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Pre-B and B cells in children on leukaemia remission maintenance treatment

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SUMMARY

The percentages of pre-B cells, mature B cells and IgM plasma cells were reduced in the marrow of children receiving continuous cytotoxic drug treatment to maintain leukaemia remission, compared with children receiving intermittent drug treatment in the UKALL V trial or untreated controls. When treatment was ended, the proportion of marrow pre-B cells rose above that of the controls and remained elevated for more than 6 months. These observations define more precisely the cellular basis of suppression of antibody immunity by cytotoxic drugs. They also suggest the existence of a complex feedback control of pre-B cell numbers and B cell differentiation during recovery.

INTRODUCTION

Severe infections resulting from immunosuppression by cytotoxic drugs are a major problem and deaths from infection during drug-maintained remission of acute lymphoblastic leukaemia (ALL) in children with good prognostic features (Hardisty & Till, 1968) are almost as common as deaths from leukaemic relapses (Medical Research Council, 1976). Experiments in animals suggest that the anti-tumour and immunosuppressive effects of different cytotoxic drugs depend on both the dose and the spacing of doses (Berenbaum, 1975). This has led to the evaluation of three different ALL remission-maintenance regimes in which the frequency and spacing, but not the total dose of the drugs were varied (Fig. 1). Patients receiving daily drugs (Group C) had significantly lower blood lymphocyte counts, lymphocyte responses to mitogens and lower plasma immunoglobulin concentrations (particularly of IgM) than the patients in Groups I and G whose treatment included drug-free periods (Rapson *et al.*, submitted for publication). Group C patients also had a higher frequency of serious infections and of death from infection. The deaths were from non-bacterial pneumonias and *Klebsiella* septicaemia. IgM antibodies are important in the defence against bacterial dissemination (Hobbs, 1975) and they probably limit the extracellular spread of viruses (Allison, 1974), so low IgM levels in children receiving cytotoxic drugs are likely to contribute to their susceptibility to infection.

In order to define more precisely the cellular basis for antibody immunosuppression and the speed of recovery, we have studied the frequency of B lymphocytes and their precursors, pre-B cells, in the bone marrow of ALL children on different treatment regimes or who had finished treatment.

MATERIALS AND METHODS

Patients. Children participating in the UKALL V trial who were in remission were studied while receiving, or after completing, maintenance chemotherapy. Only patients presenting with good prognostic features (less than 14 years old

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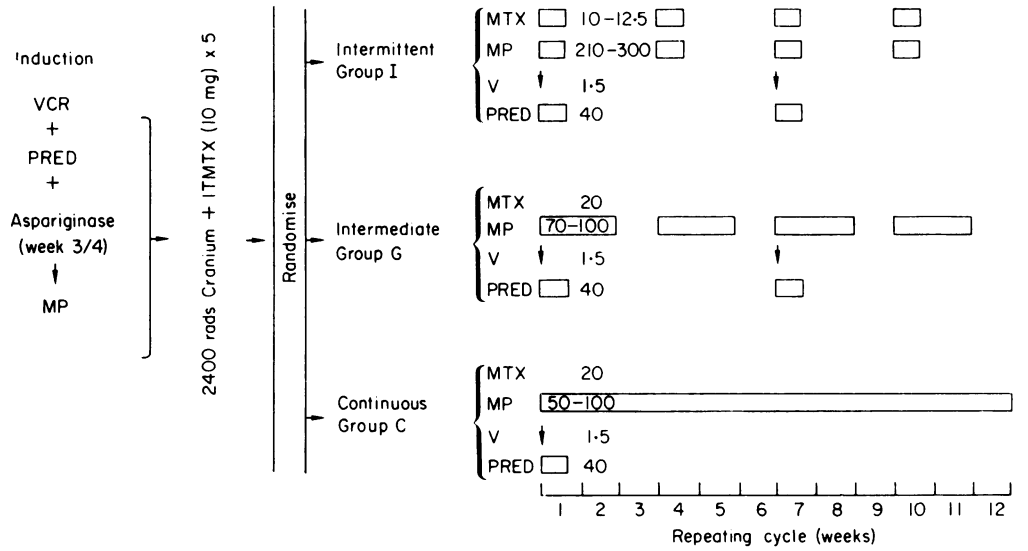

FIG. 1. Treatment schedules. UKALL V 1976. Drug doses are given in mg/M^2 of body surface area. ITMIX, Intrathecal methotrexate; MTX, methotrexate; MP, 6-mercaptopurine; V, vincristine; PRED, prednisolone.

white cell count < 20×10^9/l and no evidence of a mediastinal mass on X-ray) entered this trial. After induction of remission with prednisolone, vincristine and asparaginase and central nervous system prophylaxis (2400 rads to the cranium and five intrathecal injections of methotrexate), the children were chosen at random to receive one of the three maintenance regimes shown in Fig. 1. The median age of the patients was 6·5 years and there were no significant inter-group differences. Samples of blood and bone marrow were taken on the first day of each cycle of maintenance therapy according to the treatment protocol.

Controls. Bone marrow aspirated for diagnosis from children with a variety of illnesses was used as a control if it appeared to be morphologically normal in stained smears. Diagnosis included solid tumours, cystinosis and lipid storage disorders. The median age of the controls was 2·5 years. The study did not entail the aspiration of more marrow than was routinely collected.

Cell preparations. 0·5–1 ml of iliac crest marrow aspirate was washed with heparinized Hanks' balanced salt solution (HBSS). Erythrocytes were lysed with ammonium chloride (Boyle, 1968) at 30°C for 15 min, a procedure which was sometimes repeated. Cells were then washed and incubated in HBSS with 10% foetal calf serum (Gibco-Bio Cult, Glasgow, Scotland) for 45–60 min at 37°C to deplete cytophilic immunoglobulin (Lobo, Westervelt & Horowitz, 1975).

Lymphocytes were isolated from heparinized blood by Ficoll–Triosil centrifugation (Harris & Ukaejiofo, 1970).

Fluorescent antisera. Fluorescein-conjugated sheep anti-human IgM, IgG, IgA were purchased from Wellcome Reagents Ltd. (MF 03, 04, 05). Specificity was confirmed by staining immunoabsorbent particles (Hayward & Ezer, 1974). The anti-IgD (Nordic SWAHu/IgD/FITC 3·973) was absorbed with Sepharose-linked IgG and used at a 1:32 dilution. It did not cap surface IgM on lymphocytes.

Antibodies to purified human monoclonal IgM were raised in sheep and chickens. Immune sera were absorbed with insolubilized IgG and agammaglobulinaemic human serum and then passed over an IgM immunoabsorbent column. Anti-IgM antibodies were eluted with 2·5 M potassium thiocyanate, counter current dialysed against PBS, and then concentrated and separated on a small Sepharose 6B column. The IgG peak was conjugated to rhodamine tetramethylisothiocyanate (Morse *et al.*, 1977). Unbound dye was removed on a Sephadex G50 column equilibrated in 0·01 M phosphate buffer pH 7·6, and the conjugate was directly absorbed onto 0·01 M pre-equilibrated Whatman DE 52 for stepwise elution. In general, the 0·1–0·2 M eluates proved optimal for fluorescence staining; they had a protein rhodamine ratio of 1·5:2·2 and were used at a concentration of 100 μg/ml. The specificity of these conjugates was confirmed by staining IgG, IgA and IgM myeloma marrow preparations and by double staining with the fluorescein-conjugated antisera.

Fluorescence staining. Cells were stained for surface immunoglobulin with fluorescein-conjugated antibodies by conventional methods (Waller & MacLennan, 1977); an aliquot was examined for fluorescent cells and the remaining cells were allowed to cap for 60 min at 30°C. These cells were centrifuged onto slides (1000 rpm 7 min, Shandon cytocentrifuge), fixed in acetic acid-ethanol (acetic acid 5%, ethanol 95%) (15 min, −20°C) and stained for pre-B cells with rhodamine-conjugated anti-IgM antibodies for 15 min. The slides were rinsed in PBS and then kept for 24 hr in PBS before mounting in Uvinert aqueous (Hopkin and Williams) and examination on a Zeiss microscope with incident UV and transmitted phase contrast optics.

E-rosette test. T cells were identified as sheep erythrocyte rosettes (Waller & MacLennan, 1977).

Statistics. Positive cells were counted as a percentage and the results were log-normally distributed (Fig. 2). Tests of

differences between the groups were by *t*-test on log-transformed values unless the number of comparisons was < 10, when the rank sum test was used.

RESULTS

Identification of positive cells

The surface immunoglobulin (sIg) of B cells was stained green and red and the cytoplasmic immunoglobulin M (cIg) of pre-B cells and plasma cells was stained red. The B cells were counted as a percentage of total mononuclear cells in the suspension and on the slides. Pre-B cells were identified on the slides as cells containing small amounts of cIgM (stained red) but lacking sIgM (stained green). IgM plasma cells were clearly distinguishable from pre-B cells by their morphology, much more intense staining and the frequent presence of small amounts of sIgM.

Pre-B cells

The proportion of pre-B cells in the marrows of the various groups of patients and controls is shown in Fig. 2. The patients on maintenance treatment had significantly fewer pre-B cells than the controls

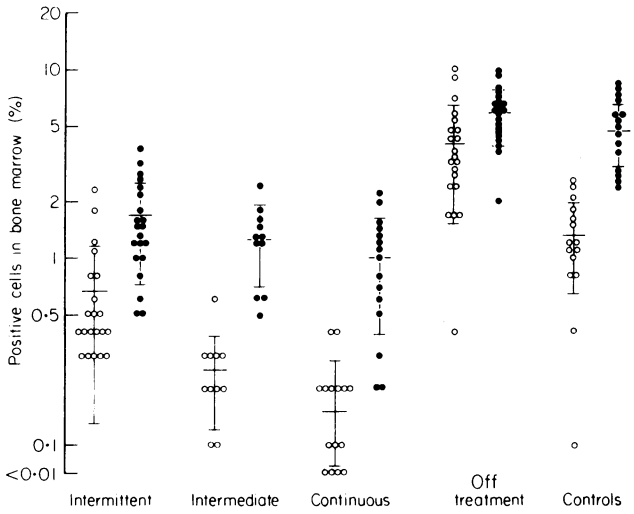

FIG. 2. Pre-B cells (O) and B cells (●) iu bone marrow. Results are expressed as a percentage of nucleated marrow cells. Horizontal lines indicate arithmetic mean ± 1 s.d.

($P<0{\cdot}001$). The patients on continuous treatment had least, followed by those on intermediate treatment, and those on intermittent treatment had most pre-B cells; this trend was statistically significant (*t*-test on log transformed values, $P<0{\cdot}001$). Patients who had finished their course of treatment had significantly more pre-B cells in their marrow than any other group. This rise was related to the length of time off treatment (Fig. 3), with a peak between 2 and 3 months.

B cells in marrow

All groups of patients receiving maintenance treatment had significantly fewer $sIgM^+$ B cells on their marrow slides than controls or patients off-treatment ($P<0{\cdot}001$). The same trend was apparent in preparations counted in suspension before capping, although there were some quantitative differences amongst the patients receiving treatment (Table 1). In particular, a higher frequency of $sIgM^+$ B cells was found in the suspension preparations from the intermittent, intermediate and continuous treatment groups than on the cytocentrifuge preparations from the same patients. In each of these groups the difference was significant ($P<0{\cdot}001$), while in the controls and patients off treatment patients there was no significant difference ($P>0{\cdot}3$).

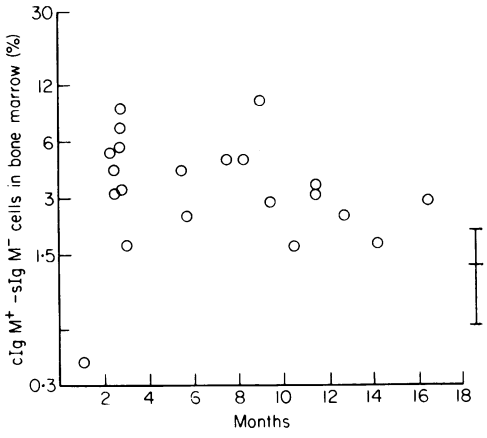

FIG. 3. Proportion of pre-B cells in the bone marrow of leukaemic children after the completion of maintenance treatment plotted against time (months) after finishing treatment. Results are expressed as in Fig. 2. The horizontal line indicates arithmetic mean ± 1 s.d. for control marrows.

TABLE 1. Frequency of T and B cells in the marrow of the various groups of patients and controls*

Patient group	Number of patients	Percentage of E-rosettes	sIgA	sIgG	sIgM	sIgD
Intermittent	18	10·5±4·8	1·4±0·8	4·9±2·1	4·7±1·5	3·8±2·0
Intermediate	8	8·4±6·0	1·6±0·3	5·4±1·9	4·0±1·1	3·4±1·3
Continuous	14	8·6±6·6	0·8±0·5	3·2±1·6	3·1±1·0	2·8±1·3
Off-treatment	18	9·2±5·3	1·9±0·8	4·8±1·8	6·7±1·7	4·2±1·4
Controls	12	13·0±6·0	1·8±0·9	4·0±1·5	5·9±1·3	3·1±1·1

* Results are expressed as mean percentage of positive cells ± 1 s.d. 200 nucleated cells were counted in each suspension preparation.

Other classes of sIg were counted on suspension preparations only. The frequency of $sIgD^+$ cells followed the same trend as $sIgM^+$ cells but the greatest depression (in the patients on continuous treatment) did not reach statistical significance. Since sIgD is found mainly on $sIgM^+$ cells, some indication of the percentage of $sIgM^+$ $sIgD^-$ cells can be obtained by comparing (as $\%\ sIgD^+ \div \%sIgM^+$) the ratio of cells stained for these two classes. The results (Table 2) suggest that there were fewer $sIgM^+$ $sIgD^-$ cells in the three treatment patients.

$sIgA^+$ cells were significantly depressed in the continuous treatment group ($P<0{\cdot}005$), as were $sIgG^+$ cells ($P<0{\cdot}02$). Other differences were not significant.

The frequency of marrow B lymphocytes was not significantly related to the pre-B cell frequency in any of the patients groups studied ($r = 0{\cdot}24$), although if patient and control results were pooled there was a weak correlation ($r = 0{\cdot}7$).

Plasma cells in marrow

Only IgM-containing plasma cells were stained in our preparations. Their frequency was low even in controls (mean 0·24% ± 0·27) and it was significantly depressed only in the patients on continuous treatment (mean 0·11% ± 0·1, $P<0{\cdot}05$).

TABLE 2. $sIgD^+/sIgM^+$ lymphocytes in the marrow of the various groups of patients and controls*

	Continuous	Intermediate	Intermittent	Off-treatment	Controls
$\frac{sIgD}{sIgM} \times 100$	99·9†	93	78·9	64	56·5
s.e.	13	35	8·4	5·2	5·3

* Results are calculated from individual observations of patients in Table 1.
† Significantly different from controls, $P < 0{\cdot}01$.

TABLE 3. Percentage of B cells with different classes of surface immunoglobulin from blood of the different patient groups*

Patient group	Number of patients	Percentage of: sIgA	sIgG	sIgM	sIgD
Intermittent	16	2·2±0·9	4·2±2·4	5·9±2·7	3·8±2·0
Intermediate	5	1·9±0·8	4·0±0·8	3·4±1·3	2·1±1·3
Continuous	9	1·1±0·6	3·1±1·4	4·1±1·6	1·9±1·1
Off-Treatment	8	4·0±1·6	6·7±2·5	15·1±2·7	11·7±4·6

* Results are expressed as in Table 1. 200 nucleated cells were counted in each cell suspension preparation.

T cells in marrow

About 10% of marrow cells made E rosettes in all the groups studied.

B cells in blood

Only patients' blood samples were studied. Within the treatment groups, the only significant difference to emerge was a reduction in $sIgA^+$ cells in the continuous treatment patients (Table 3). The off-treatment patients were an unsatisfactory reference group because only eight were studied and their percentage of sIg^+ cells was higher than that generally found in our laboratory. The ratio of % $sIgD^+$ ÷ % $sIgM^+$ cells in blood was highest in the treated patients and lowest in those who had finished treatment.

TABLE 4. Serum immunoglobulins in the patient groups*

Patient group	Number of patients	IgA	IgG	IgM
Intermittent	21	68·8±26·9	105·9±32·7	84·3±49·5
Intermediate	11	55·1±42·8	86·2±28·4	67·0±65·3
Continuous	13	40·8±25·7	89·2±31·7	62·3±38·9
Off-Treatment	7	57·7±22·6	126·1±16·8	75·5±34·4
+2 s.d.†		176	197	236
Mean†		95	87	106
−2 s.d.†		39	46	53

* Results are expressed as mean of log transformed values ± 1 s.d. in iu/ml.
† Range of normal values (6–9 years) from Hobbs (1975).

Serum immunoglobulins

The means of log transformed data from the patients are shown in Table 4. Comparison between groups and with healthy controls is more secure for IgG and IgM concentrations because of the age dependence of serum IgA in the range we have studied (J. R. Hobbs, 1975, personal communication). The continuous and intermediate groups tended to have lower serum immunoglobulins than the intermittent or off-treatment groups, although the differences only reached statistical significance for IgG ($P<0{\cdot}01$). Serum immunoglobulin concentrations were not significantly associated with pre-B cell percentages within patient groups.

DISCUSSION

Our studies were undertaken to compare the suppression of B cell function in patients treated with cytotoxic drugs according to different protocols for the maintenance of remission in ALL. Previous studies have shown that children on long-term anti-leukaemic treatment become lymphopenic, with reduced numbers of B and T cells in their blood (Sen & Borella, 1973), and may sequester PHA-responsive cells in their marrow (Borella, Green & Webster, 1972). Diminished IgM levels in patients on continuous maintenance treatment in this study and that of Rapson *et al.* (submitted for publication) might result from impaired B cell production, B cell sequestration or interference with B cell terminal differentiation. The recent identification of the B cell precursor, a pre-B cell (Gathings, Lawton & Cooper, 1977), permits an experimental approach to the first possibility and also offers insight into the effects of anti-leukaemic treatment on a spontaneously dividing population of marrow stem cells (Okos & Gathings, 1977). We found that all identifiable members of the B cell series (pre-B, B and plasma cells) were reduced in patients receiving anti-ALL maintenance treatment. The reduction was greatest in those on continuous daily treatment and least in those on intermittent (or pulsed) treatment. Intermediate group patients occupied an intermediate position. These observations indicate that the impaired antibody responses of these patients may not be due to the impairment of mature B cell function alone but may also reflect a diminished B cell production from pre-B cells. The immature B cells which are thought to be the progeny of pre-B cells have sIgM but lack other classes of sIg, particularly sIgD (Vitetta *et al.*, 1975). Our finding of a proportionally reduced number of $sIgM^+$ $sIgD^-$ cells in the marrows of all groups of patients on anti-leukaemic treatment is consistent with the view that patients with few pre-B cells make few immature B cells. On blood lymphocytes, only 57% of $sIgM^+$ cells from the combined groups of treated patients could be assumed to have sIgD, compared with 78% for the off-treatment patients and the 82% originally found in healthy adults (Rowe *et al.*, 1972). The differences in the calculated frequency of $sIgM^+$ and $sIgD^+$ cells in blood are therefore completely opposite to the findings in marrow. This could point to qualitative abnormalities in the blood B cells of patients during treatment and we are currently investigating this possibility. The different percentages of $sIgM^+$ cells found in the slide and suspension preparations of marrow cells from the patients on treatment (but not in the controls) could be interpreted in a similar way. However, a simpler explanation could be that the lower percentage of $sIgM^+$ cells in the slides was due to the shedding of cytophilically bound fluorescent antibody during the 60 min incubation period during which capping occurred. Why comparable differences were not found in the control samples is not accounted for by this mechanism.

Both the frequency of IgM plasma cells and the serum immunoglobulin concentration were lowest in the continuously treated patients, indicating an impairment of B lymphocyte terminal differentiation and the effector limb of antibody-mediated immunity. Effects such as these could be important in increasing a patient's susceptibility to septicaemia, since IgM antibodies appear to be particularly important in the defence against bacterial invasion of the blood stream (Hobbs, 1975).

Following termination of anti-leukaemia maintenance treatment, the number of pre-B cells in the marrow of all treatment groups rose. There was no clear correlation with the previous degree of pre-B cell suppression. The rise persisted for the full duration of the study and its peak was at 2–3 months post-termination. Since the change identified is one of proportion, it is possible that it reflects a reduction in other marrow cell populations. Two considerations weigh against this possibility; firstly, the frequency

of ALL antigen staining cells increases concomitantly with the pre-B cell rise (Greaves *et al.*, 1978), and secondly the post-treatment termination marrow is hypercellular, not hypocellular, by conventional morphological criteria.

There are several possible mechanisms which could account for the prolonged elevation in marrow pre-B cells in the face of normal numbers of blood B cells. Perhaps the simplest is that B cell numbers in the blood are not representative of spleen and lymph node B populations, and that repopulation at these sites takes several months to achieve. We have not tested this possibility directly but the relatively rapid return of serum immunoglobulin levels and antibody to normal after treatment was stopped (Borella *et al.*, 1972) would seem to argue against a prolonged depletion of tissue B cells. Another possibility is that the replenishment of blood B cells within 8 weeks of the cessation of treatment (Sen & Borella, 1973, and confirmed by us in a small number of patients studied) depends on the production of large numbers of pre-B cells and that once B cell recovery has occurred these pre-B cells persist in the marrow in a resting state. We are investigating this possibility; if it should prove correct it would indicate the existence of new levels of pre-B cell regulation. A more speculative interpretation of the prolonged rise in pre-B cells could be based on the need, following the cessation of treatment, to re-establish the quality as well as the quantity of circulating B cells. Prolonged anti-leukaemic treatment is likely to deplete the patient mostly of antigen-driven rather than resting B cells, the result of which could be a reduction in the antigen recognition repertoire of the patients' B cell system. Release from suppression by anti-leukaemic drugs could be followed by replenishment of the B cell pool from both the production of new B cells and from the division of cells in the existing B cell pool. Once the normal number of blood B cells has been reached it is conceivable that further elevation in pre-B cell numbers reflects the patients' need to re-establish a full range of clonal diversity. The nature of feedback control at this antigen-independent level could be a matter of fundamental interest.

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