

B cell origin of non-T cell acute lymphoblastic leukemia. A model for discrete stages of neoplastic and normal pre-B cell differentiation.

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Research Article

The expression of B cell associated and restricted antigens on tumor cells isolated from 138 patients with non-T cell acute lymphoblastic leukemia (non-T cell ALL) was investigated by flow cytometric analysis by means of a panel of monoclonal antibodies. Tumor cells from these patients could be assigned to one of four subgroups: human leukocyte antigen-DR-related Ia-like antigens (Ia) alone (4%, stage I); IaB4 (14%, stage II); IaB4CALLA (33%, stage III); and IaB4CALLAB1 (49%, stage IV). The expression of B cell-restricted antigens (B4 and B1) and rearrangements of Ig heavy chain genes provided strong evidence for the B cell lineage of stages II, III, and IV tumors. The lineage of the Ia alone group is still unknown. The B4 antigen was expressed on approximately 95% of all non-T cell ALLs tested, and given its absence on T cell and myeloid tumors, it appears to be an exceptional marker to define cells of B lineage. The demonstration that Ia alone, IaB4, IaB4CALLA, and IaB4CALLAB1 positive cells can be readily identified by dual fluorescence analysis in normal fetal and adult bone marrow provided critical support for the view that these leukemic pre-B cell phenotypes were representative of the stages of normal pre-B cell differentiation. It was interesting that the IaB4+ cell was more frequently identified in fetal bone marrow than in adult marrow. [...]

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Abstract. The expression of B cell associated and restricted antigens on tumor cells isolated from 138 patients with non-T cell acute lymphoblastic leukemia (non-T cell ALL) was investigated by flow cytometric analysis by means of a panel of monoclonal antibodies. Tumor cells from these patients could be assigned to one of four subgroups: human leukocyte antigen-DR-related Ia-like antigens (Ia) alone (4%, stage I); IaB₄ (14%, stage II); IaB₄CALLA (33%, stage III); and IaB₄CALLAB₁ (49%, stage IV). The expression of B cell-restricted antigens (B₄ and B₁) and rearrangements of Ig heavy chain genes provided strong evidence for the B cell lineage of stages II, III, and IV tumors. The lineage of the Ia alone group is still unknown. The B₄ antigen was expressed on ~95% of all non-T cell ALLs tested, and given its absence on T cell and myeloid tumors, it appears to be an exceptional marker to define cells of B lineage.

The demonstration that Ia alone, IaB₄, IaB₄CALLA, and IaB₄CALLAB₁ positive cells can be readily identified by dual fluorescence analysis in normal fetal and adult bone marrow provided critical support for the view that these leukemic pre-B cell phenotypes were representative of the stages of normal pre-B cell differentiation. It was interesting that the IaB₄⁺ cell was more frequently identified in fetal bone marrow than in adult marrow, whereas the predominant cell found in adult marrow expressed

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B Cell Origin of Non-T Cell Acute Lymphoblastic Leukemia

A Model for Discrete Stages of Neoplastic and Normal Pre-B Cell Differentiation

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the IaB₄CALLAB₁ phenotype. These data suggest that the leukemogenic event may be random, since the predominant pre-B cell leukemic phenotype appears to correspond to the normal pre-B cell phenotype present in these hematopoietic organs. Our observations provide an additional distinction between adult and childhood ALL, since these studies show that most non-T cell ALLs seen in children less than 2 yr old are of stage II phenotype, whereas the majority of non-T ALLs in adults are of stage IV phenotype. Finally, it should be noted that the present study suggests that the analysis of leukemic B cell phenotypes and their normal counterparts can provide a mechanism for the investigation and orderly definition of stages of pre-B cell differentiation in man.

Introduction

Increasing evidence based upon immunologic cell-surface phenotype and Ig gene rearrangements supports the idea that leukemic cells from patients with non-T cell acute lymphoblastic leukemia (non-T cell ALL)¹ appear to be the neoplastic counterparts of normal pre-B cells (1-13). Initially, the cell-surface expression of B lineage-associated determinants including the human leukocyte antigen-DR-related Ia-like antigens (Ia) and the common acute lymphoblastic leukemia antigen (CALLA) suggested that these tumors might be of B cell origin (1-3). Subsequently, it was observed that ~15% of these tumors contained cytoplasmic μ -heavy chains (c μ) (4-6) and 50% expressed the B₁ antigen, a B cell-restricted cell-surface determinant (7). Both sets of observations support the view that non-T cell ALLs

1. Abbreviations used in this paper: AML, acute myeloblastic leukemia; CALLA, common acute lymphoblastic leukemia; CML, chronic myeloblastic leukemia; c μ , cytoplasmic μ heavy chain; Ia, HLA-DR-related Ia-like antigen; kb, kilobase; T cell ALL, T cell acute lymphoblastic leukemia.

are pre-B cells and suggest the possibility that at least half of these tumors correspond to or precede the conventional $c\mu$ positive pre-B cell. Several lines of evidence support the hypothesis that the other 50% of non-T cell ALLs are also derived from early pre-B cells. Thus, the expression of cell surface and cytoplasmic B cell-restricted markers provided evidence that these tumors were derived from early human pre-B cells.

Recent analysis of Ig gene rearrangements has demonstrated that most, if not all, non-T cell ALLs and lymphoid chronic myeloblastic leukemia (CML)-blast crises were of B cell origin (10-13). However, several groups have demonstrated that occasional T cell and myeloid tumors, both in murine and human systems, may also display heavy-chain Ig gene rearrangements (11, 13-18). In contrast, light-chain rearrangements have thus far been restricted to cells of B cell origin. This indicates that several parameters will be necessary to characterize fully the lineage and stage of differentiation of an individual tumor.

In the present report, we have immunologically characterized tumor cells isolated from 138 patients with non-T cell ALL. We show that tumor cells isolated from these patients can be subdivided into four phenotypically defined subgroups based upon the expression or coexpression of B cell-associated antigen Ia, and of CALLA and B cell-restricted antigens B_4 and B_1 . In the studies described below, we demonstrate that four leukemic subgroups correspond to distinct stages of normal pre-B cell differentiation. Moreover, we will demonstrate that leukemic pre-B cell subgroups appear to correlate with the distribution of pre-B cell subsets in normal fetal and adult bone marrow.

Methods

Patients and sample preparations. All patients in this study were evaluated at the Dana Farber Cancer Institute, The Children's Hospital Medical Hospital, the Brigham and Women's Hospital, or the Beth Israel Hospital (all in Boston). The diagnosis of non-T cell ALL was made on the basis of standard morphologic and cytochemical criteria (19, 20) and the lack of reactivity with anti-Ig (IgM, IgG, κ , and λ), anti-T cell antibodies (anti-T₁, T₃, T₄, T₆, T₈, and T₁₁) (21) and antimyeloid antibodies (anti-MY₇, MY₈, MY₉, and Mo₁) (22-24). Heparinized peripheral blood or bone marrow was collected from leukemic patients before the administration of chemotherapeutic agents or blood products. Mononuclear cells were separated from these specimens by Ficoll-Hypaque density gradient centrifugation as previously described (25). For all tumor specimens, cytocentrifuge preparations were made and the percentage of malignant cells was determined by standard Wright-Giemsa morphology. In more than 75% of cases the tumor cells made up 75-100% of the test populations. Isolated tumor cells were studied either fresh or cryopreserved in 10% dimethylsulfoxide and 20% fetal calf serum at -196°C in the vapor phase of liquid nitrogen until the time of characterization.

Monoclonal antibodies. The preparation and characterization of monoclonal antibodies used in this study have been previously described in detail. All antibodies used in this study were ascites fluid used at saturated binding concentrations. Controls represent isotype identical nonreactive ascites. Monoclonal anti-Ia (anti-I-2) (26) defines a nonpolymorphic Ia-like antigen expressed on B cells, monocytes, and activated T cells. That anti-Ia is reactive with all B cell leukemias and

lymphomas, very few T cell ALLs, most acute myeloblastic leukemias (AMLs), and most stable phase CMLs suggests that it is a B cell-associated but not a B cell-restricted antigen (27). Monoclonal anti- B_4 has been previously shown to define a 40/80 kD-glycoprotein, which is B cell specific within the hematopoietic system and is not expressed on normal T cells, myeloid cells, erythrocytes, or platelets. The B_4 antigen is found on all normal and malignant B lymphocytes, excluding the plasma cell. The B_4 antigen has not been demonstrated on any T cell ($n = 37$) or myeloid tumor ($n = 172$) and is therefore considered to be a B cell-restricted antigen. CALLA is identified by the J₅ monoclonal antibody (28). J₅ precipitates a 100,000 kD glycoprotein, which is expressed on leukemic cells of 80% of patients with non-T cell ALL and 30% of patients with CML in lymphoid blast crisis. CALLA is also found on ~20% of patients with T cell ALL, 40% of patients with T cell lymphoblastic lymphoma, and on the tumor cells of most patients with Burkitt's lymphoma and nodular, poorly differentiated lymphocytic lymphoma (29). Moreover, it has been shown that CALLA+ cells can be isolated from normal fetal hematopoietic tissues and adult bone marrow and that varying percentages of these cells coexpress the B_1 antigen, $c\mu$, and terminal deoxynucleotidyl transferase (30). CALLA is therefore also considered to be associated with, but not restricted to, B cells. Monoclonal anti- B_1 has been previously shown to be B cell specific within the hematopoietic system and is expressed on all B cells, both normal and malignant, except for the terminally differentiated plasma cells (7, 31-33). This monoclonal antibody defines a 35-kD non-glycosylated phosphorylated protein (34). The B_1 antigen has not been demonstrated on any myeloid ($n = 220$) or T cell tumors ($n = 57$).

Fetal tissues. Fetal tissues were obtained within 1 h of prostaglandin-induced therapeutic abortion. All patients who underwent therapeutic abortion had last menstrual periods and diagnostic ultrasound imaging that suggested that the fetal age was less than 24 wk. To standardize comparison of gestational age, age determination postmortem was determined by crown-rump length and fetal foot length (35). Procurement of tissue was approved by the Brigham and Women's Hospital Committee on the Use of Human Subjects in Research, and informed consent was obtained from all patients who had therapeutic abortion.

Fetal bone marrow was obtained from femora or humeri by flushing the intramedullary cavity with media. Mononuclear cells were then isolated by Ficoll-Hypaque density sedimentation.

Adult bone marrow. Adult bone marrow was obtained by aspiration from normal healthy volunteers after their informed consent. Mononuclear fractions were again obtained by Ficoll-Hypaque density sedimentation.

Indirect immunofluorescence. Leukemic cells to be characterized were used fresh or thawed at the time of study. The viability of samples included in this study exceeded 85% in all cases. Indirect immunofluorescence staining and flow cytofluorometric analysis has been previously described in detail (36). In brief, $1-2 \times 10^6$ cells were treated with either 0.1 ml of a 1:250 dilution (for all antibodies this was a saturating dilution) of the specific monoclonal antibody to be tested or 0.1 ml of a 1:250 dilution of an unreactive control antibody of the identical isotype. These cells were incubated at 4°C for 30 min, washed, and then stained with a combination of fluorescein-conjugated goat anti-mouse IgM and goat anti-mouse IgG (Coulter Electronics Inc., Hialeah, FL) for 30 min at 4°C. Cells were then washed three times and analyzed. Antibody-coated cells were enumerated by flow cytometric analysis with either a FACS 1 (Becton Dickinson and Co., Sunnyvale, CA) or an EPICS V (Coulter Electronics Inc.). Attempts were made to examine only the leukemic cells within each specimen by appropriate size scatter gating. For each sample, 10,000 cells were analyzed with a log amplifier. A positive

reaction was considered to be when more than 20% of test cells were more fluorescent than the number of cells positive with isotype-identical monoclonal antibody that was unreactive with test cells. For each sample a quantitative assessment of the number of positive cells was made (the number of cells reactive with the test monoclonal antibody, minus the number of cells reactive with unreactive isotype identical monoclonal antibody, divided by the 10,000 total cells tested).

Dual fluorescence analysis. Monoclonal antibodies anti-I-2, anti-B₄, anti-J₅, and anti-B₁ were purified and conjugated to fluorescein or biotin by standard techniques (37, 38). To start dual fluorescence analysis, fetal or adult bone marrow cells at 2–3 × 10⁶ cells/sample were incubated with one directly fluoresceinated antibody and another directly biotinylated antibody at saturating concentrations. For example, cells were incubated with directly fluoresceinated anti-I-2 and biotinylated anti-B₁ at 4°C for 30 min. Cells were washed twice and then developed with a saturating concentration of avidin Texas red. For each of the directly biotinylated monoclonal antibodies, four test specimens were prepared and then counterstained with each of the four directly fluoresceinated monoclonal antibodies. 200 cells stained with the red fluorochrome were counted and the percentage of cells that coexpressed the green fluorochrome was enumerated with a fluorescent microscope (Carl Zeiss, Inc., New York, NY).

Cytoplasmic Ig assay. Cells that contained cytoplasmic Ig (cμ) were enumerated by indirect immunofluorescence by a modification of previously described techniques (39). Cytocentrifuge preparations of culture cells were fixed in 95% ethanol at 4°C for 20 min. Slides were then washed in PBS for 20 min, exposed to directly fluoresceinated affinity-purified rabbit anti-mouse Ig (Dako Immunology, CA) at a 1:20 dilution, and incubated for 20 min at room temperature in a humidified atmosphere. Slides were then washed for 2 h in PBS and mounted in 50% (vol/vol) glycerin in PBS, and the cells that contained cytoplasmic Ig were counted on a fluorescent microscope (Carl Zeiss, Inc.). At least 200 cells were counted per slide.

Ig-gene analysis. Detailed techniques have been previously described (11). In brief, high molecular weight DNA prepared from leukemic cells was digested with the BamHI and EcoRI restriction endonuclease, size fractionated by agarose gel electrophoresis, and transferred onto nitrocellulose paper. The nitrocellulose blots were hybridized with nick-translated ³²P-DNA probes of human Ig genes that could detect germ line and rearranged alleles. The joining heavy probe was 2.4-kilobase (kb) Sau 3a fragment, the constant κ-probe was a 2.5-kb EcoRI fragment, and the constant λ-probe was a 0.8-kb G12-EcoRI constant λ1-probe fragment. Such blots were washed at the appropriate stringencies and were visualized on autoradiograms.

Immune rosette depletion. In order to isolate the normal pre-B cell counterparts of non-T cell ALL, immune rosette depletion of fetal and adult bone marrow was undertaken by use of sheep erythrocytes bound to rabbit anti-mouse Ig as previously described (40, 41). Monoclonal antibodies used to deplete fetal and adult bone marrows of T cells, and of erythroid and myeloid precursors included anti-T₃, anti-T₁₁, anti-T₉, anti-Mo₁, and anti-Mo₂, respectively. Antibodies were first incubated with either fetal or adult bone marrow cells at excess concentrations for 30 min at 4°C and then washed. These antibody-labeled cells were then incubated with rabbit anti-mouse Ig conjugated sheep erythrocytes. After a 10-min incubation at room temperature and a 10-min centrifugation (800 rpm at 4°C), rosetting was done for 1 h at room temperature. The sheep erythrocyte pellet was gently resuspended and the mixture was subjected to Ficoll-Hypaque density sedimentation to separate the rosette negative monolayer from the positive pellet. These rosette negative cells, which are enriched for B lineage cells and which

were used in a dual fluorescent assay of the expression and/or coexpression of the B cell associated and restricted antigens.

Statistical methods. Deviation from independence in the data of Table III was assessed by treating it in a contingency table. This produced a value that is distributed as a chi-square (which, for an r × c table, has (r – 1)(c – 1) degrees of freedom). Individual elements were further tested by the construction of 2 × 2 tables from elements of the larger table, as described in the text, and by the application Fischer's exact test (42, 43).

Results

Subgroups of non-T cell ALL based upon the coexpression of the Ia, B₄, CALLA, and B₁ antigens. Tumor cells from 138 patients with non-T cell ALL were evaluated for reactivity with a panel of B cell-associated (anti-Ia and anti-CALLA) and B cell-restricted (anti-B₄ and anti-B₁) monoclonal antibodies. Four subgroups of non-T cell ALL could be identified by the expression or coexpression of Ia, B₄, CALLA, and B₁. As seen in Table I, tumor cells from all patients studied expressed the Ia antigen, more than two-thirds expressed CALLA, and approximately one-half expressed B₁. The first subgroup (Ia alone), contained only 5 of the 138 patients tested (stage I) (data for all subgroups, Fig. 1 and Table I). The second subgroup (20 patients) (Ia and B₄—stage II) demonstrated very strong Ia expression and moderate B₄ expression. The third subgroup (46 patients) coexpressed Ia, B₄, and CALLA (stage III). In this subgroup, the majority of patients demonstrated very strong Ia expression and moderate B₄ and CALLA expression. Finally, the fourth and largest subgroup, (Ia, B₄, CALLA, B₁—stage IV) (67 patients) demonstrated almost the identical antigen density of Ia, B₄, and CALLA, as was seen on leukemic cells in the third subgroup. This subgroup was defined by B₁ antigen, which varied from weak to intense expression.

In addition to the patients presented in Table I, a few patients studied expressed phenotypes that did not conform to the above four subgroups. Six patients expressed these anomalous phenotypes. Four non-T cell ALLs expressed Ia and CALLA but lacked B₄ and B₁. Two additional unique phenotypes were identified: one patient expressed Ia, CALLA, and B₁ but lacked B₄, and a second patient expressed Ia, B₄, and B₁ but lacked CALLA. When the small number of patients with these phenotypes is considered, it is unclear whether these cases represent tumors of a very small subgroups of pre-B cells or, alternatively, whether they represent aberrant phenotypes.

In the present study we evaluated the tumor cells from 40 patients with non-T cell ALL for the presence of cμ. Of these 40 patients, none expressed Ia alone; 3 expressed Ia and B₄; 15 expressed Ia, B₄, and CALLA; and 22 expressed Ia, B₄, CALLA, and B₁. Seven of 40 patients demonstrated cμ and all coexpressed B₁. These results are consistent with our earlier studies which indicated that a fraction of patients whose tumor cells express B₁ were also cμ reactive. Although not all of the patients could be examined, the results support the view that some Ia+ B₄+ CALLA+ B₁+ non-T cell ALLs produce detectable

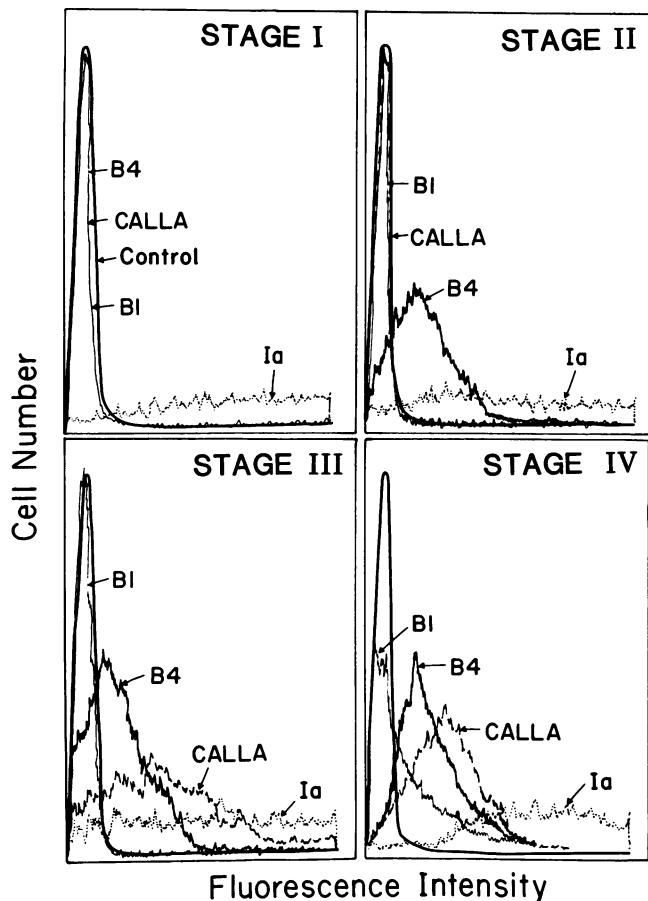


Figure 1. Phenotypic subgroups of non-T cell ALL correspond to four stages of leukemic pre-B cell differentiation. Viable tumor cells were isolated from patients with non-T cell ALL and tested for reactivity with anti-Ia, anti-B₄, anti-CALLA, and anti-B₁ by indirect immunofluorescence and flow cytometric analysis. As shown, the fluorescence profiles define four subgroups of non-T cell ALL: stage I, Ia; stage II, IaB₄; stage III, IaB₄CALLA; and stage IV, IaB₄CALLAB₁.

heavy chains and, therefore, that this group could be further subdivided into cμ+ and cμ- subsets.

Immunoglobulin gene configuration of Ia+B₄+, Ia+B₄

Table I. Subgroups of Non-T Cell ALL

Pre-B cell stage	Subgroup phenotype	Non-T cell ALL patients
		No. (%)
I	Ia	5 (4%)
II	IaB ₄	20 (14%)
III	IaB ₄ CALLA	46 (33%)
IV	IaB ₄ CALLAB ₁	67 (49%)
Total		138

+CALLA+, and Ia+B₄+CALLA+B₁+ leukemia cells. Four patients within each of the IaB₄ (stage II), IaB₄CALLA (stage III), and IaB₄CALLAB₁ (stage IV) subgroups were examined for their configuration of Ig heavy- and light-chain genes. These particular patients were chosen for analysis because they demonstrated uniformity of tumor replacement (>90%) and high antigen density for each of the subgroup antigens. None of the 12 patients demonstrated cμ. All four of the stage II patients (IaB₄) demonstrated rearranged heavy-chain genes when EcoRI and BamHI digests were examined with the joining heavy region probe (Table II). These four stage II (Ia+B₄+CALLA-) leukemias retained germline κ- and λ-light-chain genes, as has also been noted in seven Ia+CALLA- patients (11). Human Ig genes rearrange in a sequential order during differentiation in which heavy-chain rearrangements precede light-chain rearrangements and κ-light chains usually rearrange before λ. Thus, the phenotypic stage II (IaB₄) subgroup represents an early stage in the genetic maturation of B cells in which heavy-chain genes have rearranged, but light-chain gene rearrangement has not begun. All four stage III (IaB₄CALLA) leukemias had rearranged heavy chain genes and three had undergone κ-light-chain gene recombination that had either a rearrangement or a deletion of their κ-genes (Table II). Similarly, all four stage IV (IaB₄CALLAB₁) ALLs had rearranged heavy-chain genes, and

Table II. Ig-Gene Rearrangements in Subgroups of Non-T Cell ALL

Pre-B cell stage	Patient	Ig-gene rearrangement		
		Heavy chain	κ	λ
II (IaB ₄)	1	1R, 1D*	G	G
	2	2R	G	G
	3	2R	G	G
	4	1R, 1G	G	G
III (IaB ₄ CALLA)	5	2R	1G, 1D‡	G
	6	1R, 1G	G	G
	7	2R	D§	G
	8	1R, 1G	1R, 1D	G
IV (IaB ₄ CALLAB ₁)	9	2R	1G, 1D‡	G
	10	1R, 1G	G	G
	11	1R, 1G	G	G
	12	2R	D§	G

* Ig gene configurations are categorized as follows: G, germline configuration; D, deletion; R, rearranged gene.

‡ Intensity of κ-hybridization suggests that one allele has been deleted.

§ In these patients with deletions of κ-genes, long exposures of autoradiographs frequently revealed the presence of a very faint κ-band which was probably contributed by the contaminating nonleukemic cells in the preparation.

two displayed deletions of κ . The uniform presence of rearranged heavy-chain genes in all subgroups, including stage II (IaB₄) non-T cell ALLs, is further evidence for their B-cell lineage commitment. In addition, corroborating data are present for serial stages of development. All of the CALLA+ stage II (IaB₄) had germ line light-chain genes, and all cases with light-chain recombinations were CALLA+, as had been noted previously (11). However, note that the presence of CALLA and B₁ did not correlate with any specific pattern of light-chain genes in stage III and IV patients examined.

Prevalence of phenotypic subgroups according to age at diagnosis. Previous studies have shown that the response to multimodality therapy and disease-free survival in non-T cell ALL closely correlate with the age of the patient at diagnosis. Most studies demonstrate that children less than 2 yr old and adults have significantly worse prognoses than do most children (2–16 yr) (44–46). To date, no histologic, cytochemical, or immunologic criteria have explained these survival differences. To determine whether the four phenotypically defined subgroups correlated with age of diagnosis, the patients were divided into four age groups: babies (<2 yr), children (2–16 yr), young adults (16–25 yr), and adults (>25 yr). An examination of the entire patient population by age group demonstrated an age-related variation in the distribution among the four immunologically defined subgroups. As seen in Table III, the stage I subgroup had very few patients and showed no age predilection. In contrast, an examination of babies with non-T cell ALL demonstrated that a disproportionately large number (56%) expressed the stage II phenotype when compared with the other groups ($P < 0.001$). Moreover, of the 20 stage II patients studied, 10 (50%) were <2 yr old. The childhood subgroup (2–16 yr) was equally divided between the stage II and III subgroups. The young adult group

(16–25 yr) contained only 10 patients, and it is therefore difficult to determine whether a phenotypic subgroup predominates, although the distribution appears to follow that of the 2–16 yr old group. Finally, compared with the other groups, a significant ($P < 0.05$) majority (72%) of patients in the adult subgroup (>25 yr) coexpressed Ia, B₄, CALLA, and B₁ (stage IV).

Identification of stages of pre-B cell differentiation in normal fetal and adult bone marrow. With the observation that at least four phenotypically defined subgroups of non-T cell ALL could be identified, we then attempted to determine whether similar subpopulations could be identified in normal fetal and adult bone marrow. Mononuclear cells were isolated from these tissues and tested for reactivity with the monoclonal antibodies anti-Ia, anti-B₄, anti-J₅, and anti-B₁ by indirect immunofluorescence and flow cytometric analysis. As seen in Table IV, varying percentages of antigen-positive cells could be demonstrated, depending upon the bone marrow source. In the fetal bone marrows of 16–18 wk and 22–24 wk gestation, the percentage of cells that expressed Ia, B₄, CALLA, and B₁ were very similar. The major difference was a slight decrease in the number of B₄ cells and an increase in the number of B₁ positive cells with increasing gestational age. In contrast, adult bone marrow contained fewer Ia positive cells than did fetal bone marrow (17 vs. 35%) and many fewer B₄, CALLA, and B₁ positive cells (2–4 vs. 8–24%). The larger percentage expressed by adult bone marrow of Ia positive cells than of B₄, CALLA, B₁ positive cells reflects the expression of this antigen on myeloid and erythroid precursors (47, 48).

To demonstrate directly the existence of normal cellular counterparts of the four subgroups of non-T cell ALL, B cell lineage enriched fetal and adult bone marrow mononuclear populations were studied for the coexpression of the Ia, B₄, CALLA, and B₁ antigens. By 15 wk of gestation, the fetal bone marrow is the major B cell differentiative tissue (49). To enrich B lineage cells in fetal and adult bone marrow, an immune rosette technique was employed to deplete erythroid, myeloid, and T cells. As has been previously shown, this technique effectively removed unwanted cell populations within the rosette positive pellet (41). The immune rosette negative population, in contrast, was enriched five- to tenfold for B cells and B cell precursors. The B cell lineage enriched, immune rosette negative

Table III. Phenotypic Subgroups of Non-T Cell ALL: Age Distribution at Diagnosis*

Pre-B cell stage	Number of patients within each age distribution			
	<2 yr	2–16 yr	16–25 yr	>25 yr
I (Ia)	1 (6)	1 (1)	2 (20)	1 (6)
II (IaB ₄)	10 (56)‡	6 (7)	3 (30)	1 (6)
III (IaB ₄ CALLA)	3 (17)	38 (42)	2 (20)	3 (17)
IV (IaB ₄ CALLAB ₁)	4 (22)	47 (52)	3 (30)	13 (72)§
Total	18	92	10	18

Numbers in parentheses are percentages.

* Statistical analysis excluded the Ia alone group owing to the small numbers and uncertain lineage. The remainder of the table differed significantly from independence (probability of independence was <0.001).

‡ The 10 IaB₄ patients in age group 1 was greater than expected. Stages III and IV were combined and tested against stage II age groups 1 and 2 by Fischer's exact test. The probability of the 10 observations was <0.001.

§ The probability of observing 13 (stage IV) IaB₄CALLAB₁ cases in the fourth group was tested by setting up a 2 × 2 table of stages III and IV of the second and fourth groups. $P < 0.05$ by Fischer's Exact Test.

Table IV. Expression of B-Cell-associated Antigens on Mononuclear Cells Isolated from Fetal and Adult Bone Marrow

Bone marrow source	Age	No. tested	Percentage of cells expressing antigens			
			Ia	B ₄	CALLA	B ₁
Fetal	16–18 wk gestation	10	32±7	24±8	13±3	8±2
Fetal	22–24 wk gestation	7	36±8	16±6	12±6	14±3
Adult	18–35 yr	8	17±8	3±1	2±1	4±2

cells from adult and fetal bone marrow were incubated with combinations of saturating concentrations of directly fluoresceinated and biotinylated anti-Ia, anti-B₄, anti-J₅, or anti-B₁. The cell preparation was then developed with avidin Texas red, 200 red fluorescence positive cells were counted, and the cells that coexpressed green fluorescence were enumerated. In this manner it was possible to determine sequentially the number of cells that coexpressed each of the test antigens.

Table V depicts the results of three fetal bone marrows (15–17 wk gestation) compared with the results of three adult bone marrows (ages 23, 25, and 27 yr). 62% of the Ia positive cells within the rosette negative fraction of fetal bone marrow demonstrated coexpression of B₄, whereas 37% coexpressed CALLA, and only 16% coexpressed B₁. A comparison of these results with those from the rosette negative fraction of an adult demonstrates that a smaller proportion of B₄, CALLA, and B₁ cells were present in the Ia positive fraction. Further examination of the subpopulations in fetal bone marrow demonstrates that approximately half of the B₄⁺ cells coexpress CALLA and approximately one-third coexpress B₁. The fetal CALLA⁺ cells uniformly express Ia and B₄ and approximately half coexpress B₁. Finally, of the B₁⁺ cells, almost all coexpress Ia, B₄, and CALLA. These results suggest that fetal bone marrow contains a discrete population of IaB₄ positive cells but also contains cells that coexpress either Ia, B₄, and CALLA, or Ia, B₄, CALLA, and B₁.

In contrast to the fetal bone marrow, the B cell enriched immune rosette negative fraction isolated from adult bone marrows demonstrates a different pattern of B cell antigen expression. In the adult bone marrow there are significantly fewer cells of B lineage (Table IV). All B₄⁺ cells coexpress Ia and approximately three-quarters express B₄ and CALLA (Tables IV and V). The overwhelming majority of the CALLA positives coexpress Ia, B₄, and B₁. When one examines the B₁ positive cells in the adult bone marrow cells one sees a distinct difference from the fetal marrow (Tables IV and V). Although the B₁ positive cells uniformly express Ia and B₄, only two-thirds coexpress CALLA; this suggests that an Ia+B₄+CALLA-B₁⁺ cell exists in adult bone marrow. Moreover, these Ia+B₄

+CALLA-B₁⁺ cells did not appear to be mature B cells from contaminating peripheral blood, since only 10% of these cells expressed surface Ig. These observations suggest that the four subpopulations seen in non-T cell ALL are seen in adult bone marrow but that fewer total pre-B cells exist in adult bone marrow and that an additional population of Ia+B₄+CALLA-B₁⁺ mature pre-B cells can be demonstrated.

Discussion

In the present report, 138 patients with non-T cell ALL were characterized by the use of a panel of monoclonal antibodies defining B cell-associated and B cell-restricted antigens. By their differential expression of the B cell-associated antigens, Ia and CALLA, and the B cell-restricted antigens, B₄ and B₁, four phenotypically defined subgroups of non-T cell ALL could be defined. These are Ia alone (stage I, 4%); IaB₄ (stage II, 14%); IaB₄CALLA (stage III, 33%); and IaB₄CALLAB₁ (stage IV, 49%). These four phenotypically defined subgroups have provided us with both a model to study the biological and clinical heterogeneity of non-T cell ALL and a framework to identify and isolate phenotypically identical normal stages of pre-B cell differentiation.

Although there has been significant phenotypic and genetic evidence to support the idea that non-T cell ALLs are of B cell-derived neoplasms (10–13), the observation that virtually 100% of non-T cell ALLs demonstrate Ig heavy-chain rearrangements has provided compelling evidence to support this conclusion. The finding that occasional T cell ALLs, Sezary cell syndrome, AML and a number of murine T cell lymphomas also demonstrate Ig heavy-chain rearrangement reveals that heavy-chain rearrangement is not restricted solely to cells of B lineage (11, 13–18). To date, light-chain gene rearrangements have been restricted to cells of B development, but only 40% of non-T cell ALLs demonstrate light-chain recombinations (11). Thus, Ig-gene rearrangements are not sufficient to assign non-T ALLs to the B lineage. We propose that for non-T cell ALLs with rearrangements of heavy-chain alone, the B₄ antigen provides an important independent parameter with which to

Table V. Coexpression of B Cell-associated Antigens on B Cell-enriched Fractions Isolated from Fetal and Adult Bone Marrow

Fetal bone marrow rosette negative fraction (15 wk)					Adult bone marrow rosette negative fraction (25 yr)				
Initial antigen present	Cells coexpressing second antigen (%)				Initial antigen present	Cells coexpressing second antigen (%)			
	Ia-F	B ₄ -F	Calla-F	B ₁ -F		Ia-F	B ₄ -F	Calla-F	B ₁ -F
Ia (b-AVTR)	97±2	62±7	37±8	16±8	Ia (b-AVTR)	99±1	18±9	16±7	23±11
B ₄ (b-AVTR)	98±3	99±3	53±9	34±5	B ₄ (b-AVTR)	97±1	98±1	78±10	72±6
CALLA (b-AVTR)	96±3	94±5	99±2	54±8	CALLA (b-AVTR)	97±2	95±3	97±2	83±7
B ₁ (b-AVTR)	99±2	97±1	98±2	96±2	B ₁ (b-AVTR)	98±2	97±1	64±9	98±2

F, antibody directly conjugated to fluorescein isothiocyanate. b-AVTR, antibody directly conjugated to biotin and developed with avidin bound to Texas red dye. All data are mean±SD.

identify them as being of B cell origin. The B_4 antigen has not yet been detected on normal non-B cells within the hematopoietic system (9). It is, however, expressed on $\sim 95\%$ of non-T cell ALLs, 100% of B chronic lymphocytic leukemias ($n = 170$) (Nadler, L. M., R. Hardy, K. C. Anderson, K. Iida, M. P. Bates, B. Slaughenhoupt, and S. F. Schlossman, submitted for publication), and on 100% of B cell non-Hodgkin's lymphomas ($n = 103$) (50). In contrast, it has not been demonstrated on T cell (T cell ALL [$n = 15$], T cell lymphoma [$n = 14$], and T cell chronic lymphocytic leukemia [$n = 8$]) or myeloid (AML [$n = 148$] and CML [$n = 24$]) tumors (L. Nadler, unpublished data). Moreover, two human T cell leukemia lines (HSB-2) (11) and REX (E. Reinherz and O. Acuto, personal communication) which have been shown to contain rearranged Ig heavy-chain genes lack any detectable expression of B_4 (L. Nadler, unpublished data). Thus, B_4 is a B cell-restricted marker, and the presence of Ig heavy-chain rearrangements and B_4 expression together in $\sim 95\%$ of non-T cell ALLs are truly of B cell origin. The remaining 5% of morphologically and cytochemically defined non-T cell ALLs express only the Ia antigen. Whereas these tumors may represent several lineages, we propose that this subgroup represents an even earlier stage of pre-B cell differentiation than does the Ia B_4 positive subgroup. To resolve this question, additional techniques, either by genetic or cell-surface analysis, will be required to define the lineage of the Ia alone group.

It might be argued that non-T ALLs may represent aberrations of normal B cells; however, the identification of cells in normal bone marrow that express the identical cell-surface phenotype suggests that this is not true. In previous studies, the CALLA positive cell was isolated from normal fetal liver and bone marrow (30, 49) and more recently from adult bone marrow (51). Phenotypic characterization of these CALLA+ cells from fetal tissues demonstrated that more than 99% expressed Ia, 41–52% expressed B_1 , 20% expressed $c\mu$, and 15% expressed terminal deoxynucleotidyl transferase (30). This study demonstrated the existence of an Ia+CALLA+ B_1 – and an Ia+CALLA+ B_1 + normal pre-B cell. In this report, we have extended these studies by demonstrating the presence of normal cells that correspond to stage I and stage II tumors, and by confirming the existence of normal cells that correspond to stage III and stage IV tumors. The identification of subgroups of pre-B cells in normal tissues suggests that the non-T cell ALLs correspond to subgroups of normal marrow-derived pre-B cells. In this light it is interesting that of the stages II, III, and IV leukemic cells evaluated in this study for Ig-gene configurations, all had Ig heavy-chain gene rearrangements. In addition, some of the cells of stages III and IV had progressed to light-chain gene recombinations that revealed either κ -rearrangement or deletion. These observations indicate that the pre-B cell differentiation antigens of Ia, B_4 , and CALLA usually precede light-chain Ig-gene rearrangement.

The present studies suggest a tentative ordering of pre-B cell differentiation based upon the phenotypic analysis of tumor cells isolated from patients with non-T cell ALL. In previous studies we have demonstrated that the Ia+CALLA+ B_1 –

non-T cell ALL cell could be induced with phorbol myristic acid or phytohemagglutinin-leukocyte conditioned medium to express B_1 and then $c\mu$ (8). These studies established the probable order of pre-B cell differentiation, with the sequential appearance of the Ia+CALLA+ B_1 – $c\mu$ – cell which differentiates into the Ia+CALLA+ B_1 – $c\mu$ – cell and finally into the Ia+CALLA+ B_1 – $c\mu$ +. We assume that the Ia+ B_4 +CALLA– B_1 cell precedes the Ia+ B_4 +CALLA+ B_1 – cell in development, based in part on the observation that CALLA– cells have germ line light-chain genes whereas CALLA+ cells may have recombined light-chain genes (11). However, it should be noted that all efforts to induce the Ia+ B_4 +CALLA– B_1 – $c\mu$ – cell to differentiate in vitro by the use of a broad range of concentrations of phorbol myristic acid, T cell conditioned media, or dimethyl sulfoxide have been unsuccessful. The higher frequency of the Ia+ B_4 +CALLA– B_1 – ALL cell in young children with non-T cell ALL and in early fetal bone marrow provides more support for the view that this is a less mature cell. Further ordering of the stage specific steps of pre-B cell differentiation will require the development of additional techniques to induce differentiation of early pre-B cells.

It was very interesting that the distribution of these phenotypically defined subgroups of non-T cell ALL appeared to cluster according to the age of the patient at diagnosis. Most of the patients with the stage II subgroup of non-T cell ALL were children less than 2 yr old, whereas the adult non-T cell ALLs primarily expressed the stage IV phenotype. The apparent differences in subgroup frequency between young children and adults may be related to the distribution of pre-B cells in fetal and adult bone marrow. Whereas stages II, III, and IV positive cells could be identified in both fetal and adult bone marrow, it was clear that the fetal bone marrow contains a greater percentage of stage II positive cells than adult marrow. Furthermore, the adult marrow contained predominantly stage IV positive cells. The observation that for each age cluster the predominant leukemic pre-B cell subtype corresponds to the predominant normal pre-B cell counterpart in fetal and adult bone marrow might suggest that the neoplastic events are not restricted to a single stage of pre-B cell differentiation but randomly affect all stages of pre-B cell differentiation.

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