British Journal of Haematology, 2000, **110**, 139–146

Regenerating normal B-cell precursors during and after treatment of acute lymphoblastic leukaemia: implications for monitoring of minimal residual disease

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Received 29 October 1999; accepted for publication 1 March 2000

Summary. We studied 57 childhood acute lymphoblastic leukaemia (ALL) patients who remained in continuous complete remission after treatment according to the Dutch Childhood Leukaemia Study Group ALL-8 protocols. The patients were monitored at 18 time points during and after treatment [640 bone marrow (BM) and 600 blood samples] by use of cytomorphology and immunophenotyping for the expression of TdT, CD34, CD10 and CD19. Additionally, 60 BM follow-up samples from six patients were subjected to clonality assessment via heteroduplex polymerase chain reaction (PCR) analysis of immunoglobulin VH-JH gene rearrangements. We observed substantial expansions of normal precursor B cells in regenerating BM not only after maintenance therapy but also during treatment. At the end of the 2-week intervals after consolidation and reinduction treatment, B-cell-lineage regeneration was observed in BM with a large fraction of immature $CD34^+/TdT^+$ B cells. In contrast, in regenerating BM after cessation of maintenance treatment, the more mature $CD19^+/CD10^+$ B cells were significantly increased, but the fraction of immature $CD34^+/TdT^+$ B cells was essentially smaller. Blood samples showed a profound B-cell lymphopenia during treatment followed by a rapid normalization of blood B cells after treatment, with a substantial $CD10^+$ fraction (10–30%). Heteroduplex PCR analysis confirmed the polyclonal origin of the expanded precursor B cells in regenerating BM. This information regarding the regeneration of BM is essential for the correct interpretation of minimal residual disease studies.

Keywords: ALL, regenerating bone marrow, B-cell precursors, immunophenotyping, minimal residual disease.

The lymphoid compartment of bone marrow (BM) in healthy individuals contains a small fraction of early B-lineage cells with immature lymphoblastic morphology or, more frequently, with more mature cytomorphological lymphocytic characteristics (Muehleck *et al*, 1983; Longacre *et al*, 1989). These cells exhibit a precursor B-cell immunophenotype with expression of TdT, CD10 and CD34 in addition to B-lineage markers such as CD19, CD22 and/or CD79a (Janossy *et al*, 1979; Greaves *et al*, 1983; Loken *et al*, 1987; Hurwitz *et al*, 1992; Dworzak *et al*, 1998a). The proportion of such cells in children can be quite marked, ranging from 5% to 15% of mononuclear cells (MNCs) in BM aspirates, and the number of early B-cell precursors progressively decreased with age in children (Caldwell *et al*, 1991; Rego *et al*, 1998; Lucio *et al*, 1999).

Apparently, 80–85% of children with acute lymphoblastic leukaemia (ALL) have an early B-cell phenotype. These precursor B-ALL are generally regarded as malignant counterparts of immature precursor B cells in BM. The standard procedure for evaluating the results of treatment in ALL is cytomorphological analysis of BM and peripheral blood (PB) smears. When less than 5% of blasts are present upon this investigation, a complete remission of disease is assumed. But by cytomorphology alone, it is virtually impossible to distinguish between normal haematopoietic precursors or residual leukaemic cells (Van Dongen *et al*, 1996; Coustan-Smith *et al*, 1998). Distinction between normal and malignant cells is particularly important for

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regenerating BM in precursor B-ALL cases because the levels of committed B-cell progenitors (TdT^+ or TdT^-) were anecdotally reported to be as high as 50% of MNCs in BM samples after treatment (Borella *et al*, 1972; Smedmyr *et al*, 1991; Fukushima *et al*, 1998).

Most reports on regenerating BM after chemotherapy in ALL are based on small patient groups and concern single time points (Kluin-Nelemans *et al*, 1986; Van den Doel *et al*, 1988; Smedmyr *et al*, 1991; Duval *et al*, 1997; Fukushima *et al*, 1998). To assess the normal frequencies of precursor B-cells in PB and BM during and after treatment and the potential interference of these cells with the detection of minimal residual disease (MRD), a study by morphological and immunological methods was started in 1991. We report the follow-up results of 57 precursor B-ALL patients in continuous complete remission (CCR) with a median follow-up time of 5 years. Significantly increased frequencies of morphologically suspicious cells with precursor B-cell immunophenotype were not only found after treatment but also during the initial treatment phases at the end of 2-week intervals in therapy. We applied heteroduplex polymerase chain reaction (PCR) analysis of immunoglobulin heavy chain (*IGH*) gene rearrangements in order to exclude the presence of a clonal malignant precursor B-cell population at the time of BM regeneration.

MATERIALS AND METHODS

Patients. The study group consisted of 57 ALL patients aged 0–16 years (0–5 years $n = 42$; 6–16 years $n = 15$). They were treated according to the Dutch Childhood Leukaemia Study Group (DCLSG) ALL-8 protocol (intake period of the 57 patients was 1991–95), which had a BFM (Berlin–Frankfurt–Münster) backbone. The patients were stratified into three branches: standard risk group (SRG), medium risk group (MRG) and high-risk group (HRG), mainly according to the leukaemic cell mass (risk factor; RF) and the treatment response (Reiter *et al*, 1994). Only SRG and MRG patients with precursor B-ALL were considered for this study. SRG: $< 1{\cdot}0 \times 10^9$/l PB blasts on day 8, RF $< 0{\cdot}8$, no central nervous system (CNS) disease, no mediastinal mass, no T-ALL. MRG: $< 1{\cdot}0 \times 10^9$/l PB blasts on day 8, RF $\geq 0{\cdot}8$, or RF $< 0{\cdot}8$ and CNS disease and/or presence of a mediastinal mass and/or T-ALL.

The first part of the induction protocol was identical for all patients. For SRG patients, the induction protocol was directly followed by the consolidation protocol. MRG patients received a 4-week longer induction treatment and continued with the consolidation protocol, after a 2-week break in therapy. After the consolidation protocol, all patients (SRG and MRG) had a 2-week interval before starting the reinduction treatment and another 2-week interval before entering maintenance therapy. Therapy ended 2 years after diagnosis. All 57 children subjected to the study were in continuous complete remission with a median follow-up time of 5 years. The median observation time by use of cytomorphology and immunophenotyping was 179 weeks for the SRG patients ($n = 23$) and 173 weeks for the MRG ($n = 34$) patients.

Cell samples. BM and PB samples of Dutch children with ALL were sent to the central laboratory of the DCLSG for confirmation and classification of diagnosis. Samples were sent by express mail and processed within 24 h of collection. If the diagnosis of ALL was confirmed and sufficient cells were available, the patients were followed at 18 well-defined time points (T1 to T18; see Fig 1) during and after treatment by use of cytomorphology and immunophenotyping. A total of 640 BM samples (243 SRG and 397 MRG) and 600 PB samples (227 SRG and 373 MRG) from the 57 ALL patients were analysed by these techniques. Relapsed patients were excluded from the current study. All procedures were performed according to the local guidelines of the Medical Ethics Committees and samples were obtained after informed consent.

Cytomorphology. BM and PB smears at diagnosis and during follow-up were stained by standard techniques, including May–Grünwald–Giemsa (all time points) and periodic acid–Schiff reagents, Sudan black B, myeloperoxidase, a combined naphthol AS-D chloroacetate esterase and α-naphthylacetate esterase (only at diagnosis).

Immunophenotyping. MNCs were isolated from BM and PB by Ficoll–Paque centrifugation (density 1·077 g/cm^3; Pharmacia, Uppsala, Sweden) and washed three times. Washing of cells was performed with phosphate-buffered saline supplemented with 1% bovine serum albumin (PBS/BSA 1%, pH 7·8). Erythrocytes were lysed if necessary by 0·83% ammonium chloride. The viability was assessed by trypan blue dye exclusion and was $> 85\%$ in all samples studied. The following monoclonal antibodies (mAbs) were used: CD10 (J5) and CD19 (B4) from Beckman Coulter Corporation (Fullerton, CA, USA); CD34 from Becton Dickinson (San Jose, CA, USA); and rabbit anti-TdT antiserum from Supertechs (Bethesda, MD, USA).

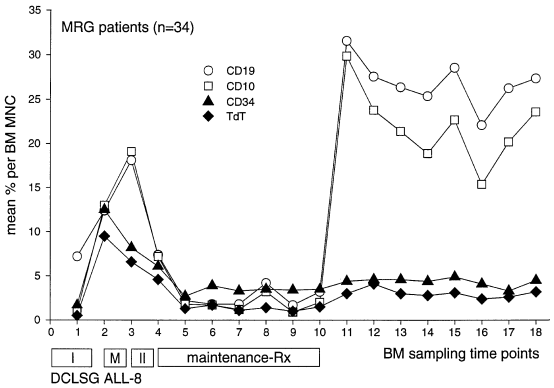

Fig 1. Median percentages of TdT^+, $CD10^+$, $CD19^+$ and $CD34^+$ cells during chemotherapy (sampling points 1–10) and after treatment cessation (sampling points 11–18) for childhood ALL patients treated according to the MRG schedule of the DCLSG ALL-8 protocol. After a relatively short pause of 2 weeks during initial treatment (points 2 and 3), an increase of both immature and more mature B-cell precursor subsets is observed. After completion of treatment, a major expansion of more mature $CD10^+/CD19^+$ cells is observed, whereas percentages of immature $CD34^+$ and TdT^+ cells show a minor increase (2–6%). I, induction; M, consolidation; II, reinduction; maintenance-Rx, maintenance treatment.

For flow cytometric immunophenotyping, the concentration of MNC suspension was adjusted to 10^7/ml. For single labelling with CD10 and CD19, a two-step procedure was used. Therefore, 50 μl of MNC suspension was incubated (4°C, 30 min) with 50 μl of the relevant mAb, washed and subsequently incubated with 50 μl of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Ig) antiserum (4°C, 30 min) and washed again. CD34 was used as a phycoerythrin (PE)-conjugated mAb in a one-step procedure. After labelling, a total of 15 000 cells were acquired and analysed on a FACScan flow cytometer using LYSYS II software (Becton Dickinson). Gating was set on forward- (FCS) and side-scatter (SSC) parameters to exclude cellular debris. The frequency of the cells expressing the relevant marker was calculated as a percentage of MNCs analysed.

Cytocentrifuge preparations were made with a cell concentration of 10^6/ml (50 μl per preparation), fixed in methanol (4°C, 30 min), washed, incubated with 15 μl rabbit anti-TdT antiserum in a moist chamber (room temperature, 30 min), washed, subsequently incubated with 15 μl FITC-conjugated goat anti-rabbit Ig antiserum and washed again. For double TdT and CD10 detection, MNCs in suspension were labelled with CD10 and a tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse Ig antiserum. The labelled cells were spun onto slides, fixed and subjected to TdT staining. All preparations were mounted in glycerol with 1 mg *p*-phenylenediamine/ml and analysed on a Zeiss fluorescence microscope equipped with phase contrast facilities (Zeiss, Oberkochen, Germany) (Van Dongen *et al*, 1987). At least 200 TdT-positive cells were analysed for expression of the relevant markers. If less than 200 TdT-positive cells were present, the whole slide was screened.

Southern blot analysis. DNA samples from precursor B-ALL patients were subjected to molecular analysis of the *IGH* gene. DNA was isolated from fresh or frozen MNC fractions as described previously (Van Dongen & Wolvers-Tettero, 1991). Fifteen micrograms of DNA was digested with *Bgl*II or *Bam*HI–*Hin*dIII restriction enzymes (Pharmacia), size separated in 0·7% agarose gels and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) and *IGH* gene configuration was analysed by hybridization of the filters with the IGHJ6 probe (Dako Corporation, Carpinteria, CA, USA) (Van Dongen & Wolvers-Tettero, 1991; Beishuizen *et al*, 1993).

PCR amplification. PCR amplification of complete *IGH* gene rearrangements was essentially performed as described previously (Pongers-Willemse *et al*, 1999; Szczepański *et al*, 1999a). For identification of clonal VH-JH rearrangements at diagnosis, 0·1 μg DNA, 12·5 pmol of the 5′ and 3′ primers and 1 U *Taq* polymerase (PE Biosystems, Foster City, CA, USA) were used in a 100-μl PCR reaction. For analysis of follow-up samples, 1 μg DNA, 30 pmol of the 5′ and 3′ oligonucleotide primers and 2·5 U *TaqGold* polymerase (PE Biosystems) were used in a 100-μl PCR reaction. DNA dilutions of diagnostic BM in polyclonal MNCs were amplified in parallel to determine the sensitivity of the assay. All primers were synthesized on an ABI 392 DNA synthesiser (PE Biosystems) using the solid phase phosphotriester method. PCR conditions after an initial 10-min step of denaturation and *TaqGold* polymerase activation comprised: 45 s at 92°C, 90 s at 60°C and 2 min at 72°C for 40 cycles using a Perkin-Elmer thermal cycler (PE Biosystems). After the last cycle, an additional extension step of 10 min at 72°C was performed. Appropriate positive and negative controls were included.

Heteroduplex analysis of PCR products. For heteroduplex analysis, the PCR products were denatured at 94°C for 5 min after the final cycle of amplification and subsequently cooled to 4°C for 60 min to induce duplex formation (Langerak *et al*, 1997; Szczepański *et al*, 1998). After duplex formation, the hetero- and/or homoduplexes were immediately loaded on 6% non-denaturing polyacrylamide gels in 0·5× Tris/boric acid/EDTA (TBE) buffer, run at room temperature and visualized by ethidium bromide staining. A 100-bp DNA ladder (Promega Corporation, Madison, WI, USA) was used as size marker.

RESULTS

BM B-cell precursors during ALL treatment

Until day 33 of the induction treatment, all patients received the same therapy. At this time (T1) the induction response of all patients was evaluated. SRG and MRG patients were found to have similarly low frequencies (0·3–2%) of cells positive for TdT, CD34, CD10 and somewhat higher percentages of $CD19^+$ cells (~ 7%) (Table I and Fig 1).

After this time point, SRG patients immediately entered the consolidation treatment and were evaluated at T3, just before the reinduction treatment, and again at T4, just before starting maintenance treatment. Both samples were taken after a 2-week break in the treatment. At T3, a rise in cells positive for TdT (mean 3·9 ± 2·6%), CD34 (mean 4·6 ± 4%), CD10 (mean 8·0 ± 7·2%) and CD19 (mean 9·6 ± 7·4%) was observed compared with T1. At T4, these percentages were still higher than at the end of the induction phase (T1).

In contrast to SRG patients, MRG patients had a 2-week interval in treatment after longer induction therapy. The results of this phase were evaluated at T2. Compared with SRG patients at T3 and T4, an even higher rise in cells positive for TdT (mean 9·5 ± 6·9%), CD34 (mean 12·5 ± 7·2%), CD10 (mean 13·0 ± 9·4%) and CD19 (mean 12·4 ± 9·2%) was found at T2 in MRG patients (Table I).

During maintenance treatment (T5 to T10), the frequencies of cells positive for CD19, CD10, TdT and CD34 were similarly low (1–5%) in the SRG and MRG patients.

BM B-cell precursors after treatment cessation

The first post-treatment BM sample evaluation was taken at T11, i.e. about 3 months after stopping chemotherapy. At that time point, the $CD10^+$ and $CD19^+$ cells were found in high percentages, i.e. 29·9 ± 15·6% and 31·6 ± 15·9%, respectively, for SRG patients. Only minor differences were found between SRG and MRG patients (Table I and Fig 1). Although $CD34^+$ (mean 5·7 ± 2·6%) and $CD10^+/TdT^+$

Table I. Percentages of MNCs expressing TdT, CD10, CD34 and CD19 in BM during and after chemotherapy for children with ALL in the MRG ($n = 34$).

Sampling time point*	Average time from diagnosis (weeks)	Number of patients analysed	Morphological blasts (mean ± SD)	% of $CD34^+$ (mean ± SD)	% of TdT^+ (mean ± SD)	% of $CD10^+$ (mean ± SD)	% of $TdT^+/CD10^+$ (mean ± SD)	% of $CD19^+$ (mean ± SD)
T1	5	30	0·7 ± 0·7	1·7 ± 2·1	0·5 ± 1·0	0·9 ± 3·0	0·1 ± 0·3	7·2 ± 5·5
T2	14	27	2·1 ± 1·4	12·5 ± 7·2	9·5 ± 6·9	13·0 ± 9·4	8·0 ± 6·1	12·4 ± 9·2
T3	24	31	2·0 ± 1·4	8·2 ± 6·0	6·6 ± 4·4	19·1 ± 12·7	5·6 ± 4·4	18·1 ± 12·1
T4	34	32	1·8 ± 1·4	6·1 ± 4·6	4·6 ± 4·2	7·2 ± 9·1	3·4 ± 3·7	7·4 ± 9·3
T5	46	22	0·6 ± 0·5	2·7 ± 2·1	1·3 ± 1·2	1·8 ± 5·9	0·6 ± 1·1	2·2 ± 5·7
T6	59	26	0·7 ± 0·7	3·9 ± 2·4	1·7 ± 1·4	1·7 ± 4·0	0·6 ± 0·8	1·8 ± 2·0
T7	72	27	0·8 ± 0·8	3·3 ± 2·3	1·1 ± 1·0	1·2 ± 1·9	0·7 ± 1·7	1·8 ± 1·9
T8	84	27	0·9 ± 0·7	3·5 ± 2·2	1·4 ± 1·3	3·2 ± 8·4	0·7 ± 1·0	4·2 ± 7·5
T9	94	11	0·7 ± 0·6	3·4 ± 2·5	1·0 ± 0·8	0·9 ± 1·2	0·2 ± 0·3	1·7 ± 1·2
T10	102	26	1·0 ± 0·7	3·5 ± 5·7	1·5 ± 1·3	2·0 ± 8·9	0·6 ± 0·9	3·2 ± 7·7
T11	116	27	1·3 ± 0·7	4·4 ± 3·0	3·0 ± 2·2	29·9 ± 15·6	3·0 ± 1·7	31·6 ± 15·9
T12	130	21	1·0 ± 0·7	4·6 ± 1·9	4·1 ± 3·6	23·8 ± 11·3	3·4 ± 3·1	27·6 ± 10·4
T13	143	22	1·3 ± 0·6	4·6 ± 2·0	3·0 ± 1·7	21·4 ± 13·5	2·8 ± 1·6	26·4 ± 13·8
T14	157	24	1·1 ± 0·6	4·4 ± 1·4	2·8 ± 2·1	18·9 ± 9·5	2·5 ± 1·9	25·4 ± 11·2
T15	168	17	1·1 ± 0·7	4·9 ± 2·1	3·1 ± 1·2	22·7 ± 12·5	2·9 ± 1·2	28·6 ± 10·5
T16	181	15	0·9 ± 0·6	4·1 ± 2·3	2·4 ± 1·6	15·4 ± 9·0	2·3 ± 1·6	22·1 ± 8·6
T17	195	6	1·3 ± 1·3	3·3 ± 2·1	2·6 ± 1·4	20·2 ± 11·1	2·3 ± 1·2	26·3 ± 11·5
T18	207	6	1·3 ± 0·5	4·5 ± 1·4	3·2 ± 1·6	23·6 ± 12·8	3·1 ± 1·5	27·4 ± 13·0

*See Fig 1 for relation to treatment phase.

Table II. Percentages of MNCs expressing TdT, CD10 and CD19 in PB for children with ALL in the MRG ($n = 34$).

Sampling time point*	Average time from diagnosis (weeks)	Number of patients analysed	% of TdT^+ (mean ± SD)	% of $CD10^+$ (mean ± SD)	% of $CD19^+$ (mean ± SD)
T1	5	28	0·3 ± 0·9	0 ± 0·2	8·6 ± 5·5
T2	14	25	0·3 ± 0·4	0·2 ± 0·5	1·2 ± 1·4
T3	24	30	0·1 ± 0·2	0·3 ± 0·6	3·2 ± 2·9
T4	34	31	0·1 ± 0·2	0·2 ± 0·4	0·8 ± 0·9
T5	46	22	0·1 ± 0·1	0·1 ± 0·3	1·3 ± 1·9
T6	59	23	0·1 ± 0·2	0·1 ± 0·4	2·4 ± 3·4
T7	72	25	0·1 ± 0·3	0·3 ± 0·8	1·8 ± 1·9
T8	84	27	0 ± 0·1	1·6 ± 4·9	3·7 ± 5·1
T9	94	10	0 ± 0·1	0·2 ± 0·4	1·6 ± 1·6
T10	102	23	0·1 ± 0·5	0·3 ± 0·7	3·0 ± 1·8
T11	116	27	0·1 ± 0·3	4·8 ± 4·4	15·9 ± 7·4
T12	130	20	0·2 ± 0·7	4·7 ± 7·1	21·1 ± 6·8
T13	143	19	0·1 ± 0·1	5·1 ± 7·8	21·3 ± 8·4
T14	157	23	0 ± 0·1	3·8 ± 4·5	21·1 ± 8·6
T15	168	15	0·1 ± 0·3	3·2 ± 3·8	21·5 ± 15·3
T16	181	14	0 ± 0·1	3·5 ± 4·6	18·3 ± 5·5
T17	195	6	0	2·2 ± 2·6	23·5 ± 14·5
T18	207	5	0·3 ± 0·4	1·9 ± 2·1	21·8 ± 5·8

*See Fig 1 for relation to treatment phase.

cells (mean 3·8 ± 2·1%) also slightly increased after treatment cessation, these frequencies were now significantly lower than the frequencies of $CD19^+$ or $CD10^+$ cells (Table I). The high values for $CD19^+$ and $CD10^+$ precursor B cells were consistently found at all later time points, at least until 2 years after stopping the therapy.

PB B-cell precursors during ALL treatment and after treatment cessation

The mean frequencies of PB $CD19^+$ cells were persistently low throughout consolidation, reinduction and maintenance treatment phases, ranging from 1% to 5% of MNCs. Moreover, the most immature $TdT^+/CD10^+$ B-cell precursors were absent or very low (< 0·1%) in PB throughout the different treatment phases as well as after treatment cessation (Table II). After chemotherapy cessation, the percentages of PB B cells rapidly increased to normal values; at T11, $CD19^+$ cells already comprised 15·9 ± 7·4% in MRG patients. Interestingly, a substantial proportion (10–30%) of these $CD19^+$ cells (2–5% of PB MNCs) was co-expressing the CD10 molecule. The differences between SRG and MRG patients were not significant.

Southern blotting and heteroduplex PCR analysis

Southern blotting of diagnosis DNA from six patients (three SRG and three MRG) revealed clonal *IGH* gene rearrangements in all cases. Heteroduplex PCR analysis showed a single monoclonal VH-JH product in five patients and two homo- and heteroduplexes in one patient, indicating biallelic *IGH* gene rearrangements (Langerak *et al*, 1997; Szczepański *et al*, 1998). The sensitivity of heteroduplex PCR analysis for these clonal markers ranged from 1% to 5%. A total of 60 BM follow-up samples were analysed, 10 per patient on average. None of the follow-up samples contained monoclonal PCR-detectable complete *IGH* gene rearrangements identical to those found at diagnosis. The intensity of heteroduplex smears derived from polyclonal B cells correlated with the percentages of B-lineage cells in the sample (Fig 2).

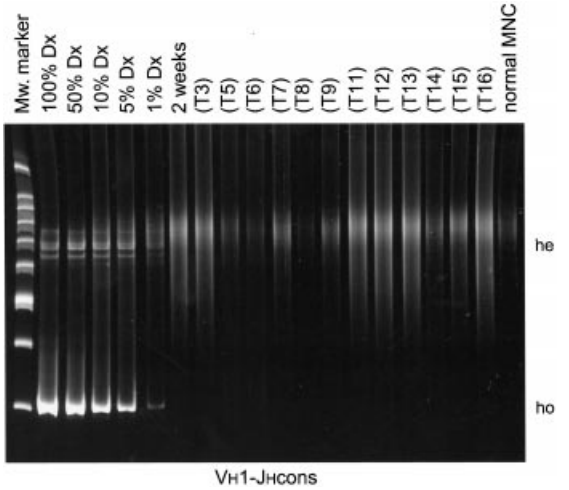

Fig 2. Malignant lymphoblasts at diagnosis contained a monoclonal VH1-JH gene rearrangement, as demonstrated by heteroduplex PCR analysis. Dilution of the diagnosis BM DNA into polyclonal MNC DNA derived from healthy blood donors showed a sensitivity threshold of approximately 1%. None of the follow-up samples contained clonal homoduplexes. The density of heteroduplex smears is related to the percentages of polyclonal $CD19^+$ cells in the analysed samples. he, heteroduplexes; ho, homoduplexes; Mw marker, 100-bp molecular weight marker.

DISCUSSION

The BM MNC compartment of healthy individuals contains a subset of precursor B cells characterized by the expression of markers such as CD19, CD10, TdT and CD34 (Janossy *et al*, 1979; Greaves *et al*, 1983; Loken *et al*, 1987). Such cells constitute 5–15% of MNCs in BM aspirates from healthy children. Increases in the proportion of normal B-cell precursors have been reported in children with benign diseases (Longacre *et al*, 1989; Fukushima *et al*, 1998). Even the immature $CD10^+/TdT^+$ cells can occur in unusually high frequencies and may raise suspicion for malignancy, particularly in BM regenerating after chemotherapy (Smedmyr *et al*, 1991; Duval *et al*, 1997; Dworzak *et al*, 1997; Fukushima *et al*, 1998).

Our study of 640 BM samples and 600 PB samples from a homogeneous large group of 57 precursor B-ALL patients demonstrated that even after a relatively short pause of 2 weeks during treatment regeneration of suppressed B-cell development takes place. After such short breaks in treatment, particularly immature $CD10^+/TdT^+$ and $CD34^+$ cells can be found in BM at frequencies as high as 20% of MNCs. The type and extent of B-cell regeneration seems to be proportional to the aggressiveness of preceding treatment, as was shown by the differences in marker positivity between the two treatment arms. For example, at T3 in SRG patients, the immature TdT^+ cells comprised $3{\cdot}9 \pm 2{\cdot}6\%$ of MNCs, but in MRG patients $6{\cdot}6 \pm 4{\cdot}4\%$ of MNCs. Similar differences appeared to be present for $CD34^+$, $CD19^+$ and $TdT^+/CD10^+$ cells.

After cessation of maintenance chemotherapy, we observed bulky polyclonal recovery of precursor B cells in BM. Three months after treatment withdrawal, the B-cell compartment markedly increased from approximately 5% to approximately 30% of BM MNCs (Table I and Fig 1). The detected frequencies of around 30% (± 16%) of BM MNCs for $CD10^+$ and $CD19^+$ cells were significantly higher than those found in healthy donors (5–15% of BM MNCs) (Caldwell *et al*, 1991; Rego *et al*, 1998). It is not surprising to see such massive B-cell regeneration after chemotherapy for ALL bearing in mind that this type of treatment is accompanied by profound B-cell lymphopenia, which was also clearly demonstrated in this study. This is in contrast to T-cell numbers and T-cell immunophenotype in PB, which remained virtually normal (Caver *et al*, 1998). Moreover, long-term survivors of ALL have been shown to lack protective antibody levels specific for common paediatric bacterial or viral pathogens, despite prior vaccinations (Smith *et al*, 1995). Among regenerating B-cell progenitors in BM after maintenance chemotherapy, we observed an obvious predominance of the more mature $CD19^+/CD10^+/TdT^-/CD34^-$ population, which is also predominant in the normal paediatric BM B-lineage cells (Lucio *et al*, 1999). Furthermore, we and colleagues (Fukushima *et al*, 1998) found that the decline to normal percentages of $CD10^+$ and $CD19^+$ BM cells did not occur within 2 years from stopping the chemotherapy (T18).

We observed a rapid normalization of the blood B-cell counts after treatment cessation. However, a substantial subset of these $CD19^+$ cells (10–30%) was co-expressing CD10, which is markedly higher than normal PB, where $CD19^+/CD10^+$ cells comprise less than 1% of MNCs (Ryan *et al*, 1984; Calado *et al*, 1999). This more mature $CD19^+/CD10^+/TdT^-$ precursor B-cell population apparently reflects the massive regeneration in BM at this time point and may be perceived as the release of this more mature precursor B-cell subset into the blood during the first 2 years after treatment (Table II). Apparently, the interaction between stromal cells and more mature $CD19^+/CD10^+/TdT^-$ precursor B cells in regenerating BM is less stringent than in immature $CD19^+/CD10^+/TdT^+$ B-cell precursors because this subset was hardly found in the PB. This also implies that the finding of an immature $CD19^+/CD10^+/TdT^+$ B-cell subset in PB of ALL patients is very suggestive of ALL cells.

The presence of early B-cell precursors in normal BM as well as their polyclonal expansion in 'regenerating' BM should be taken into account when immunophenotypic MRD studies are performed in precursor B-ALL. On one hand, moderately increased frequencies of $CD19^+/CD10^+/TdT^+$ BM cells should never be directly interpreted as relapse of ALL, particularly during the high-dose treatment phases. On the other hand, substantial increase in B-cell precursors after chemotherapy, particularly with a homogenous immunophenotype, may herald a relapse of the disease (Farahat *et al*, 1995; Vervoordeldonk *et al*, 1996; Griesinger *et al*, 1999). Alternatively, malignant cells in precursor B-ALL patients can be distinguished from normal early B-lineage cells based on atypical immunological characteristics. This concerns tumour-associated ectopic expression of antigens such as TdT, CD66c or NG2, cross-lineage antigen expression, maturational asynchronous antigen expression, antigen overexpression or loss of markers expressed in normal B-cell precursors (Van Dongen *et al*, 1996; Dworzak *et al*, 1998b; Ciudad *et al*, 1999; Sugita *et al*, 1999). Such characteristics can be detected with currently available four-colour flow cytometry in at least 85% of the cases (Wells *et al*, 1998; Campana & Coustan-Smith, 1999; Lucio *et al*, 1999; Weir *et al*, 1999).

In problematic cases, molecular studies are warranted to ensure that B-lineage cells really represent regenerating normal BM precursors and are not the first sign of an imminent relapse. For this purpose, we applied heteroduplex PCR analysis, which we routinely use for identification of clonal Ig and TCR gene rearrangements as MRD targets (Langerak *et al*, 1997; Szczepański *et al*, 1998, 1999b). Heteroduplex PCR analysis of *IGH* gene rearrangements in a total of 60 remission BM follow-up samples demonstrated that B-cell precursors in regenerating BM indeed had a polyclonal origin. Furthermore, the high density of polyclonal heteroduplex smears was related to the abundance of B-cell precursors at the same time points (Fig 2). Although heteroduplex PCR analysis has a limited sensitivity (1–5%) and cannot be used for MRD monitoring (Langerak *et al*, 1997), it is sufficiently sensitive to determine clonality in samples containing high frequencies of 'regenerating' B cells. Thus, heteroduplex PCR analysis can be easily applied to confirm the polyclonal origin of the precursor B cells in regenerating BM.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Professor Dr R. Benner for continuous support, and Mr T. M. van Os for preparation of the figures.

The board members of the DCLSG are I. M. Appel, H. van den Berg, J. P. M. Bökkerink, M. C. A. Bruin, J. J. Groot-Loonen, S. S. N. de Graaf, K. Hählen, P. M. Hoogerbrugge, W. A. Kamps, F. A. E. Nabben, A. Postma, J. A. Rammeloo, T. Revesz, A. Y. N. Schouten-van Meeteren, A. J. P. Veerman, M. van Weel-Sipman and R. S. Weening.

This work was supported in part by the Dutch Cancer Foundation/Koningin Wilhelmina Fonds (grant EUR 94-852) and the Ank van Vlissingen Foundation.

REFERENCES

Beishuizen, A., Verhoeven, M.A., Mol, E.J., Breit, T.M., Wolvers-Tettero, I.L.M. & van Dongen, J.J.M. (1993) Detection of immunoglobulin heavy-chain gene rearrangements by Southern blot analysis: recommendations for optimal results. *Leukemia*, **7**, 2045–2053.

Borella, L., Green, A.A. & Webster, R.G. (1972) Immunologic rebound after cessation of long-term chemotherapy in acute leukemia. *Blood*, **40**, 42–51.

Calado, R.T., Garcia, A.B. & Falcão, R.P. (1999) Age-related changes of immunophenotypically immature lymphocytes in normal human peripheral blood. *Cytometry*, **38**, 133–137.

Caldwell, C.W., Poje, E. & Helikson, M.A. (1991) B-cell precursors in normal pediatric bone marrow. *American Journal of Clinical Pathology*, **95**, 816–823.

Campana, D. & Coustan-Smith, E. (1999) Detection of minimal residual disease in acute leukemia: by flow cytometry. *Cytometry*, **38**, 139–152.

Caver, T.E., Slobod, K.S., Flynn, P.M., Behm, F.G., Hudson, M.M., Turner, E.V., Webster, R.G., Boyett, J.M., Tassie, T.L., Pui, C.H. & Hurwitz, J.L. (1998) Profound abnormality of the B/T lymphocyte ratio during chemotherapy for pediatric acute lymphoblastic leukemia. *Leukemia*, **12**, 619–622.

Ciudad, J., San Miguel, J.F., Lopez-Berges, M.C., Garcia Marcos, M.A., Gonzalez, M., Vazquez, L., del Canizo, M.C., Lopez, A., Van Dongen, J.J.M. & Orfao, A. (1999) Detection of abnormalities in B-cell differentiation pattern is a useful tool to predict relapse in precursor-B-ALL. *British Journal of Haematology*, **104**, 695–705.

Coustan-Smith, E., Behm, F.G., Sanchez, J., Boyett, J.M., Hancock, M.L., Raimondi, S.C., Rubnitz, J.E., Rivera, G.K., Sandlund, J.T., Pui, C.H. & Campana, D. (1998) Immunological detection of minimal residual disease in children with acute lymphoblastic leukaemia. *Lancet*, **351**, 550–554.

Duval, M., Fenneteau, O., Cave, H., Gobillot, C., Rohrlich, P., Guidal, C., Lescoeur, B., Legac, S., Schlegel, N., Sterkers, G. & Vilmer, E. (1997) Expansion of polyclonal B-cell precursors in bone marrow from children treated for acute lymphoblastic leukemia. *Hematology and Cell Therapy*, **39**, 139–147.

Dworzak, M.N., Fritsch, G., Fleischer, C., Printz, D., Froschl, G., Buchinger, P., Mann, G. & Gadner, H. (1997) Multiparameter phenotype mapping of normal and post-chemotherapy B lymphopoiesis in pediatric bone marrow. *Leukemia*, **11**, 1266–1273.

Dworzak, M.N., Fritsch, G., Froschl, G., Printz, D. & Gadner, H. (1998a) 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. *Blood*, **92**, 3203–3209.

Dworzak, M.N., Fritsch, G., Fleischer, C., Printz, D., Froschl, G., Buchinger, P., Mann, G. & Gadner, H. (1998b) Comparative phenotype mapping of normal vs. malignant pediatric B-lymphopoiesis unveils leukemia-associated aberrations. *Experimental Hematology*, **26**, 305–313.

Farahat, N., Lens, D., Zomas, A., Morilla, R., Matutes, E. & Catovsky, D. (1995) Quantitative flow cytometry can distinguish between normal and leukaemic B-cell precursors. *British Journal of Haematology*, **91**, 640–646.

Fukushima, T., Sumazaki, R., Koike, K., Tsuchida, M., Matsui, A. & Nakauchi, H. (1998) Multicolor flow-cytometric, morphologic, and clonogenic analysis of marrow CD10-positive cells in children with leukemia in remission or nonmalignant diseases. *Journal of Pediatric Hematology and Oncology*, **20**, 222–228.

Greaves, M.F., Hariri, G., Newman, R.A., Sutherland, D.R., Ritter, M.A. & Ritz, J. (1983) Selective expression of the common acute lymphoblastic leukemia (gp 100) antigen on immature lymphoid cells and their malignant counterparts. *Blood*, **61**, 628–639.

Griesinger, F., Pirò-Noack, M., Kaib, N., Falk, M., Renziehausen, A., Troff, C., Grove, D., Schnittger, S., Büchner, T., Ritter, J., Hiddemann, W. & Wörmann, B. (1999) Leukaemia-associated immunophenotypes (LAIP) are observed on 90% of adult and childhood acute lymphoblastic leukaemia: detection in remission marrow predicts outcome. *British Journal of Haematology*, **105**, 241–255.

Hurwitz, C.A., Gore, S.D., Stone, K.D. & Civin, C.I. (1992) Flow cytometric detection of rare normal human marrow cells with immunophenotypes characteristic of acute lymphoblastic leukemia cells. *Leukemia*, **6**, 233–239.

Janossy, G., Bollum, F.J., Bradstock, K.F., McMichael, A., Rapson, N. & Greaves, M.F. (1979) Terminal transferase-positive human bone marrow cells exhibit the antigenic phenotype of common acute lymphoblastic leukemia. *Journal of Immunology*, **123**, 1525–1529.

Kluin-Nelemans, H.C., Bolhuis, R.L., Lowenberg, B., Campana, D. & Sizoo, W. (1986) Characterization of normal and regenerating bone marrow cells with a panel of monoclonal antibodies. *Scandinavian Journal of Haematology*, **36**, 71–78.

Langerak, A.W., Szczepański, T., van der Burg, M., Wolvers-Tettero, I.L.M. & van Dongen, J.J.M. (1997) Heteroduplex PCR analysis of rearranged T cell receptor genes for clonality assessment in suspect T cell proliferations. *Leukemia*, **11**, 2192–2199.

Loken, M.R., Shah, V.O., Dattilio, K.L. & Civin, C.I. (1987) Flow cytometric analysis of human bone marrow. II. Normal B lymphocyte development. *Blood*, **70**, 1316–1324.

Longacre, T.A., Foucar, K., Crago, S., Chen, I.M., Griffith, B., Dressler, L., McConnell, T.S., Duncan, M. & Gribble, J. (1989) Hematogones: a multiparameter analysis of bone marrow precursor cells. *Blood*, **73**, 543–552.

Lucio, P., Parreira, A., van den Beemd, M.W., van Lochem, E.G., van Wering, E.R., Baars, E., Porwit-MacDonald, A., Bjorklund, E., Gaipa, G., Biondi, A., Orfao, A., Janossy, G., Van Dongen, J.J.M. & San Miguel, J.F. (1999) Flow cytometric analysis of normal B cell differentiation: a frame of reference for the detection of minimal residual disease in precursor-B-ALL. *Leukemia*, **13**, 419–427.

Muehleck, S.D., McKenna, R.W., Gale, P.F. & Brunning, R.D. (1983) Terminal deoxynucleotidyl transferase (TdT)-positive cells in bone marrow in the absence of hematologic malignancy. *American Journal of Clinical Pathology*, **79**, 277–284.

Pongers-Willemse, M.J., Seriu, T., Stolz, F., d'Aniello, E., Gameiro, P., Pisa, P., Gonzalez, M., Bartram, C.R., Panzer-Grumayer, E.R., Biondi, A., San Miguel, J.F. & van Dongen, J.J.M. (1999) Primers

and protocols for standardized MRD detection in ALL using immunoglobulin and T cell receptor gene rearrangements and *TAL1* deletions as PCR targets. Report of the BIOMED-1 Concerted Action: Investigation of minimal residual disease in acute leukemia. *Leukemia*, **13**, 110–118.

Rego, E.M., Garcia, A.B., Viana, S.R. & Falcao, R.P. (1998) Age-related changes of lymphocyte subsets in normal bone marrow biopsies. *Cytometry*, **34**, 22–29.

Reiter, A., Schrappe, M., Ludwig, W.D., Hiddemann, W., Sauter, S., Henze, G., Zimmermann, M., Lampert, F., Havers, W., Niethammer, D., Odenwald, E., Ritter, J., Mann, G., Welte, K., Gadner, H. & Riehm, H. (1994) Chemotherapy in 998 unselected childhood acute lymphoblastic leukemia patients. Results and conclusions of the multicenter trial ALL-BFM 86. *Blood*, **84**, 3122–3133.

Ryan, D.H., Mitchell, S.J., Hennessy, L.A., Bauer, K.D., Horan, P.K. & Cohen, H.J. (1984) Improved detection of rare CALLA-positive cells in peripheral blood using multiparameter flow cytometry. *Journal of Immunological Methods*, **74**, 115–128.

Smedmyr, B., Bengtsson, M., Jakobsson, A., Simonsson, B., Oberg, G. & Totterman, T.H. (1991) Regeneration of CALLA (CD10+), TdT+ and double-positive cells in the bone marrow and blood after autologous bone marrow transplantation. *European Journal of Haematology*, **46**, 146–151.

Smith, S., Schiffman, G., Karayalcin, G. & Bonagura, V. (1995) Immunodeficiency in long-term survivors of acute lymphoblastic leukemia treated with Berlin–Frankfurt–Munster therapy. *Journal of Pediatrics*, **127**, 68–75.

Sugita, K., Mori, T., Yokota, S., Kuroki, M., Koyama, T.O., Inukai, T., Iijima, K., Goi, K., Tezuka, T., Kojika, S., Shiraishi, K., Nakamura, M., Miyamoto, N., Karakida, N., Kagami, K. & Nakazawa, S. (1999) The KOR-SA3544 antigen predominantly expressed on the surface of Philadelphia chromosome-positive acute lymphoblastic leukemia cells is nonspecific cross-reacting antigen-50/90 (CD66c) and invariably expressed in cytoplasm of human leukemia cells. *Leukemia*, **13**, 779–785.

Szczepański, T., Langerak, A.W., Wolvers-Tettero, I.L.M., Ossenkoppele, G.J., Verhoef, G., Stul, M., Petersen, E.J., de Bruijn, M.A.C., van't Veer, M.B. & van Dongen, J.J.M. (1998) Immunoglobulin and T cell receptor gene rearrangement patterns in acute lymphoblastic leukemia are less mature in adults than in children: implications for selection of PCR targets for detection of minimal residual disease. *Leukemia*, **12**, 1081–1088.

Szczepański, T., Pongers-Willemse, M.J., Langerak, A.W., Harts, W.A., Wijkhuijs, J.M., van Wering, E.R. & van Dongen, J.J.M. (1999a) Ig heavy chain gene rearrangements in T-cell acute lymphoblastic leukemia exhibit predominant DH6-19 and DH7-27 gene usage, can result in complete V-D-J rearrangements, and are rare in T-cell receptor αβ lineage. *Blood*, **93**, 4079–4085.

Szczepański, T., Beishuizen, A., Pongers-Willemse, M.J., Hählen, K., van Wering, E.R., Wijkhuijs, J.M., Tibbe, G.J.M., De Bruijn, M.A.C. & van Dongen, J.J.M. (1999b) Cross-lineage T-cell receptor gene rearrangements occur in more than ninety percent of childhood precursor-B-acute lymphoblastic leukemias: alternative PCR targets for detection of minimal residual disease. *Leukemia*, **13**, 196–205.

Van den Doel, L.J., Pieters, R., Huismans, D.R., van Zantwijk, C.H., Loonen, A.H., Broekema, G.J., de Waal, F.C. & Veerman, A.J. (1988) Immunological phenotype of lymphoid cells in regenerating bone marrow of children after treatment for acute lymphoblastic leukemia. *European Journal of Haematology*, **41**, 170–175.

Van Dongen, J.J.M. & Wolvers-Tettero, I.L.M. (1991) Analysis of immunoglobulin and T cell receptor genes. Part I. Basic and technical aspects. *Clinica Chimica Acta*, **198**, 1–91.

Van Dongen, J.J.M., Adriaansen, H.J. & Hooijkaas, H. (1987) Immunological marker analysis of cells in the various hematopoietic differentiation stages and their malignant counterparts. In: *Application of Monoclonal Antibodies in Tumor Pathology* (ed. by D.J. Ruiter, G.J. Fleuren & S.O. Warnaar), pp. 87–116. Martinus-Nijhoff, Dordrecht.

Van Dongen, J.J.M., Szczepański, T., de Bruijn, M.A.C., Van den Beemd, M.W.M., de Bruin-Versteeg, S., Wijkhuijs, J.M., Tibbe, G.J.M., Van Gastel-Mol, E.J., Groeneveld, K. & Hooijkaas, H. (1996) Detection of minimal residual disease in acute leukemia patients. *Cytokines in Molecular Therapy*, **2**, 121–133.

Vervoordeldonk, S.F., Merle, P.A., Behrendt, H., Steenbergen, E.J., Van Leeuwen, E.F., Van den Berg, H., dem Borne, A.E., Van der Schoot, C.E. & Slaper-Cortenbach, I.C. (1996) Triple immunofluorescence staining for prediction of relapse in childhood precursor B acute lymphoblastic leukaemia. *British Journal of Haematology*, **92**, 922–928.

Weir, E.G., Cowan, K., LeBeau, P. & Borowitz, M.J. (1999) A limited antibody panel can distinguish B-precursor acute lymphoblastic leukemia from normal B precursors with four color flow cytometry: implications for residual disease detection. *Leukemia*, **13**, 558–567.

Wells, D.A., Sale, G.E., Shulman, H.M., Myerson, D., Bryant, E.M., Gooley, T. & Loken, M.R. (1998) Multidimensional flow cytometry of marrow can differentiate leukemic from normal lymphoblasts and myeloblasts after chemotherapy and bone marrow transplantation. *American Journal of Clinical Pathology*, **110**, 84–94.