Abstract
The existence of subpopulations of clonal lymphocytes in patients with low grade lymphoproliferative disorders, with regard to both cell size and bcl-2 protein concentration is reported. Flow cytometric analysis showed that the lymphocytes with higher levels of bcl-2 corresponded to a subset of larger lymphocytes. Statistical analysis suggested that the increased concentration of bcl-2 was not accounted for by the increase in cell size and it is possible that these cells form a functionally distinct component of the clonal proliferation. One patient, analysed in greater detail during treatment with a purine analogue, showed the subpopulations to exhibit a differential sensitivity to chemotherapy.
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Keywords: bcl-2; low grade lymphoproliferative disorders; purine analogue treatment; cell size

Heterogeneity of clonal lymphocytes with regard to bcl-2 protein concentration and cell size

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The bcl-2 protein has been shown to be overexpressed in low grade lymphoproliferative disorders. In follicular non-Hodgkin's lymphoma (NHL) this is believed to result from the 14;18 translocation placing the bcl-2 gene under the control of the immunoglobulin heavy chain gene. It is now known that overexpression of bcl-2 in lymphoproliferative disorders is not restricted to lymphomas bearing the t(14;18), and that other mechanisms exist for the increased expression of this protein. In B cell chronic lymphocytic leukaemia it has been proposed that hypomethylation of the bcl-2 gene contributes to its increased expression. It is suggested that disruption of translational and post-translational control mechanisms play a role in follicular NHL and a comparative genomic hybridisation study of centrocyclic/centroblastic NHL has shown amplification of the bcl-2 gene.

Bcl-2 is an intracellular protein, localising to the mitochondrial membrane, the endoplasmic reticulum, and the nuclear membranes. It is a complicated protein that appears to possess multiple independent functions resulting in roles both as an ion channel and as an adaptor or docking protein. Overexpression of bcl-2 in a pre-B cell leukaemia cell line has been shown to block chemotherapy induced apoptosis. Successful treatment of low grade lymphoproliferative disorders with purine analogues (fludarabine, 2-chlorodeoxyadenosine (2-CdA) and deoxycoformycin) is dependent on the induction of apoptosis in lymphocytes, hence there has been speculation as to whether the concentration of bcl-2 in lymphocytes could predict the outcome of treatment in these patients.

In the process of investigating bcl-2 in B cells of patients with lymphoproliferative disorders, we identified some patients with appreciable heterogeneity in cell size and bcl-2 concentration. Other investigators have noted the occurrence of a population of larger lymphocytes in some patients with chronic lymphocytic leukaemia and reported that these cells had significantly more nucleoside transporter protein than normal sized lymphocytes. These observations raise the possibility that these cells form a functionally distinct component of the underlying clonal population.

Methods
FLOW CYTOMETRY
Peripheral blood was obtained from patients with low grade lymphoproliferative disorders. Mononuclear cells were separated on lymphocyte separation medium (Nycomed, Oslo, Norway) according to the manufacturer's instructions. Cells were fixed in 0.5% formaldehyde (diluted directly before use from a 4% stock solution) and stored at 4°C. The cells were spun out of formaldehyde and permeabilised by incubating at 37°C in phosphate buffered saline (PBS)/0.2% tween 20 for 15 minutes before staining.

Cells were dual stained with anti-CD19/PE and anti-bcl-2/FITC monoclonal antibodies (Dako, High Wycombe, Bucks, UK). The isotype control was a mouse IgG1-RD1/mouse IgG1-FITC (Coulter, Luton, Beds, UK). Flow cytometry was performed on a FACSscan flow cytometer (Becton Dickinson, Oxford, UK) using a 488 nm beam from an argon ion laser. FITC fluorescence was collected after a 530 nm (band width 30 nm) filter, and PE fluorescence collected after a 585 nm (band width 42 nm) filter. Both were measured using...
logarithmic amplification. Ten thousand events were collected after gating on CD19 positive cells and the data analysed using Flowmate software (Dako). Calibration and sensitivity were checked using fluorescent labelled beads (Calibrite beads; Becton Dickinson).

**STATISTICAL ANALYSIS**

Linear regression was performed using SPSS PC+ software. For analysis the square root of forward scatter (FSC) and the cube root of green fluorescence (FL1) was used to account for FSC and FL1 being proportional to cell surface area (radius^2) and volume (radius^3), respectively. This assumes that lymphocytes approximate to a spherical shape.

**Results**

In a series of 127 patients with low grade lymphoproliferative disorders it was observed that a proportion (n = 20) appeared to have populations of CD19+ lymphocytes showing different levels of bcl-2. Fifteen of these patients had previously been treated and this observation can be explained by detection of the re-emergence of the malignant clone against a background of normal cells; however, the other five patients were not in this situation with virtually all of their B cells being of clonal origin (as shown by surface immunophenotyping). The CD19+ lymphocytes in these patients all showed more intense staining than normal B cells with anti-bcl-2 (data not shown). In all cases the more intensely staining bcl-2 populations corresponded to a subset of larger lymphocytes and in three of the five cases these cells formed a separate population on staining with CD19 (fig 1).

Statistical analysis on one patient (DL) showed R^2 for linear regression of the square

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*Figure 1* Typical results from dual staining with anti-CD19-PE and anti-bcl-2-FITC. (A) Dot plot forward scatter versus side scatter. Region 1 corresponds to normal sized lymphocytes. (B) FITC fluorescence (bcl-2) histogram ungated. (C) FITC fluorescence (bcl-2) histogram, overlay (black) corresponds to events in region 1. (D) PE fluorescence (CD19) histogram ungated. (E) PE fluorescence (CD19) histogram, overlay (black) corresponds to events in region 1.
root of the forward scatter against the cube root of the green fluorescence to be 0.69 for the total cell population. When the cells were split into two populations on the basis of green fluorescence (high and low) (fig 1B) and the regression repeated, R2 for high was 0.04 and for low it was 0.33; suggesting that the relation between cell size and bcl-2 concentration does not persist within the subpopulations.

This patient was monitored during chemotherapy with 2-CdA, after just one course elimination of the high bcl-2 cells present before treatment was observed. This was followed by a reduction in the lower bcl-2 cells after further courses, accompanied by re-emergence of a lymphocyte population showing a level of bcl-2 protein equivalent to that of normal lymphocytes (fig 2). This re-emergence of normal B cells was supported by flow cytometric analysis of clonality. The disappearance of the high bcl-2 cells was also noted in two patients on treatment with chlorambucil and prednisolone, and in one patient not receiving any treatment.

Discussion
It is generally presumed that cells with a high bcl-2 concentration should be more resistant to chemotherapy induced apoptosis. The data presented here conflicts with this theory and raises the question of whether bcl-2 concentration in a cell is related to cell size. Little attention appears to have been given to assessing whether larger cells require more bcl-2 to achieve an equivalent anti-apoptotic effect. Consideration of antigens expressed on the cell surface is relatively straightforward—the larger the cell, the greater the surface area and therefore the higher the fluorescence intensity expected. However, bcl-2 is an intracellular protein so it is not obvious whether a similar argument should be applied. Other investigators assessing the prognostic significance of bcl-2 concentration in acute myeloid leukaemia compensated for size difference simply by dividing by mean forward scatter. However, if bcl-2 concentration is related to cell size it should be dependent on cell volume rather than surface area.

We have attempted to assess the relation between cell volume and bcl-2 concentration by comparing CD19+ subpopulations. Although statistical analysis showed a strong relation between cell size and bcl-2 in the overall cell population, when the cells were divided into two populations based on their bcl-2 concentration, the correlation with cell volume was less obvious, particularly for the high bcl-2 population. This implies that, although the larger cells do indeed have more bcl-2, within each subpopulation the relation between these two variables is poor and the increase in cell size cannot account for the increase in bcl-2.

This was supported by a study of patient DL's lymphocytes, which showed a differential sensitivity to 2-CdA with treatment. The higher bcl-2/larger lymphocytes were more sensitive to drug exposure, suggesting the existence of a physiological difference between the two populations. The tendency of cells to undergo apoptosis is now thought to be dependent on the ratio of Bax:bcl-2 rather than the absolute level of bcl-2, and this may explain the finding of differential drug sensitivity.

These observations highlight the heterogeneity possible in clonal lymphocyte populations and raise a note of caution for techniques that, unlike flow cytometry, do not alert the user to this. A physiological difference is apparent between these subpopulations of lymphocytes that has not been fully explained by examination of cell size and levels of bcl-2 alone.

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