Four-Color Flow Cytometric Investigation of Terminal Deoxynucleotidyl Transferase–Positive Lymphoid Precursors in Pediatric Bone Marrow: CD79a Expression Precedes CD19 in Early B-Cell Ontogeny

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Terminal deoxynucleotidyl transferase (TdT)-positive cells in human bone marrow (BM) are a phenotypically inhomogeneous population of precursor cells. In their majority, these TdT$^+$ cells are unambiguously committed to the B lineage, as evidenced by CD19 expression. However, TdT$^+$ precursors that lack CD19 also exist and these may encompass a differentiation potential for the B as well as for other lineages. Because recent data suggested that CD19 expression is not the earliest differentiation event in B-cell ontogeny, we sought to reevaluate TdT$^+$ lymphoid precursors in pediatric BM to define the phenotypic denominator of B-lineage affiliation upstream of CD19. Using four-color flow cytometry, we focused on the assessment of the CD79a antigen, which is highly B-cell specific and which may also be expressed very early in B-cell ontogeny. We found that a majority of TdT$^+$ cells coexpressed CD19 and CD79a in addition to CD10 and CD34, whereas, in all investigated samples, some TdT$^+$ precursors lacked CD19 but expressed CD79a, which suggestively indicates also their B-lineage affiliation. In contrast to the CD19$^+$ precursors, which were usually CD10^{hi} and CD79b$^+$, these CD19$^-$CD79a$^+$ putative B-cell precursors preferentially expressed CD10 at low levels and were CD79b$^+$ in only 41%. About 17% of these TdT$^+$CD19$^-$CD79a$^+$ precursors also coexpressed CD33 and CD7, but not myeloperoxidase, CD14, or cytoplasmic CD3, which is discussed in the light of cellular activation rather than lineage promiscuity. Our data confirm that the earliest differentiation stages of B cells can be dissected upon expression of the lineage antigens CD79a and CD19 and imply that CD79a is earlier expressed than CD19. This raises the chance to follow the sequential events heralding B-cell commitment in the most immature precursors by correlating phenotypic and genetic differentiation markers.

THE SEQUENCE OF antigen acquisition during early B-cell development has been a recurrent issue of investigation. From studies of neoplastic cells it was initially suggested that CD19 is the first B-cell antigen to appear on terminal deoxynucleotidyl transferase (TdT)-positive lymphoid progenitors, thus heralding lineage-commitment.[1,2] Investigations of normal human bone marrow (BM) led to the conclusion that, on physiologic TdT$^+$/CD34$^+$ precursors, CD19 and CD10 appear essentially at the same time.[3-5] Bright CD10 expression was recognized typical for these earliest B-cell precursors (BCPs).[3,4] More recent publications documented that the proportions of CD10$^+$ cells exceeded those of CD19$^+$ cells in CD34$^+$/TdT$^+$ precursor populations.[6-9] CD19$^+$ B cells could be grown from such CD10$^+$CD19$^-$ progenitors derived from fetal liver as well as from adult BM, which corroborates the current concept that CD10 is the upstream marker in early B-cell ontogeny.[10,11] However, CD10 is not B-cell specific,[12] and it has been documented that the CD10$^+$CD19$^-$ progenitor pool harbors also cloning potential for T, natural killer (NK), and dendritic cells.[11] Quantitative relations of these different capacities have remained elusive, not at least due to the in vitro culture conditions.

In an independent approach aimed at the assessment of CD99 expression in B-cell development, we have recently observed that early (CD34$^+$/TdT$^+$) lymphoid precursors in the BM can be dissected according to their levels of CD10 expression.[13] Aside from a major CD10^{hi}CD19$^+$ population, a CD10^{lo} subset was found that only partially coexpressed CD19. The CD10^{lo}CD19$^-$ cells were considered to correspond to the CD10$^+$CD19$^-$ progenitors claimed in the above-mentioned studies. We speculated that, due to their uniformity in high CD99 expression and in light scattering patterns and due to their regeneration kinetics in parallel with B-cell outgrowth after chemotherapy, the majority of the CD10^{lo} progenitors may be committed to the B lineage, irrespective of CD19 expression.[14] To test this hypothesis, we reevaluated in the present study the marker coexpression patterns of TdT$^+$ progenitors in BM. Special interest was dedicated to the analysis of CD79a expression in these cells, because this marker has been claimed to be of extraordinary B-lineage specificity.[15-17] Our data document for the first time that many of those TdT$^+$ progenitors that lack CD19 express CD79a in addition to CD10, which corroborates the concept that these cells are committed to the B lineage and which implies that CD79a precedes CD19 in the earliest differentiation events of B-cell ontogeny.

MATERIALS AND METHODS

Sample description. BM samples were obtained from 9 children: n = 5 were BM donors for transplantation (2 years and 7 months, 3 years and 7 months, 8 years and 11 months, 12 years and 4 months, and 12 years and 11 months), n = 1 was a boy (3 years and 10 months old) after autologous peripheral stem cell transplantation for neuroblastoma without current evidence of disease, n = 1 was a girl (1 year and 10 months old) regenerating after chemotherapy for Langerhans cell histiocytosis (LCH) not involving BM and without signs of disease-associated hematological dysfunction, n = 1 was a boy (2 months old) suffering from neuroblastoma without BM infiltration, and n = 1 was a girl (4 years and 1 month old) with bcr/abl-positive common acute lymphoblastic leukemia (ALL) that was in remission at the time of immunologic investigation (polymerase chain reaction-negative for bcr/abl). Data concerning these patients were obtained from the Austrian study centers of the international Berlin-Frankfurt-Münster (BFM) study group, of the international LCH trials, and of the national neuroblastoma study NB-94.

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Antibodies. Fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, allophycocyanin (APC)-labeled, or unconjugated pure monoclonal antibodies (MoAbs) were used: CD3 (UCHT1-PE), CD10 (SS2/36-PE), CD14 (TÜK4-PE), CD19 (HD37-PE, pure), CD33 (WM54-PE), CD79a (HM57-PE, pure), and CD79b (SN8 pure) were all from Dako (Glostrup, Denmark); CD3 (SK7-APC), CD19 (SJ25C1-APC), and CD34 (HPCA2-PE, -APC) were from Becton Dickinson (BD; Sunnyvale, CA); CD7 (3A1-RD1-PE) was from Coulter Immunology (Krefeld, Germany); antihuman TdT (H Tdt-1,3,4-FITC) was from Immunotech (Marseille, France); and antihuman myeloperoxidase (H-43-5-PE) was from An der Grub (Kaumberg, Austria). Murine irrelevant isotype-matched fluorescent MoAbs were purchased from Dako and BD.

Immunofluorescence staining procedure. Mononuclear cells (MNCs) were isolated from the BM specimens by density gradient centrifugation and washed twice in RPMI 1640 medium containing 2% fetal calf serum (FCS; both from GIBCO, Paisley, UK) before immunofluorescence labeling. Staining protocols used four-color investigations in all experiments, except for the cell sorting (see below). All antibodies were used at concentrations titrated for optimal staining. Cellular permeabilization (for the investigation of CD79a, CD79b, TdT, myeloperoxidase, and cytoplasmic CD3) was usually performed as the first step of a five-step labeling cascade, because CD79a detection was performed using preferentially the unconjugated HM-57 MoAb. In brief, 5 to 10 $\times$ 10^5 MNCs per analysis were permeabilized as recently described using a commercially available formaldehyde-based erythrocyte lysing solution (BD) supplemented with Triton-X100 (0.015%).[14] The concentration of the latter had been titrated for optimal permeabilization together with a good preservation of light scattering and fluorescence properties. Next, the cells were incubated with the unconjugated MoAbs and then with biotinylated rabbit $F(ab')_2$ antimouse Ig antibodies (from Dako). To exclude nonspecific staining via unsaturated binding sites of the second step reagent, this was followed by incubation with unlabeled irrelevant murine IgG_1 (MOPC21; Sigma, St Louis, MO). Without washing, the directly labeled MoAbs were then added simultaneously and together with streptavidin-Peridinin Chlorophyll Protein (PerCP; BD). Murine irrelevant isotype-matched fluorescent MoAbs (from Dako as well as from BD) were included in the staining protocols as negative controls. Antibody incubations were performed at 4°C in the dark over 30 minutes and were followed by washing steps using phosphate-buffered saline (0.13 mol/L NaCl, 7 mmol/L Na_2HPO_4, 3 mmol/L NaH_2PO_4) with 2% FCS. Before flow cytometry, cell suspensions were passed through a 30-µm nylon mesh (Swiss Silk Bolting Manufacturing Co, Zurich, Switzerland).

Flow cytometry. We used a FACSCalibur (BD) equipped with an Argon laser tuned to 488 nm and a diode laser with 635nm emission. Calibration beads (Calibrite; BD) were routinely used for monitoring and optimizing the instrument settings. Data were acquired with the CELL Quest software (BD). Forward light scattering (FSC), transformed orthogonal light scattering (T-SSC), and fluorescence signals (FL-1-FITC, FL-2-PE, FL-3-PerCP, and FL-4-APC) were stored in listmode data files. Samples were first analyzed conventionally (30,000 cells), and then gated acquisition was performed using the same material for a better definition of the small TdT^+ subset of MNCs. Live gates were set in the appropriate fluorescence versus SSC correlations and gated events acquired to maximum yield (mean, 8,550; range, 1,750 to 30,000; n = 9). All data were analyzed using PAINT-A-GATE software (BD).

Cell sorting was performed on a FACStar Plus (BD). Using a CD19/CD10/CD34 staining, $CD10^+CD34^+$ cells were sorted into the $CD19^+$ and $CD19^-$ fractions. Correspondence of these cell populations to the $CD79a^+TdT^+CD19^{+\text{ or }-}$ cells was proven in parallel conventional four-color analyses of the same material using various MoAb combinations. Cytospin preparations of the sorted subsets were made on a Shandon Cytospin II (Southern Product Ltd, Astmoor, UK) at 30*g* for 10 minutes. Slides were stained with Pappenheim stain.

RESULTS

Analysis of TdT^+ precursors in the BM. The TdT expression in the BM of 9 probands was evaluated after fixation/permeabilization of MNCs using a triple-pool of FITC-labeled MoAbs (IgG2a and IgG1). Pooled irrelevant isotype-matched FITC MoAbs were used to correct for background fluorescence in the FITC channel. A mean of 3.6% of MNCs (on event basis in dot plots) were TdT^+ (range, 0.6% to 13.6%). To assess the proportions of TdT^+ precursors coexpressing CD10, CD19, CD34, and CD79a, gated TdT^+ events were acquired in all 9 experiments. The background positivity in the fluorescence channels used for quantification of marker coexpression was also evaluated with appropriately labeled, irrelevant, isotype-matched control MoAbs. The proportions of cells positive for CD34, CD10, and CD79a were in the same range, whereas slightly less TdT^+ cells were found to be $CD19^+$. A mean of 86% (range, 75% to 93%) of TdT^+ precursors coexpressed the stem cell antigen CD34, 87% (range, 66% to 96%) were $CD10^+$, and 84% (range, 65% to 93%) were $CD79a^+$, but only 68% were $CD19^+$ (range, 48% to 92%). It is noteworthy that, even in individual BM samples, the proportions of cells positive with CD79a (or CD10) were always higher than those positive with CD19, regardless of whether they were derived from normal or postchemotherapy donors (Table 1). This implied that CD19 was not expressed on all $CD79a^+TdT^+$ precursors. To investigate this correlation with respect to the earliest phenotypic signs of B-lineage commitment, we studied, as a next step, TdT^+ cells that coexpressed CD79a or CD19 (data are presented in the following section). However, according to the aim of our study, we did not investigate in detail the few TdT^+ cells that lacked signs of B-lineage commitment ($CD79a^-CD19^-$). Most of the latter cells were TdT^{dim}, $CD34^{hi}$ and lacked myeloperoxidase (MPO), but were positive with CD33, thus resembling myeloid precursors. Some TdT^{dim} precursors (which lacked CD19 and CD79a) also coexpressed CD7. In another small proportion of cells, TdT expression was ambiguous by being marginal to the negative area. These cells stained strongly with CD33 as well as MPO, but lacked CD34, and were considered to be myelomonocytic.

Table 1. TdT^+ Precursors in BM and Their Marker Coexpression Patterns

Proband (age)	% TdT^+ (of MNC)	% of TdT^+ Cells Positive With Marker			
		CD34	CD10	CD19	CD79a
1. 2 mo	4.2	82	87	66	91
2. 1 yr 10 mo	2.7	90	79	49	65
3. 2 yr 7 mo	3.9	93	95	74	89
4. 3 yr 7 mo	2.9	90	92	84	92
5. 3 yr 10 mo	1.9	80	96	92	93
6. 4 yr 1 mo	13.6	90	91	75	93
7. 8 yr 11 mo	0.7	ND	85	48	81
8. 12 yr 4 mo	0.6	ND	66	60	70
9. 12 yr 11 mo	1.9	75	89	60	80

Values were derived from gating experiments as described in Materials and Methods.

Abbreviation: ND, not determined.

Analysis of TdT$^+$ precursors with respect to CD79a and CD19 expression. CD19 was expressed on only 78% of gated CD79a$^+$TdT$^+$ precursors (range, 61% to 92%) from 9 BM specimens. Reversely, de facto, all CD19$^+$TdT$^+$ BCPs were CD79a$^+$ (mean, 97%; range, 91% to 100%). Next, we assessed the antigen coexpression patterns of these two putatively B-lineage–committed CD79a$^+$TdT$^+$ precursor subsets as dissected by CD19 expression. These analyses were also performed on gated CD79a$^+$TdT$^+$ populations. Quantitative data on the marker correlations are given in Table 2, and a comprehensive phenotypic analysis is shown in Fig 1. With respect to CD34 expression, there was no difference between the CD19$^+$ and CD19$^-$ subsets, because almost all CD79a$^+$TdT$^+$ precursors were uniformly CD34$^+$. CD10 was also expressed on almost all of the cells, but not homogeneously, which is in accordance with recent observations.[13,14] Most CD79a$^+$TdT$^+$ precursors were CD10^{hi}, and fewer cells expressed the antigen only at low levels (CD10^{lo}). In coanalyzing the CD79a$^+$TdT$^+$ cells for CD19 and levels of CD10, we found that the CD19$^-$ precursors preferentially resided in the CD10^{lo} subset (Fig 1). In detail, 61% (range, 33% to 78%) of CD10^{lo}CD79a$^+$TdT$^+$ cells, but only 19% (range, 4% to 39%) of CD10^{hi} precursors, lacked CD19.

We analyzed the CD79a$^+$TdT$^+$ precursor subsets, dissected by CD19 expression, for coexpression of myeloid and T-cell markers (CD33, MPO, CD14, CD7, and cytoplasmic CD3). As shown in detail in Table 2, we could hardly recognize expression of these markers on CD19$^+$ populations. However, CD19$^-$CD79a$^+$TdT$^+$ precursors showed significant coexpression of CD7 and, in particular, of CD33 (see Fig 1), but not of MPO, CD14, and CD3. The expression levels of the former two antigens were usually low.

In three experiments, we also included an MoAb to CD79b. We found that this antigen was also expressed in the cytoplasm of a proportion of TdT$^+$ cells (mean, 57%; range, 51% to 65%). Whereas de facto all CD79b$^+$TdT$^+$ cells were CD79a$^+$ (mean, 95%; range, 94% to 96%) and most were CD19$^+$ (mean, 88%; range, 78% to 98%), only a proportion of CD79a$^+$TdT$^+$ precursors were CD79b$^+$ (mean, 69%; range, 62% to 82%). In detail, the CD19$^+$CD79a$^+$TdT$^+$ BCPs were mostly CD79b$^+$ (mean, 85%; range, 78% to 92%), whereas the CD19$^-$CD79a$^+$TdT$^+$ precursors were CD79b$^+$ in only 41% (range, 35% to 47%).

We found also that the CD19$^+$ and CD19$^-$ subsets of the CD79a$^+$TdT$^+$ precursors differed in their light scattering properties as well as in their morphology (Fig 2). To prepare cytospin preparations for microscopic analysis, MNCs of one BM sample were stained with CD10, CD19, and CD34, and the CD10$^+$CD34$^+$ cells were sorted by flow cytometry into a CD19$^+$ and a CD19$^-$ fraction. CD10 and CD34 were used as surrogate markers substituting for CD79a and TdT to avoid the cellular fixation and permeabilization step that is obligatory for CD79a and TdT detection. We proved the correspondance of these populations to CD79a$^+$TdT$^+$CD19$^{+\text{ or }-}$ cells by parallel four-color analyses, finding that similar proportions (1) of TdT$^+$CD19$^-$ cells were CD10$^+$ or CD79a$^+$ (23% and 25% of TdT$^+$) and (2) of CD79a$^+$CD19$^-$ cells were CD34$^+$ or TdT$^+$ (74% and 77%). With respect to morphology, the majority of the CD19$^+$ precursors were of typical small lymphocyte size (also defined by their very low FSC and T-SSC properties), had very scant cytoplasm, had relatively dense chromatin, and had no or inconspicuous nucleoli. In contrast, the CD19$^-$ precursors were a relatively homogeneous population of intermediately sized, blastoid cells with large nuclei (in part lobulated, possibly as a cytospin artifact), had evenly dispersed, relatively fine chromatin with prominent nucleoli, and displayed a comparatively wider, basophilic cytoplasm and focal juxtanuclear areas of clearing. In keeping with their morphology, these CD19$^-$ precursors showed elevated FSC and T-SSC properties as compared with the majority of the CD19$^+$CD79a$^+$TdT$^+$ cells.

DISCUSSION

Recent evidence suggests that expression of CD19 is not the first differentiation event in human B-cell ontogeny. B cells have been grown from progenitors expressing CD10 but lacking CD19,[10,11] and activation of the IgH locus, a prerequisite for rearrangement, has been shown to precede CD19 expression.[18] However, CD10 is not restricted to B cells,[12] and T, NK, and dendritic cells have also been cloned from CD10$^+$CD19$^-$ progenitors.[11] Hence, the human precursor population in which B-lineage commitment first occurs is still phenotypically ill defined. Therefore, we sought to subcharacterize TdT$^+$ progenitors using CD79a, which seemed a good candidate antigen to specifically delineate early B-lineage affiliation. The CD79 antigen is a heterodimer containing one subunit each of the MB-1 (CD79a) and B29 (CD79b) molecules that are noncovalently associated with Igs on the surface membrane of mature B cells.[19,20] By linking the antigen-recognition structure to intracellular protein tyrosin kinases, CD79 exerts a key role in signal transduction and cellular activation of B cells.[21] Unique in function, the antigen has been claimed to be completely B-cell specific.[15-17,22,23] Specificity was related particularly to the protein level, because a few myeloid and T-cell lines were found to contain CD79a/CD79b RNA transcripts, but no antigen.[17] To date, the only observation challenging the B-cell specificity of CD79 was a report on positivity in paraffin sections of cases of acute promyelocytic leukemias.[24] However, these data were not corroborated by investigations of the Ig rearrangement status of these leukemias. In another study dealing with acute myeloid

Table 2. Coexpression Patterns of CD79a$^+$TdT$^+$ Precursors

	Mean % (range; n = 9) of CD79a$^+$TdT$^+$ Cells Positive With Marker										
	CD19	CD34*	CD10	CD10^{lo}	CD10^{hi}	CD33	CD7	MPO*	cytoCD3*	CD14*	Control
Total	78 (61-92)	93 (88-97)	96 (93-99)	26 (17-33)	73 (67-83)	5 (1-8)	6 (2-10)	3 (2-6)	3 (1-6)	3 (1-8)	2 (1-4)
CD19$^+$		ND	ND	14 (7-17)	87 (83-93)	3 (1-4)	4 (2-6)	3 (2-5)	3 (1-4)	2 (1-6)	2 (1-3)
CD19$^-$		ND	ND	61 (30-79)	39 (21-70)	18 (2-29)	17 (5-37)	6 (3-12)	5 (2-14)	5 (2-13)	4 (1-8)

Values were derived from gating experiments as described in Materials and Methods.

Abbreviations: n, number of investigated normal samples; ND, not determined.

*n = 5 investigated samples.

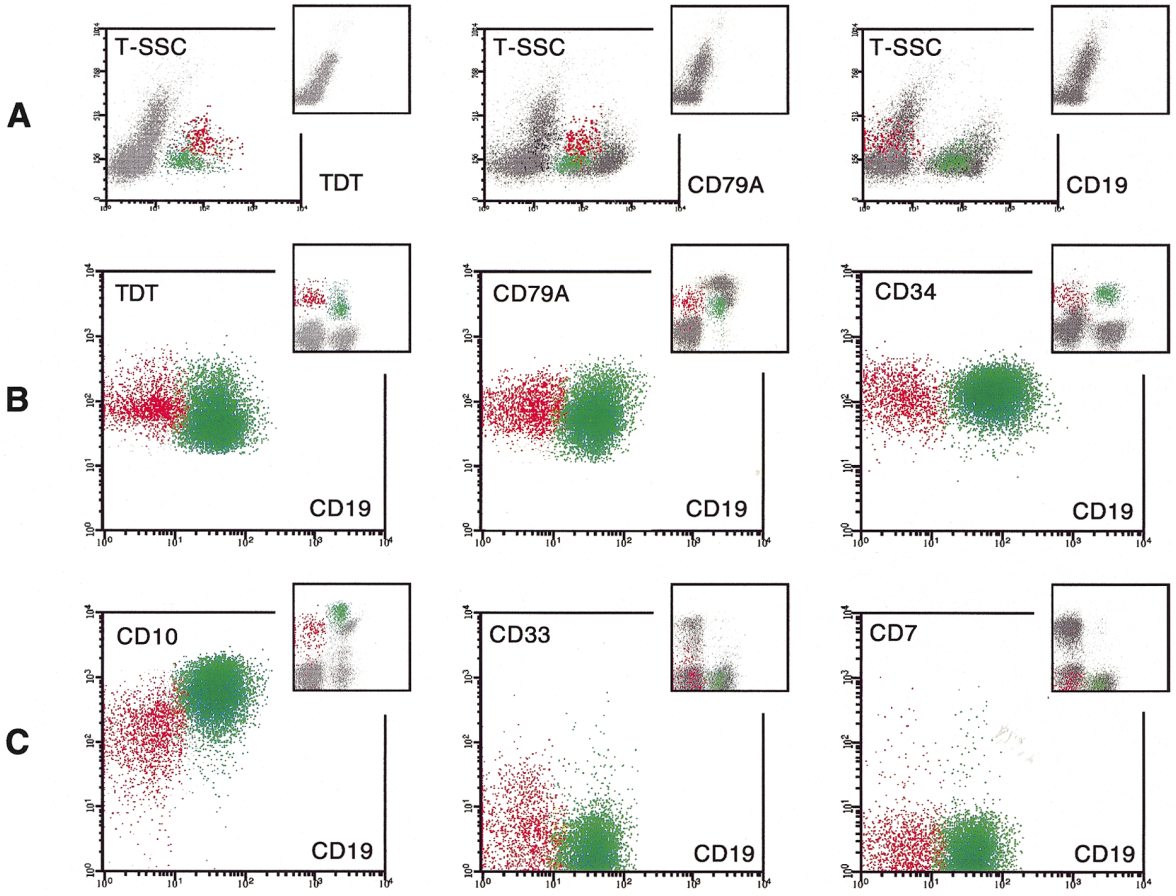

Fig 1.

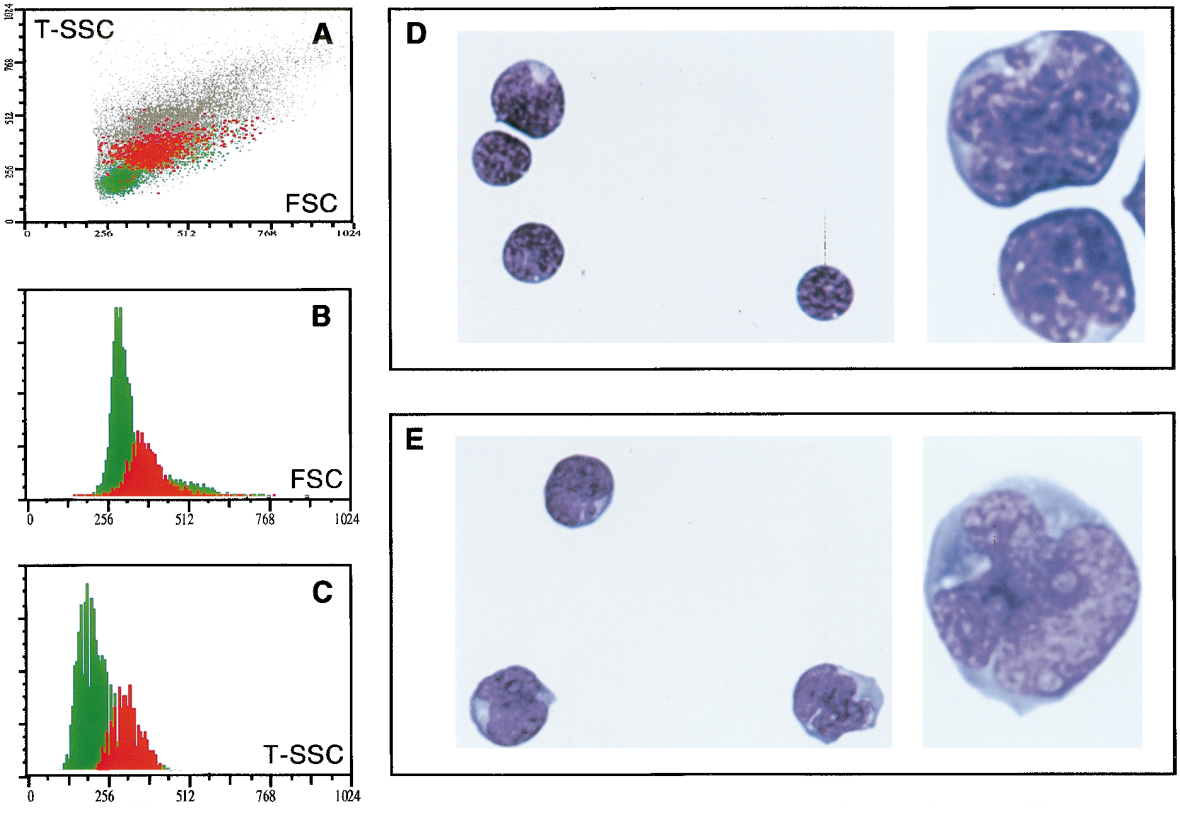

Fig 2.

leukemias, 5 of 77 cases were found to be $CD79^+$, all of which showed Ig rearrangements and were therefore considered biphenotypic.[16] CD79 showed the highest correlation with molecular data and, in this regard, was found to be superior to other B-cell antigens such as CD19 or CD22. Along with the latter finding, the CD79 heterodimer chains seem therefore the most specific markers of B-lineage commitment currently known. Expression of both the CD79a and CD79b genes starts early in B-cell development, suggestively even before Ig gene rearrangement, as indicated by studies in the mouse and by experiments with three human cell lines with germline Ig genes.[17,23,25] In the mouse, both the CD79a and CD79b genes have been shown to be expressed on the RNA level before upregulation of CD19 in the earliest BM B-lineage cells.[26] However, such data on human BCPs are lacking. Only one recent study dealt with the CD79 expression in normal human BM.[15] It showed that the antigen is expressed, before cytoplasmic µ-chains, already on TdT^+ BCPs, but concluded that CD79 may not be expressed as early as CD19, although evidence for the latter issue was weak.

In the present study, we focused on the analysis of CD79a, which has been considered to be expressed earlier in human BCPs than CD79b.[27] We found that many TdT^+ precursors in the BM that lack CD19 express CD79a, which strongly indicates B-lineage commitment independently of CD19. Conspicuously, it may also indicate that CD79a precedes CD19 in human B-cell ontogeny. Alternatively, the existence of $TdT^+CD79a^+CD10^+$ progenitors that are $CD19^+$ or $CD19^-$ might reflect parallel pathways of differentiation, as postulated for B lymphopoiesis.[28] However, due to (1) phenotypic patterns indicative of a phenotypic drift ($CD19^-CD10^{lo}CD34^+ > CD19^+CD10^{lo}CD34^+ > CD19^+CD10^{hi}CD34^+$) in early BCPs,[13,14] (2) characteristic patterns of CD99 expression in these populations,[13] (3) a sequential evolution of $CD19^+$ B cells from $CD19^-CD10^+$ progenitors,[10,11] and (4) other (molecular) signs heralding definitive B-lineage commitment before CD19 expression,[18] we find the first interpretation more likely. By examining the molecular status of the Ig genes in these immature B-cell stages, as phenotypically dissected on the basis of CD79a and CD19 expression, it should now be possible to order the sequence of molecular differentiation events of early B cells upstream to what has recently been published.[18,29] This may also formally prove that CD79a precedes CD19 in B-cell ontogeny.

It is noteworthy that we document also that CD10 is not homogeneously expressed on TdT^+ precursors. Given the proposed new concept of phenotypic maturation steps in early BCPs, our data may indicate that CD10 is expressed first at low levels in the suggestively most immature BCPs ($TdT^+CD79a^+CD19^-$), followed by gradual upregulation to high levels in more differentiated TdT^+CD19^+ BCPs. In the classical studies on early B-cell differentiation, CD10 was found to be expressed first at high levels, followed by downregulation along with loss of expression of TdT and CD34.[3,4] However, these studies only considered the numerically more prominent (and suggestively more mature) $CD10^{hi}CD19^+TdT^+$ BCPs, whereas the $CD10^{lo}TdT^+$ putative BCPs, which are characterized by CD79a expression rather than by CD19-positivity, have elapsed description not at least due to their paucity. Hence, our data do not principally reverse the established patterns of CD10 expression in early B-cell development, but rather add a detail upfront. With respect to this proposed sequence of phenotypic maturation steps, we found also that most $CD19^+CD79a^+TdT^+$ cells expressed CD79b, whereas the latter antigen was positive in only a proportion of the $CD79a^+TdT^+$ BCPs that lacked CD19. Essentially all $CD79b^+TdT^+$ cells were $CD79a^+$. Preliminarily (because derived from only 3 experiments), these data indicate that, in human B-cell ontogeny, CD79b is expressed later than CD79a, but before CD19. This finding contrasts suggestively with the situation in the mouse, where CD79b expression, at least on the RNA level, seems to precede that of CD79a and CD19.[26]

Surprisingly, we found also that the myeloid and T-cell markers CD33 and CD7 were expressed at low intensity on a proportion of the $CD19^-CD79a^+TdT^+$ putative BCPs, which could be regarded as a challenge for the unambiguous lineage-

Fig 1. Four-color flow cytometric analysis of TdT^+ precursors with respect to expression of CD79a and CD19. BM MNCs of a healthy girl (2 years and 7 months old) were prepared from a specimen collected at BM donation for her diseased sister. Cells were stained with CD19, CD79a, and TdT, together with either CD10, CD34, CD33, or CD7. Isotype controls were also included in the experiment. First, 30,000 total events of each individual sample were acquired. Next, live gates were set in parameter correlations engaging T-SSC, TdT, and CD79a, and approximately 10,000 TdT^+CD79a^+ events were acquired from the same material. Dot plots of relevant parameter correlations (logarithmic scale, except for T-SSC: linear scale) show aspects of total MNCs in row A (large plots). The small inserted plots in A visualize the respective isotype controls for each antigen. The large dot plots of rows B and C show gated events, whereas the small inserted plots display the same marker correlations in ungated mode. Only TdT^+ precursors are shown in colors: the subset characterized by coexpression of CD79a and CD19 is depicted in green, whereas $CD79a^+$ precursors lacking CD19 are painted red. The few TdT^+ cells that lack both CD79a and CD19 are painted black (see A). All other TdT^- cells appear grey. Note that $CD19^-CD79a^+TdT^+$ cells (red) are $CD34^+$, display slightly elevated TdT expression as well as T-SSC properties, and express CD10 at a lower level compared with $CD19^+$ precursors (green). Weak CD33 positivity can also be seen on some cells of the former subset, whereas CD7 expression is very rare in either subset of this sample.

Fig 2. Light scattering properties and morphologic appearence of $CD79^+TdT^+$ precursors that express or lack CD19. A correlation of the FSC and T-SSC properties of a BM sample stained with CD19, CD79a, and TdT is shown in dot plot A. TdT^+ precursor cells that express CD79a are depicted in colors: the $CD19^+$ subpopulation of these cells is painted green, whereas $CD19^-CD79a^+TdT^+$ cells appear red. Histograms of the same material, correlating the FSC (B) or the T-SSC (C) in the x-axis with a relative measure of the cell number (y-axis) show only gated TdT^+CD79a^+ cells. Note that $CD19^-CD79a^+TdT^+$ cells (red) exhibit comparatively higher FSC and SSC properties than the majority of $CD19^+$ precursors (green). To study the morphology of these subsets, the same sample was stained with CD10, CD19, and CD34, and the $CD10^+CD34^+$ cells were sorted into a $CD19^+$ and a $CD19^-$ fraction. CD10 and CD34 were used as surrogate antigens substituting for CD79a and TdT to avoid cellular alterations by the fixation/permeabilization reagents (the correspondance of these markers was proven in parallel experiments). Pappenheim-stained cytospin preparations of sorted cells are shown in (D; $CD19^+$ subset) and (E; $CD19^-$ subset). Note that the majority of $CD19^+$ precursors are small lymphoid cells, whereas $CD19^-$ precursors are intermediately sized blasts with large nuclei and prominent nucleoli and display a wider and basophilic cytoplasm with focal juxtanuclear areas of clearing.

assignment of this subset. However, cross-lineage expression, especially of myeloid markers, is a well-recognized phenomenon in malignant and normal B lymphopoiesis. CD13 and CD33 are frequently expressed on BCP-ALLs.[30,31] Normal immature $CD19^+$ BCPs as well as $CD34^+/TdT^+$ lymphoid progenitors in the BM have also been reported to express these myeloid antigens.[7,28,32-35] Likewise, CD7 and other early T-cell antigens lack lineage fidelity, because they may also be expressed on myeloid as well as on B-lineage precursors,[28,36] although expression of CD7 on $CD19^-CD34^+/TdT^+$ BM progenitors has primarily been interpreted as evidence for T-lineage affiliation.[8,37-39] Interestingly, cloning experiments proved a T-cell differentiation capacity in the $CD34^+$ progenitor compartment of adult BM[40,41] and targeted this potential to progenitors that expressed as well as that lacked T-cell markers[40,42] and to $CD10^+Lin^-$ progenitors.[11,43] However, there are no absolute numbers in the literature that relate this T-cell differentiation capacity to the pool of $CD34^+/TdT^+$ progenitors devoid of CD19 expression, and CD7 was recently discouraged as a reliable criterion for an unambiguous delineation of a genuine T-precursor subset in BM.[36,39] Hence, positivity of CD33 and CD7 on some of the putative very immature BCPs, which are characterized by lack of CD19 and by expression of the highly lineage-specific antigen CD79a, may not per se preclude B-lineage affiliation. Alternatively, it may just indicate cellular activation, as has recently been considered for both antigens, CD33 and CD7.[36,44] Lack of expression of the more specific lineage markers of myeloid and T cells, MPO, CD14, and cytoplasmic CD3, in the $CD19^-CD79a^+TdT^+$ putative BCPs corroborates this interpretation.

In summary, we believe that a subcharacterization of TdT^+ progenitors with the CD79a marker should allow to order the earliest differentiation events that herald B-lineage commitment. It may also permit a more specific dissection of B-lymphoid differentiation capacity in the BM from that of the T-lineage and from multilineage potential.

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