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Growth of pre-B cells in cultures of bone marrow from children with acute lymphoblastic leukaemia and other diseases

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SUMMARY

Pre-B cells from the bone marrow of children with acute lymphoblastic leukaemia (ALL) survived up to 144 hr after the completion of treatment and divided in culture with maximum cell numbers at 24 hr. There was no rise in B cell number and no evidence of differentiation from pre-B to B cells. Binucleated pre-B cells in cultures containing cytochalasin B confirmed that pre-B cell division was occurring. Cycloheximide reduced cell numbers in culture but bromodeoxyuridine did not. Pre-B cell numbers also increased in culture of morphologically normal marrows from treated and untreated patients with solid tumours, and probably in normal marrows from patients with non-malignant diseases.

INTRODUCTION

The pre-B cells of human bone marrow, thought to be B cell precursors, are characterized by small amounts of μ chains in the cytoplasm and no detectable surface immunoglobulin (Gathings, Lawton & Cooper, 1977). We previously found that the percentage of pre-B cells was high in marrow samples from children with acute lymphoblastic leukaemia (ALL) who had completed remission maintenance treatment more than 3 months earlier (Paolucci, Hayward & Rapson, 1979). The percentage remained high for 6 or more months after the numbers of B cells in blood had returned to normal. To investigate the mechanisms of this increase we have compared the survival in short-term tissue cultures of pre-B cells from off-treatment ALL patients, other patients with childhood malignancies and controls who had received no cytotoxic drug treatment.

MATERIALS AND METHODS

Patients and marrow samples. Eighteen children who had completed remission maintenance treatment for ALL 1–16 months previously were studied. Their median age when studied was 7·5 years (range 4–14). At diagnosis they had no mediastinal mass and total WBC $< 20 \times 10^9$/l. These criteria were mainly selected for patients with common ALL (non-T, non-B, ALL antigen-positive, [Greaves *et al.*, 1977]). All patients had completed 3 years of remission maintenance treatment, in the UK ALL V trial, comprising vincristine, methotrexate, 6 MP and prednisolone (Rapson *et al.*,

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1980). A further five ALL patients were studied while receiving this treatment (median age 5·2 years, range 3–8). Three other patient groups were studied: five children had non-malignant diseases (malabsorption, chronic hiccough, hepatorenal failure, biliary atresia and congenital heart defect); their median age was 3·5 years (range 0·5–6) and they had not received cytotoxic drugs. Four children (median age 10 years, range 7–14) with other malignancies (two T cell lymphosarcomas, one neuroblastoma, one rhabdomyosarcoma) who had received radiotherapy and chemotherapy 1 to 9 months prior to study, and 4 children (median age 3·1 years, range 0·8–5) who had not had radiotherapy or chemotherapy (three neuroblastoma and one teratoma) were also studied.

Bone marrow was taken for diagnosis or for treatment control; all samples were morphologically normal apart from hypocellularity in those on cytotoxic drug treatment. The samples we studied were the residue after haematological preparations had been made and additional samples or volume were not aspirated for experimental purposes. Insufficient cells were recovered from some samples for all the *in vitro* studies.

Cell culture. Marrow samples were washed in heparinized Hanks' balanced salt solution and red cells were lysed with ammonium chloride as described by Paolucci *et al.* (1979). The remaining cells were resuspended in RPMI 1640 medium with 10% foetal calf serum (GIBCO BIOCULT), 2 mM glutamine and 50 μg/ml gentamicin (Roussell Laboratories) at 10^6 cells/ml. Some of this suspension (0·6 ml) was set aside for study (time 0) and the remaining cells were cultured in 0·2 ml volumes (Falcon 3040). These conditions had been established as optimal in previous studies for survival of both pre-B and B cells. Incubation was at 37°C in a humidified atmosphere with 5% CO_2. Triplicate wells were harvested after 4, 18, 24, 48, 72, 96, 120 and 144 hr and the number of viable (trypan blue-excluding) cells counted in a haemocytometer. The cells were washed and stained for surface IgM with fluorescein-labelled antibodies (Wellcome MF04), then cytocentrifuged, fixed and stained for cytoplasmic IgM with rhodamine-labelled antibodies as described by Paolucci *et al.* (1979). The percentage of pre-B, B and plasma cells was counted with a fluorescence microscope, and the absolute number of these cells per culture was calculated from the number of cells recovered. To selected cultures were added cytochalasin B (Sigma) in foetal calf serum, cycloheximide (Sigma) in saline, 5 bromo-2-deoxyuridine (BUdR, Sigma) or colchicine in saline. Endocytosis of IgM-containing antigen–antibody complexes on cell surfaces was induced by adding 60 μg/ml of sheep anti-human IgM to cultures for 24 hr after which the cells were washed.

Statistics. Previous studies (Paolucci *et al.*, 1979) showed that the percentage of pre-B cells in off-treatment ALL marrows was log-normally distributed. Comparisons between groups were therefore made with a paired *t*-test on log-transformed data or, where the sample number was small, with the Mann–Witney *U*-test.

RESULTS

Pre-B and B cells in cultures of off-treatment ALL marrows

The calculated number of pre-B cells in marrow cultures increased significantly ($P < 0·02$) during the first 24 hr of culture (Fig. 1) and gradually fell thereafter. The number of B cells recovered fell throughout the culture period. The total number of viable cells fell slowly so that 80% of the original number remained at 24 hr. Cells classified as pre-B cells were amongst the large and small mononuclear populations at time 0 whilst after 24 hr of culture only small pre-B cells were seen. Possible explanations for the rise in pre-B cell numbers include cell division and differentiation; these possibilities were tested using specific inhibitors. The appearance of multinucleate pre-B cells in cultures containing cytochalasin B (which prevents daughter cell separation in telophase [Smith, Ridler & Faunch, 1967]) was dose-related (Table 1), and at 96 hr a few pre-B cells had as many as four nuclei. Colchicine, which blocks mitosis at metaphase through interference with microtubule formation, prevented a rise in the number of pre-B cells in culture at the only dose tested. Cycloheximide (an inhibitor of protein synthesis) caused a dose-dependent reduction in the numbers of pre-B cells in culture (Table 1). The effect of BUdR on the number of pre-B cells was significant only at concentrations which greatly reduced the viability of the cells generally; at the concentrations shown in Table 1, the number of pre-B cells was not significantly reduced.

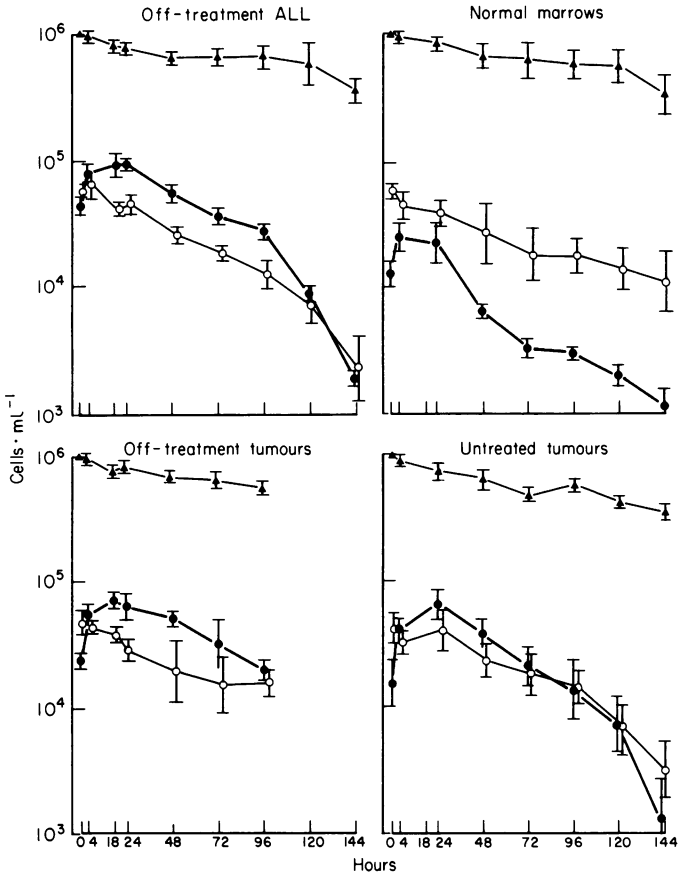

Fig. 1. Numbers of pre-B cells (•), B cells (o) and viable cells (▲) during 6 days of cultures of marrows from 18 patients with ALL off-treatment, five patients with non-malignant diseases, four solid tumours off-treatment, and four with untreated solid tumours. Results are expressed as antilog of mean of logged data ± s.e.m.

Erroneous identification of B cells as pre-B cells (as a result of endocytosis of surface μ–anti-μ complexes) was unlikely because the criteria for pre-B cells excluded cells with fluorescein staining. To exclude the possibility that cells which had ingested IgM for other reasons might be mistaken for pre-B cells, we induced the endocytosis of surface IgM by culturing marrow cells with unlabelled anti-μ for 24 hr. After staining with rhodamine-conjugated anti-μ, some cells with irregular clumps of IgM in their cytoplasm were seen. The quality of this staining was clearly different from pre-B cells in which the cytoplasmic IgM is uniformly distributed throughout the cytoplasm. Marrow cultures from ALL patients receiving remission maintenance treatment had few pre-B cells (mean $2{\cdot}2 \times 10^3$, s.e. $0{\cdot}5 \times 10^3$) or B cells (mean $1{\cdot}1 \times 10^4$, s.e. $0{\cdot}3 \times 10^4$) after 24 hr and the cell viability was only 50%. Very few pre-B or B cells were seen after 48 hr of culture.

Pre-B and B cells in cultures of normal marrows

Marrow from the five children with non-malignant diseases who had not received cytotoxic drugs contained four-fold fewer pre-B cells than the off-treatment ALL patients at time 0 ($P < 0{\cdot}001$); the number of B cells was similar in the two groups. There was a trend towards higher numbers of pre-B cells after 4 and 24 hr of culture but this did not reach statistical significance in the small number studied ($P = 0{\cdot}1$). The limited availability of these normal marrows precluded culture with cytochalasin B.

Table 1. Numbers of pre-B cells ($\times 10^4 \cdot ml^{-1}$) and % binucleated cells in 18-hr cultures of off-treatment ALL marrows treated with cytochalasin B (CB), cycloheximide (CH), bromodeoxyuridine (BUdR) and colchicine (Col)

		CB			CH			BUdR		Col	
	Control	2·5*	5	10	2·5	5	10	3	10	0	0·5
Cell number	12	7·9†	4·8‡	2·4‡	4·8‡	2·4‡	1·0‡	8·0	7·0	9·0	2·2‡
($\times 10^4 \cdot ml^{-1} \pm 1$ s.e.m.)	(10–14)	(7·3–8·4)	(3·9–5·8)	(1·7–3·3)	(3·9–5·8)	(2·1–2·8)	(8–1·3)	(6·2–9·8)	(5·0–9·4)	(7–11)	(1·0–3·2)
Binucleated cells (%)	0	5 (±0·7)	9·8 (±1·4)	11·6 (±1·7)							

* Concentration (μg/ml).
† Significance of difference from controls, $P < 0{\cdot}01$.
‡ Significance of difference from controls, $P < 0{\cdot}001$.
Results expressed as geometric mean (and range ±1 s.e.m.).

Pre-B and B cells in cultures of marrows from solid tumour patients

The percentage of pre-B and B cells at time 0 was similar to the off-treatment ALL patients, whether the tumour patients had been treated with cytotoxic drugs and irradiation or not. The results of culture also resembled the off-treatment ALL patients in that there was a significant ($P < 0{\cdot}05$) rise in the number of pre-B cells to a level higher than B cells in both groups.

DISCUSSION

The increase in absolute number (and percentage) of cells with the immunofluorescence staining characteristics of pre-B cells during 24 hr of *in vitro* culture and the appearance of multinucleate pre-B cells in the presence of cytochalasin B suggest that these cells divide in culture. Colchicine, cytochalasin B and cycloheximide all prevented the rise in the number of pre-B cells seen in control cultures of marrow from off-treatment ALL patients, suggesting that both cell division and protein synthesis are required. BUdR, which interferes with cell differentiation at low concentrations (Janossy, Snajdr & Simak-Ellis, 1976) did not affect the number of pre-B or B cells recovered from cultures unless high doses were used. We therefore did not obtain evidence for the differentiation of pre-B cell precursors into pre-B cells, or pre-B cells into B cells, during the first 18 hr of culture. Nevertheless, the magnitude of the rise in the number of pre-B cells during the first 4 hr of culture of off-treatment ALL marrow suggests that they may be generated by differentiation as well as cell division.

Okos & Gathings (1977) found uptake of tritiated thymidine by autoradiography of cultured adult marrow pre-B cells although Pearl *et al.* (1978) found no increase in pre-B cell number. The rise we found in normal marrow was small compared with off-treatment marrows, which could suggest that *in vitro* proliferation of pre-B cells occurs more actively in regenerating marrow following cytotoxic treatments. The possibility that proliferation by malignant pre-B cells contributed to our results seems unlikely since only 20–25% of ALL has pre-B cell characteristics (Vogler *et al.*, 1978), and the children we studied have remained well for up to 18 months without relapse of their leukaemia.

The percentage of pre-B cells in marrow from children with non-malignant diseases was less than that from the off-treatment ALL patients, confirming our previous observation (Paolucci *et al.*, 1979). Though the increase in the number of pre-B cells during culture of normal marrows was not statistically significant, some division may have occurred. Nonetheless, the peak was lower than in all malignancy groups and, unlike them, the pre-B cell numbers remained less than the B cell numbers. It seems unlikely that pretreatment with cytotoxic drugs is the only stimulus to increase pre-B cell numbers and proliferation as both of these were high in the untreated children with malignancies. It is conceivable that response to malignancy or even an aetiologic agent such as a virus might have contributed to increased marrow pre-B cell activity.

Absence of a significant rise in B cell numbers in any of our cultures suggests that few, if any, pre-B cells differentiated into B cells under the conditions we used. This result contrasts with the *in vitro* differentiation of B cells in culture of foetal rabbit liver (Hayward *et al.*, 1977) and mouse liver following termination of anti-μ suppression (Burrows *et al.*, 1978). *In vitro* B cell differentiation in cultures of normal mouse marrow has also been observed (Fairchild & Cohen, 1978), though in these studies the identity of the precursors was not established. Our results may be relevant to the failure to induce *in vitro* B cell differentiation in cultures of marrow from boys with congenital X-linked agammaglobulinaemia (Pearl *et al.*, 1978), even in the presence of agents acting on cyclic nucleotides, despite the presence of normal percentages of pre-B cells in these patients. The existing evidence that pre-B cells are precursors of B cells is only circumstantial, but if it is accepted our results suggest that pre-B cell division is not inevitably linked to the production of B cells and that a second signal may be required. If this is so, then the primary defect in congenital X-linked agammaglobulinaemia might involve a failure to produce this signal, as an alternative to an intrinsic abnormality of the patients' pre-B or B cells. We have no indication of a possible nature for this hypothetical second signal, although a possible suppressor signal for pre-B cell activity is the subject of another paper (Paolucci *et al.*, 1981).

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