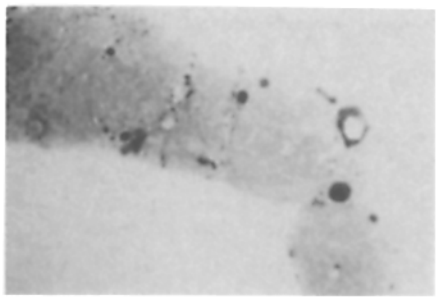
Patient 3



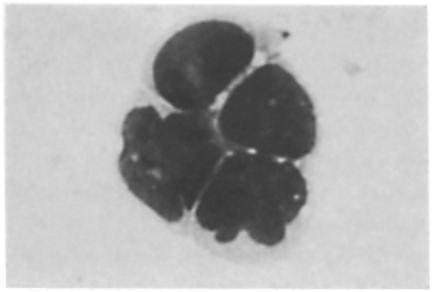


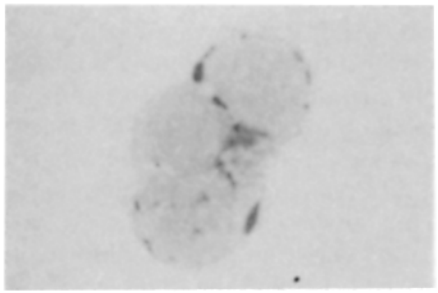
Patient 7



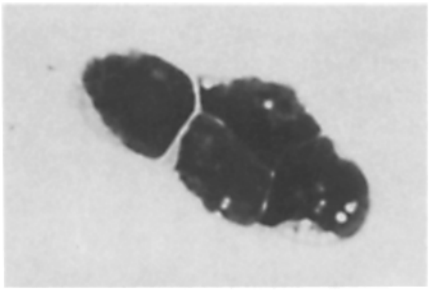


Patient 10





Patient 11



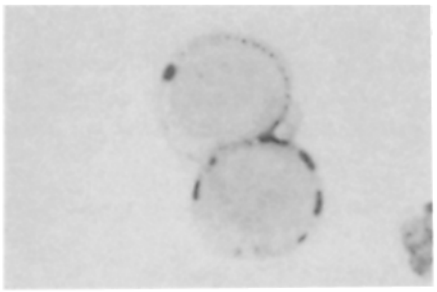


TABLE V
Immunological Marker Profiles of Cultured Cells Pooled from Day 7 Blast Colonies

Patient	Cells (%) reactive with T-lineage markers			Cells (%) reactive with B-lineage markers				Cells (%) reactive with myeloid/erythroid markers	
	CD2 35.1	CD5 T101	CD7 3A1	CD9 BA-2	CD10 BA-3	B43	CD24 BA-1	anti-MY 8	anti-glycophorin A (R10)
1	1	2	0	97	0	98	25	2	0
2	0	NT	NT	95	67	94	92	0	NT
3	2	0	0	90	40	90	35	1	NT
4	1	NT	NT	65	84	55	34	0	NT
5	0	NT	NT	85	0	66	40	0	NT
6	0	NT	NT	98	78	95	93	0	0
7	0	0	2	99	58	85	91	3	1
8	0	NT	NT	86	66	75	90	NT	NT
10	1	0	0	49	27	74	85	1	0
11	0	NT	0	95	0	90	10	0	0

Surface marker analyses using indirect immunofluorescence were performed on acetone-fixed cytospin preparations. The number of cells expressing each marker was determined using a Zeiss fluorescent microscope. Quantitative data were obtained from examination of 100–200 cells. Numbers represent percentage of colony blasts reactive with the mAb. NT, not tested. No colony formation of any type was noted among cells from patients 9 and 12.

sion of B-lineage–associated surface determinants (Table V). In 3 of 10 cases (patients 2, 6, and 7) the immunological phenotype of colony blasts was identical to that of the bulk blast population before culture, and in the remaining 7 cases it differed from the preculture evaluation. Two major immunological groups could be identified based on the reactivity pattern with BA-2 (anti-CD9), BA-3 (anti-CD10), BA-1 (anti-CD24), and B43 mAb. The first group was comprised of four cases (patients 2, 6–8). Colony blasts in this group had BA-2$^+$, BA-3$^+$, BA-1$^+$, B43$^+$ marker profiles. Thus, their immunological phenotype was consistent with that of bone marrow lymphoblasts before culture. In particular, 58–78% of cultured blasts expressed CD10/CALLA in this group (Table V). The second major group was comprised of three cases (patients 1, 5, and 11) in which the cells in the blast colonies did not express CD10/CALLA. In this group, the expression of CD24 was also significantly greater on the uncultured initial blast population than on the cells pooled from day 7 blast colonies (Table V). In the remaining three cases (patients 3, 4, and 10), cultured blasts had marker profiles that differed from the evaluation before culture, as well as from the immunological phenotype observed in the two major groups described above. There was no apparent correlation between the expression of B-lineage–associated antigens on cultured blasts and the number or size of blast colonies.

In all 10 cases with successful cultures, the colony blasts did not express T-lineage–associated antigens (i.e., CD2, CD5, and CD7) or M/E markers (i.e., MY8 and glycophorin A) but were stained with at least two different mAb directed against distinct B-lineage–associated surface determinants (Table V). Despite considerable heterogeneity, the marker profiles detailed in Table V are consistent with B-lineage ALL. Importantly, in all cases, a large fraction of

TABLE VI

Effect of Immunotoxin Treatment on Common B-lineage ALL Progenitor Cells

Patient	Mean number of blast colonies per 10^5 BMMNC						Inhibition (%) of blast progenitor cells				
	No treatment	B43 mAb + PAP (8 h)	B43-PAP (8 h)	B43-PAP (4 h)	3A1-PAP (8 h)	T101-PAP (8 h)	B43 mAb + PAP (8 h)	B43-PAP (8 h)	B43-PAP (4 h)	3A1-PAP (8 h)	T101-PAP (8 h)
1	1,850	1,925	0	0	1,710	NT	0.00	≥99.95	≥99.95	7.57	NT
2	160	155	0	0	210	NT	3.13	≥99.38	≥99.38	0.00	NT
3	1,150	983	0	0	1,001	NT	14.52	≥99.91	≥99.91	12.96	NT
4	90	132	0	NT	103	95	0.00	≥98.89	NT	0.00	0.00
5	210	205	2.5	NT	253	213	2.38	98.81	NT	0.00	0.00
6	197	163	0	NT	NT	208	17.26	≥99.49	NT	NT	0.00
7	2,631	2,540	0	NT	2,701	2,230	3.46	≥99.95	NT	0.00	15.24
8	113	125	9	NT	NT	102	0.00	92.00	NT	NT	9.73
10	514	502	NT	0	NT	487	2.33	NT	≥99.81	NT	5.25
11	505	643	NT	0	NT	525	0.00	NT	≥99.80	NT	0.00

After IT treatment (4×10^{-8} M) at 37°C for the indicated time periods, the survival of leukemic B-lineage progenitor cells was measured in a colony assay system as described in Materials and Methods. Results were expressed as the mean number of blast colonies per 10^5 BMMNC obtained from treated and untreated duplicate samples. The percent inhibition of blast progenitor cells was calculated as 100 − (mean number of blast colonies per 10^5 treated cells) × 100/(mean number of blast colonies per 10^5 untreated cells).

NT, not tested. No colony growth of any type was noted among cells from patients 9 and 12.

cultured blasts in day 7 colonies expressed the B-lineage–associated surface determinant defined by B43 mAb, which has been used as a carrier transport protein for the plant hemitoxin PAP in our study.

The immunological phenotype of cells pooled from secondary blast colonies was identical to that of blasts in the primary colonies (Table IV).

B43-PAP-induced Inhibition of Common B-lineage ALL Progenitor Cells. Given the heterogeneity in antigen expression and toxin sensitivity among leukemia cell populations, combined with the current lack of knowledge about the immunological profile or PAP sensitivity of clonogenic ALL blasts, measurements of [^{3}H]leucine incorporation on the bulk population alone might not permit a precise determination of the frequency at which blast progenitor cells escape killing. Therefore, it was important to evaluate the immunotherapeutic potential of B43-PAP in a clonogenic assay system. Table VI shows the efficacy of B43-PAP against leukemic bone marrow progenitor cells from common B-lineage ALL patients. No blast colonies were detected in six of eight leukemic bone marrow samples treated with 4×10^{-8} M B43-PAP for 8 h at 37°C. 4 h incubation with B43-PAP proved as effective as 8 h incubation, and no residual blast colonies were detected in IT-treated samples from any of five patients tested (Table VI). In summary, colony formation was completely inhibited in 8 of 10 cases by B43-PAP (4- or 8-h incubations at 37°C). The inhibition of leukemic progenitor cells ranged from 92% (patient 8) to ≥99.96% (patient 7), with a mean value of ≥98.9%. In contrast to the toxicity of B43-PAP, a mixture of 4×10^{-8} M free B43 mAb and 8×10^{-8} M unconjugated PAP did not elicit a significant inhibition of blast progenitor cells. Control IT 3A1-PAP and T101-PAP only minimally affected the colony forming ability of common B-lineage ALL progenitor cells (Table VI).

Effect of B43-PAP on Normal Monopotent (CFU-GM, CFU-E, CFU-MK) and Pluripotent (CFU-GEMM) Bone Marrow Progenitor Cells. BMMNC from two healthy individuals were examined for B43-PAP reactivity by indirect immuno-

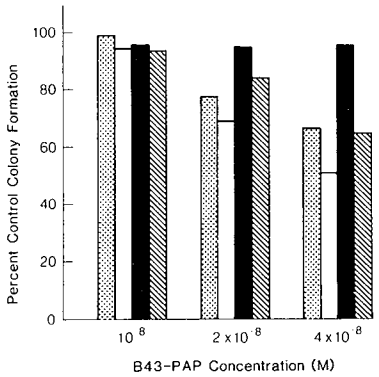


FIGURE 5. Effects of B43-PAP on normal myelomonocytic (CFU-GM), erythroid (CFU-E), megakaryocytoid (CFU-MK), and pluripotent (CFU-GEMM) progenitor cells. Normal BMMNC from two healthy donors were incubated in triplicate for 8 h at 37°C in the presence of various concentrations of B43-PAP. After treatment cells were assayed for hematopoietic colony formation in methylcellulose cultures. Results are expressed as the mean percent control colony formation. CFU-GM, ▩; CFU-E, □; CFU-MK, ■; CFU-GEMM ▧.

fluorescence and flow cytometry as described in Materials and Methods. The percentage of B43-PAP-labeled BMMNC was 9.1 and 8.1%, respectively. Fig. 5 shows the effect of 8-h B43-PAP treatment on in vitro progenitor cell recovery from these bone marrow samples. Data are expressed as the mean percent of colony forming units (CFU) recovered per 10^5 treated cells compared with untreated control cultures. Control cultures yielded 159 (range, 150–170) GM, 118 (range, 105–131) E, 12.8 (range, 8–20) pure MK, and 19 (range, 16–21) multilineage (GEMM) colonies per 10^5 BMMNC. At 10^{-8} M B43-PAP, monopotent and pluripotent progenitors were only minimally affected. At 4×10^{-8} M, 33% of CFU-GM, 48.5% of CFU-E, and 35.5% of CFU-GEMM were inhibited. CFU-MK were inhibited only 4%. As shown in Table VI, B43-PAP eliminated up to 99.96% of common B-lineage ALL progenitor cells at this IT concentration.

Discussion

In our previous studies, we have evaluated the effects of cyclophosphamide congeners (24), multiple antibodies and complement (44), IT (13–18), and combination protocols (15, 25, 44) against established leukemia cell lines. Our findings revealed a marked heterogeneity in the sensitivity of different target lymphoblastic leukemia cell lines to various reagents. This was attributed to considerable differences in cell cycle kinetics, cellular drug metabolism, complement sensitivity, immunological phenotype, and toxin sensitivity. Considering our lack of knowledge about the clonogenic blasts from ALL patients with respect to the above biological features, we concluded that the therapeutic potential of purging strategies has to be critically analyzed against leukemic progenitor cells obtained directly from ALL patients.

In this study, we used a novel colony assay system to evaluate the cytotoxicity of B43-PAP, a human B cell–directed IT, against sIg^- B-lineage leukemic bone marrow progenitor cells freshly obtained from ALL patients. A maximum kill

of ≥99.96% of leukemic progenitor cells was achieved after treatment with B43-PAP under conditions in which <40% of normal pluripotent hematopoietic progenitor cells (CFU-GEMM) were inhibited. B43-PAP completely inhibited primary blast colony formation in 8 of 10 common B-lineage ALL cases. These data establish that the B-lineage–associated surface determinant recognized by B43 mAb is expressed on common B-lineage ALL progenitor cells in sufficient quantities to allow effective binding and internalization of the IT. Our findings further illustrate that B-lineage blast progenitors are sensitive to PAP at the ribosomal level. Previous reports from our group have established the immunological stability (42) as well as the therapeutic potential (46) of PAP IT in vivo. Together with our earlier findings, the results presented here indicate that IT containing PAP may be useful for in vivo elimination of residual leukemic progenitor cells from patients with common B-lineage ALL, as well as for *ex vivo* purging of autologous marrow grafts in these patients before autologous BMT.

The cytotoxicity of B43-PAP was not limited to B-lineage progenitor cells only, and notably resulted in substantial inhibition of cellular protein synthesis in the bulk leukemic bone marrow blast population of all 12 common B-lineage ALL patients. The actual percent inhibition of B-lineage blasts measured in protein synthesis and colony assays slightly differed. However, this is not surprising, since the protein synthesis assay measures a short-term (24 h) inhibitory effect whereas colony assays evaluate long-term (7 d) inhibition. Furthermore, protein synthesis assays measure the inhibitory effects of IT on the bulk population of leukemic cells, whereas the relatively small (0.09–2.63%) clonogenic fraction of common B-lineage ALL cell populations may differ from the bulk population in surface antigen expression, IT entry and degradation, as well as PAP sensitivity.

To our knowledge, B43-PAP is the first IT to prove effective against sIg^- common B-lineage ALL cells. By comparison, ricin A-chain conjugates reactive with CALLA, gp26, and B1 were ineffective in inhibiting the growth of Burkitt's lymphoma cell lines (47, 48). Polyclonal rabbit anti–human Ig antibodies linked to ricin A-chain were selectively toxic against the sIg^+ Daudi cell line (10). However, leukemic blasts from common B-lineage ALL patients do not express sIg (22, 23, see Table II), and therefore could not be eliminated by anti–human Ig IT. Similarly, mAb-toxin conjugates directed against Ia, albeit inhibitory to Burkitt's lymphoma cell lines (47), show little promise for clinical application because of crossreactivity with normal monopotent and pluripotent progenitor cells (49).

Surprisingly, the PAP conjugate of T101 mAb (anti-CD5) displayed a moderate toxicity against common B-lineage ALL cells in the protein synthesis inhibition assay. This observation was unexpected, since <10% of B-lineage blasts were $T101^+$ as measured by indirect immunofluorescence using FACS. However, it is possible that FACS results may not accurately reflect antigen number. Hence, the CD5 surface determinant may indeed be present on some B-lineage leukemic cells in small but sufficient quantities to allow T101-PAP binding and internalization. This T cell associated antigen is also found on B-lineage chronic lymphocytic leukemia cells bearing sIg (31). $>2 \times 10^6$ IT molecules/cell were present at 4×10^{-8} M concentration of T101-PAP. Therefore, the observed toxicity may

be partially attributed to nonspecific uptake of T101-PAP by pinocytosis. If this is true, the variability of T101-PAP toxicity, which ranged from 0 to 32% might simply reflect differences in the pinocytotic activity of common B-lineage ALL blast populations. Similarly, the differences seen in B43-PAP induced inhibition of normal bone marrow progenitor cells can also be explained by differences in nonspecific endocytosis of the IT. Alternatively, the amount of IT may be comparable, but pinocytosed cells may differ with respect to their PAP sensitivity at the ribosomal level.

The colony assay system described in this paper, as well as the data concerning blast colony formation in vitro, differ from those reported by Izaguirre et al. (50) in several respects. These authors used a cell culture method that requires special incubator chambers with low oxygen tension, extensive depletion of T lymphocytes, and presence of irradiated feeder cells. It is noteworthy that these investigators obtained no blast colonies in the absence of T lymphocyte feeders and concluded that, besides growth-promoting cytokines, immediate cell-cell communications are also required for colony formation. In contrast, using the culture technique reported here, we found that neither feeder cells nor special low-oxygen incubator chambers are required for reproducible growth of freshly obtained leukemic B-lineage bone marrow blasts. We are currently studying the correlations between various clinical parameters and colony formation in our assay system. Notably, in 21 of 30 common B-lineage ALL bone marrow samples evaluated in experiments thus far, successful cultures with blast colony formation were obtained in the absence of T lymphocyte feeders (Uckun, Kersey, Haag, Gajl-Peczalska, Heerema, Arthur, LeBien, Vallera; manuscript in preparation). Hence, our experience with highly enriched bone marrow blast populations does not support the contention that immediate cell-cell interactions between leukemic B-lineage blasts and normal T lymphocytes are essential for blast colony formation.

In agreement with the findings of Izaguirre et al. (50), we found that blast colony formation requires the presence of PHA-LCM. However, the use of human plasma and FCS is also essential for B-lineage blast colony formation in our assay system. Only the combined use of these three supplements reproducibly supported the in vitro proliferation of leukemic bone marrow blasts from patients with common B-lineage ALL. The use of human plasma has the additional advantage that it increases the viscous support in methylcellulose cultures. This decreases cell migration in marrow cultures and increases the likelihood that blast colonies are derived from single leukemic progenitor cells. Blast colony formation was linear with respect to numbers of cultured cells between 10^3 and 10^5 leukemic blasts per milliliter. This linear pattern of colony growth is consistent with near to optimal culture conditions, and also suggests a single cell origin of B-lineage blast colonies.

The primary plating efficiency of blast progenitor cells varied between 0.09 and 2.63%. This variation might represent fluctuations in blast progenitor physiology occuring at random during the course of leukemia. Alternatively, the primary plating efficiency might be a stable blast progenitor property unique to each case. If the latter is true, our findings indicate a marked heterogeneity in proliferative potential among the common B-lineage ALL blast populations. In

each ALL case with successful cultures, day 7 colonies displayed a significant variation in cell number. This finding suggests that the clonogenic fraction of the leukemic blast population in each patient contains cells that are heterogenous with respect to their cell cycle kinetics.

The capacity of blast progenitors to undergo self-renewal results in the maintainence of cycling blast populations. The evaluation of the secondary plating efficiency yields valuable information about the self-renewal property of blast progenitor cells (51). Therefore, we attempted to determine the self-renewal ability of leukemic B-lineage progenitor cells in replating experiments, and found that the secondary plating efficiencies did not exceed 7.12%. The majority of B-lineage lymphoblasts pooled from primary day 7 colonies and recultured did not proliferate and form secondary colonies. Thus, only a very small fraction of the progeny of blast progenitor cells displayed progenitor cell characteristics. These data are consonant with previous findings obtained by Izaguirre et al. (50). Similar findings were reported by Buick et al. (52), who evaluated the self-renewal property of myeloid-lineage blast progenitor cells in replating experiments.

Morphological, cytochemical, and immunological analyses of cells pooled from primary day 7 colonies provided the evidence that they are B-lineage lymphoblasts. Izaguirre et al. (50) reported that cells within blast colonies have the same immunological phenotype as the cells in marrow or blood of the patients from whom the colonies were propagated. In contrast, in 7 of 10 common B-lineage ALL cases with successful marrow cultures presented in this paper, the immunological phenotype of day 7 colony blasts differed significantly from the evaluation of the bulk bone marrow blast population before culture. In 3 of 10 cases, the colony blasts did not express CD10/CALLA. In one of these three cases, colony blasts were recultured to evaluate their self-renewal ability. Cells harvested from secondary blast colonies were found to be also CD10/CALLA-negative. Our findings may be interpreted as an indication of the existence of CD10/CALLA-negative leukemic progenitor cells in the bone marrow of patients with common B-lineage ALL. This hypothesis is supported by the phenotypic shifts observed during the clinical course of common B-lineage ALL. Greaves et al. (53) reported that, in relapse ALL, CD10/CALLA may be lost as compared with the phenotype at diagnosis. Such alterations in immunological phenotype were also observed in a more recent clinical study at our institution (19). Since ALL blast populations are most likely subject to selective pressure and clonal evolution, the phenotypic shifts in common B-lineage ALL during leukemia progression or relapse may be due to a change in the balance of the determining influences with time, or after chemotherapy. In the aforementioned group of three ALL cases with CALLA-negative colony blasts, the expression of CD24 determinant defined by BA-1 mAb was also significantly greater on the preculture blast population than on the cultured blasts. Although evidence of monoclonality for the colony blasts would have been highly desirable, we were unable to use chromosome abnormalities or rearrangements of Ig genes as clonal markers because of the limited number of cells obtained from day 7 B-lineage blast colonies. After detailed morphological, cytochemical and immunological

marker analyses, we did not have sufficient colony cells left to evaluate their clonality.

Unlike CD10/CALLA and CD24, the CD9 surface determinant recognized by BA-2, as well as the antigen recognized by B43 mAb were strongly expressed in all cases by the vast majority of cultured blasts. The conserved expression of these B-lineage markers might be taken to indicate that they may represent surface receptors functionally involved in cell-cell interactions, proliferation, and clonogenic growth control of B-lineage ALL blast populations.

In conclusion, we have described a new assay system that allows reproducible proliferation and colony formation of freshly cultured bone marrow blasts from patients with common B-lineage ALL. Experimental data produced in this model system of leukemic B-lineage progenitor cells will likely provide information of value in understanding the underlying biological events that influence growth progression and control of leukemic hematopoietic stem cells in common B-lineage ALL. A major finding of clinical significance presented in this paper is that B-lineage leukemic progenitor cells can be effectively eradicated by an IT containing PAP. These experiments provide a paradigm for the critical evaluation of the therapeutic potential of an antileukemic treatment strategy in common B-lineage ALL.

Summary

We report a novel colony assay for B-lineage progenitor cells in acute lymphoblastic leukemia (ALL). The primary plating efficiency of blast progenitors freshly obtained from common B-lineage ALL patients varied between 0.09 and 2.63%. Morphological, cytochemical, and immunological analyses of cells from day 7 colonies provided the evidence that they are B-lineage lymphoblasts. Immunological marker analyses of cultured blasts using BA-2 (anti-CD9), BA-3 (anti-CD10), BA-1 (anti-CD24), and B43 mAb have allowed us to define two distinct immunological groups. The first group had BA-2$^+$, BA-3$^+$, BA-1$^+$, B43$^+$ marker profiles, consistent with the phenotype of uncultured bone marrow blasts. The second group differed in that the cells in the blast colonies were BA-3 (anti-CD10)–negative, although many of the cells in the bulk population were BA-3$^+$ before culture. Blasts from both groups were able to proliferate and form secondary colonies when recultured.

A pan-B immunotoxin was synthesized by linking B43, a human B cell–specific mAb, to pokeweed antiviral protein (PAP). This study showed that B43-PAP can effectively eradicate leukemic progenitor cells freshly obtained from patients with common B-lineage ALL. B43-PAP eliminated >99.96% of blast progenitors under conditions in which only minimal inhibition of normal bone marrow progenitor cells (CFU-GM, CFU-E, CFU-MK, CFU-GEMM) was observed. Our results establish that the surface determinant recognized by B43 is expressed on B-lineage progenitor cells in ALL, and that these cells are sensitive to PAP at the ribosomal level. To our knowledge, B43-PAP is the first IT to prove effective against common B-lineage ALL cells.

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References

1. Dicke, K., G. Spitzer, and A. R. Zander, editors. 1985. Clinical autologous bone marrow transplantation studies in leukemia. *In* Autologous Bone Marrow Transplantation. Proceedings of the First International Symposium. Gulf Coast Printing, Houston, TX. 1–125.
2. Lowenberg, B., and A. Hagenbeek, editors. 1984. Autologous bone marrow transplantation in the treatment of acute leukemia. *In* Minimal Residual Disease in Acute Leukemia. Martinus Nijhoff Publishers, Rotterdam, The Netherlands. 165–287.
3. Dicke, K., G. Spitzer, and A. R. Zander, editors. 1985. Marrow purging methods. *In* Autologous Bone Marrow Transplantation. Proceedings of the First International Symposium. Gulf Coast Printing, Houston, TX. 373–471.
4. Kaizer, H., and G. W. Santos. 1982. Bone marrow transplantation in acute leukemia. *Semin. Hematol.* 19:227.
5. Uckun, F. M., M. Korbling, and W. Hunstein. 1984. In vitro chemotherapy as a prelude to autologous marrow transplantation in high grade malignant non-Hodgkin's lymphomas. *J. Cancer Res. Clin. Oncol.* 107:47.
6. Bast, R. C., J. Ritz, J. M. Lipton, M. Feeney, S. E. Sallan, D. G. Nathan, and S. F. Schlossman. 1983. Elimination of leukemic cells from human bone marrow using monoclonal antibody and complement. *Cancer Res.* 43:1389.
7. Bast, R. C., P. DeFabritiis, J. Lipton, R. Gelber, C. Maver, L. Nadler, S. Sallan, and J. Ritz. 1985. Elimination of malignant clonogenic cells from human bone marrow using multiple monoclonal antibodies and complement. *Cancer Res.* 45:499.
8. LeBien, T. W., R. C. Ash, E. D. Zanjani, and J. H. Kersey. 1983. In vitro cytoreduction of leukemia cells in human bone marrow using a cocktail of monoclonal antibodies. *In* Modern Trends in Human Leukemia. R. Neth, R. C. Gallo, M. F. Greaves, M. A. S. Moore, K. Winkler, editors. Springer-Verlag, New York. p. 112.
9. LeBien, T. W., D. E. Stepan, R. M. Bartholomew, R. C. Stong, and J. M. Anderson. 1985. Utilization of a colony assay to assess the variables influencing elimination of leukemic cells from human bone marrow with monoclonal antibodies and complement. *Blood.* 65:945.
10. Muirhead, M., P. J. Martin, B. Torok-Storb, J. W. Uhr, and E. S. Vitetta. 1983. Use of an antibody–ricin A chain conjugate to delete neoplastic B cells from human bone marrow. *Blood.* 62:327.
11. Vallera, D. A., R. C. Ash, E. D. Zanjani, J. H. Kersey, T. W. LeBien, P. C. L. Beverley, D. M. Neville, and R. J. Youle. 1983. Anti-T-cell reagents for human bone marrow transplantation: Ricin linked to three monoclonal antibodies. *Science (Wash. DC).* 222:512.
12. Vallera, D. A. 1986. The use of immunotoxins in bone marrow transplantation: Eradication of T cells and leukemic cells. *In* Immunoconjugates. C.-W. Vogel, editor. Oxford University Press. In press.

13. Ramakrishnan, S., and L. L. Houston. 1984. Inhibition of human acute lymphoblastic leukemic cells by immunotoxins: Potentiation by chloroquine. *Science (Wash. DC).* 223:58.
14. Uckun, F. M., S. Ramakrishnan, and L. L. Houston. 1985. Immunotoxin-mediated elimination of clonogenic tumor cells in the presence of human bone marrow. *J. Immunol.* 134:2010.
15. Uckun, F. M., R. Stong, R. J. Youle, and D. A. Vallera. 1985. Combined ex vivo treatment with immunotoxins and mafosfamid: A novel immunochemotherapeutic approach for elimination of neoplastic T-cells from autologous marrow grafts. *J. Immunol.* 134:3504.
16. Stong, R. C., F. M. Uckun, R. J. Youle, J. H. Kersey, and D. A. Vallera. 1985. Use of multiple T-cell directed intact ricin immunotoxins for autologous bone marrow transplantation. *Blood.* 66:627.
17. Vallera, D. A., R. J. Youle, D. M. Neville, Jr., and J. H. Kersey. 1982. Bone marrow transplantation across major histocompatibility barriers. V. Protection of mice from lethal graft vs. host disease by pretreatment of donor cells with monoclonal anti-Thy-1.2 coupled to the toxic lectin ricin. *J. Exp. Med.* 155:949.
18. Vallera, D. A., D. E. Myers, J. H. Kersey, A. H. Filipovich, and F. M. Uckun. 1986. Immunotoxins (IT) in bone marrow transplantation (BMT): Eradication of T cells/leukemia cells and potential for treatment of residual disease. *J. Cell. Biochem.* 10(Suppl.):A–D.
19. Ramsay, N., T. W. LeBien, M. Nesbit, P. McGlave, D. Weisdorf, P. Kenyon, D. Hurd, A. Goldman, T. Kim, and J. H. Kersey. 1985. Autologous bone marrow transplantation for patients with acute lymphoblastic leukemia in second or subsequent remission: Results of bone marrow treated with monoclonal antibodies BA-1, BA-2, BA-3 plus complement. *Blood.* 66:508.
20. Kaizer, H., R. K. Stuart, R. Brookmeyer, W. E. Beschorner, H. G. Braine, W. H. Burns, D. J. Fuller, M. Korbling, K. F. Mangan, R. Saral, L. Sensenbrenner, R. K. Shadduck, A. C. Shende, P. J. Tutschka, A. M. Yeager, W. H. Zinkham, O. M. Colvin, and G. W. Santos. 1985. Autologous bone marrow transplantation in acute leukemia: A phase I study of in vitro treatment of marrow with 4-hydroperoxycyclophosphamide to purge tumor cells. *Blood.* 65:1504.
21. Bast, R. C., S. E. Sallan, C. Reynolds, J. Lipton, and J. Ritz. 1985. Autologous bone marrow transplantation for CALLA-positive acute lymphoblastic leukemia: An update. *In* Autologous Bone Marrow Transplantation. Proceedings of the First International Symposium. K. Dicke, G. Spitzer, A. R. Zander, editors. Gulf Coast Printing. p. 3.
22. Kersey, J. H., A. Goldman, C. Abramson, M. Nesbit, G. Perry, K. Gajl-Peczalska, and T. W. LeBien. 1982. The clinical utility of monoclonal antibody phenotyping in childhood acute lymphoblastic leukemia. *Lancet.* 2:1419.
23. Sobol, R. E., I. Royston, T. W. LeBien, J. Minowada, K. Anderson, F. R. Davey, J. Cuttner, C. Schiffer, R. R. Ellison, and C. D. Bloomfield. 1985. Adult acute lymphoblastic leukemia phenotypes defined by monoclonal antibodies. *Blood.* 65:730.
24. Uckun, F. M., S. Ramakrishnan, and L. L. Houston. 1985. Ex vivo elimination of lymphoblastic leukemia cells from human marrow by mafosfamid. *Leukemia Res.* 9:83.
25. Uckun, F. M., S. Ramakrishnan, and L. L. Houston. 1985. Increased efficiency in selective elimination of leukemia cells by a combination of a stable derivative of cyclophosphamide and a human B-cell specific immunotoxin containing pokeweed antiviral protein. *Cancer Res.* 45:69.
26. Jansen, J., J. H. F. Falkenburg, D. E. Stepan, and T. W. LeBien. 1984. Removal of

neoplastic cells from autologous bone marrow grafts with monoclonal antibodies. *Semin. Hematol.* 21:164.
27. Stong, R. C., and J. H. Kersey. 1985. In vitro culture of leukemic cells in t(4;11) acute leukemia. *Blood.* 66:439.
28. Miller, D., S. Leikin, V. Albo, H. Sather, and D. Hammond. 1981. Prognostic importance of morphology (FAB classification) in childhood acute lymphoblastic leukemia. *Br. J. Hematol.* 48:199.
29. McKenna, R. W., R. K. Brynes, M. E. Nesbit, C. D. Bloomfield, J. H. Kersey, E. Spanjers, and R. D. Brunning. 1979. Cytochemical profiles in acute lymphoblastic leukemia. *Am. J. Ped. Hemeatol. Oncol.* 3:263.
30. Martin, P. J., G. Longton, J. A. Ledbetter, W. Newman, M. P. Braun, P. G. Beatty, and J. Hansen. 1983. Identification and functional characterization of two distinct epitopes on the human T cell surface protein Tp50. *J. Immunol.* 131:180.
31. Royston, I., J. A. Majda, S. M. Baird, B. L. Meserve, and J. C. Griffiths. 1980. Human T-cell antigens defined by monoclonal antibodies: The 65,000 dalton antigen of T-cells (T65) is also found on chronic lymphocytic leukemia cells bearing surface immunoglobulin. *J. Immunol.* 125:725.
32. Bernard, A., L. Boumsell, J. Dausset, C. Milstein, and S. F. Schlossman. 1984. Leukocyte Typing: Human leukocyte differentiation antigens detected by monoclonal antibodies. Springer-Verlag, Berlin. p. 25.
33. Kersey, J. H., T. W. LeBien, C. S. Abramson, R. Newman, R. Sutherland, and M. Greaves. 1981. p24: A human leukemia associated and lymphohemopoietic progenitor cell surface structure identified with monoclonal antibody. *J. Exp. Med.* 153:726.
34. LeBien, T. W., D. R. Boue, J. G. Bradley, and J. H. Kersey. 1982. Antibody affinity may influence antigenic modulation of the common acute lymphoblastic leukemia antigen in vitro. *J. Immunol.* 129:2287.
35. Abramson, C. S., J. H. Kersey, and T. W. LeBien. 1981. A monoclonal antibody (BA-1) primarily reactive with cells of human B lymphocyte lineage. *J. Immunol.* 126:83.
36. Griffin, J. D., and S. F. Schlossman. 1984. Expression of myeloid differentiation antigens in acute myeloblastic leukemia. *In* Leukocyte Typing: Human Leukocyte Differentiation Antigens Detected by Monoclonal Antibodies. Springer-Verlag, New York. p. 404.
37. Greaves, M. F., C. Sieff, and P. A. W. Edwards. 1983. Monoclonal antiglycophorin as a probe for erythroleukemias. *Blood.* 61:645.
38. Houston, L. L., S. Ramakrishnan, and M. A. Hermadson. 1983. Seasonal variation in different forms of pokeweed antiviral protein, a potent inactivator of ribosomes. *J. Biol. Chem.* 25:9601.
39. Ramakrishnan, S., and L. L. Houston. 1984. Comparison of the selective cytotoxic effects of immunotoxins containing ricin A chain or pokeweed antiviral protein and anti-Thy-1.1 monoclonal antibody. *Cancer Res.* 44:201.
40. Ramakrishnan, S., M. R. Eagle, and L. L. Houston. 1982. Radioimmunoassay of ricin A- and B-chains applied to samples of ricin A-chain prepared by chromatofocussing and by DEAE Bio-Gel A chromatography. *Biochim. Biophys. Acta.* 719:341.
41. Cawley, D. B., M. L. Hedblom, and L. L. Houston. 1979. Protection and rescue of ribosomes from the action of ricin A chain. *Biochemistry.* 17:2648.
42. Uckun, F. M., D. A. Vallera, and S. L. Wee. 1985. B lymphocyte regulation of human hematopoiesis. *J. Immunol.* 135:3817.
43. Ramakrishnan, S., F. M. Uckun, and L. L. Houston. 1985. Anti-T cell immunotoxins containing pokeweed antiviral protein: Potential purging agents for human autologous bone marrow transplantation. *J. Immunol.* 135:3616.

44. LeBien, T. W., J. M. Anderson, D. A. Vallera, and F. M. Uckun. 1986. Increased efficacy in selective elimination of leukemic cell line clonogenic cells by a combination of monclonal antibodies BA-1, BA-2, BA-3 + complement and mafosfamid (ASTA Z 7557). *Leukemia Res.* In press.
45. Ramakrishnan, S., and L. L. Houston. 1985. Immunological and biological stability of immunotoxins in vivo: Clearance of disulfide-linked pokeweed antiviral protein-antibody conjugates from blood. *Cancer Res.* 45:2031.
46. Ramakrishnan, S., and L. L. Houston. 1984. Prevention of growth of leukemia cells in mice by monoclonal antibodies against Thy-1.1 antigen disulfide linked to two ribosomal inhibitors: Pokeweed antiviral protein or ricin A chain. *Cancer Res.* 44:1398.
47. Bast, R. C., P. DeFabritiis, M. Bregni, V. Raso, C. Reynolds, J. M. Lipton, J. S. Greenberger, L. M. Nadler, S. E. Sallan, and J. Ritz. 1985. Removal of malignant cells from human bone marrow using monoclonal antibodies and complement. *In* Autologous Bone Marrow Transplantation. Proceedings of the First International Symposium. K. Dicke, G. Spitzer, A. R. Zander, editors. Gulf Coast Printing. p. 373.
48. Raso, V., J. Ritz, M. Basala, and S. F. Schlossman. 1982. Monoclonal antibody-ricin A chain conjugate selectively cytotoxic for cells bearing the common acute lymphoblastic leukemia antigen. *Cancer Res.* 42:457.
49. Szer, J., H. Joachim Deeg, F. R. Appelbaum, and R. Storb. 1985. Failure of autologous marrow reconstitution after cytolytic treatment of marrow with anti-Ia monoclonal antibody. *Blood.* 65:819.
50. Izaguirre, C. A., J. Curtis, H. Messner, and E. A. McCulloch. 1981. A colony assay for blast cell progenitors in non-B non-T (common) acute lymphoblastic leukemia. *Blood.* 57:823.
51. McCulloch, E. A. 1983. Stem cells in normal and leukemic hemopoiesis. *Blood.* 62:1.
52. Buick, R. N., M. D. Minden, and E. A. McCulloch. 1979. Self-renewal in culture of proliferative blast progenitor cells in acute myeloblastic leukemia. *Blood.* 54:95.
53. Greaves, M. F., A. Paxton, G. Janossy, C. Pain, S. Johnson, and T. A. Lister. 1980. Acute lymphoblastic leukemia associated antigen. III. Alterations in expression during treatment and in relapse. *Leukemia Res.* 4:1.