Clin. exp. Immunol. (1985) **62,** 95–103.

Surface glycoproteins as markers of the cellular status of B chronic lymphocytic leukaemia lymphocytes

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(Accepted for publication 8 May 1985)

SUMMARY

The phenotype of B CLL cells is investigated with respect to their surface glycoproteins. These glycoproteins are identified by vectorial tritiation followed by 1 and 2 dimensional gel electrophoresis, and by lectin and MoAb binding using immunoprecipitation and flow cytometry. The profiles of the CLL cells are compared with those of normal B cells, prepared from tonsils, and T cells from peripheral blood. The CLL cells show many similarities with T cells, particularly the expression of glycoproteins which bind the MoAbs gpL 115, F10-44-2 and EZB 52, and a complex set of binding sites for *Helix pomatia* lectin. The significance of these observations in terms of the cellular origins of the leukaemic lymphocytes is discussed.

Keywords chronic lymphocytic leukaemia surface glycoproteins monoclonal antibodies leukaemic phenotype

INTRODUCTION

Elucidation of the molecular nature of the exterior surface of cells depends initially on the identification of the molecules exposed at the surface by labelling those molecules with impermeant tags, either impermeable chemical labels or antibodies raised against the surface antigens. Combination of the wide range of chemical probes now available with the rapidly expanding library of monoclonal antibodies (MoAb) against the cell surface antigens facilitates the detailed investigation of the molecular architecture of the surface. The relative contributions of the two approaches varies from cell to cell, and, not surprisingly, in the case of the lymphocyte immunological methods have predominated. In this communication we aim to bring together both lines of study and exploit them to analyse the particular problem of the surface changes associated with the neoplastic transformation of the chronic lymphocytic leukaemia (CLL) lymphocyte.

CLL involves a monoclonal expansion of lymphocytes, usually B lymphocytes, and has an unpredictable course, ranging from a relatively benign form which remains stable over several years to a more aggressive type with relatively rapid progression. If these varieties can be distinguished by differences in the surface glycoproteins then such an analysis could have direct clinical significance in addition to contributing to the more general question of the nature of surface changes associated with a malignant transformation. The surface glycoproteins have been examined by other authors and many of the anomalous glycoproteins which characterize the leukaemia are apparent in their

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publications (Andersson *et al.*, 1979; Axelsson, Hammarström & Mellstedt, 1979; Gahmberg & Andersson, 1982; Tötterman *et al.*, 1983). Recent advances in the identification of lymphocyte surface molecules have enabled us to identify the anomalous features of the CLL cell in terms of the surface molecules found in normal B and T cells. In an earlier publication (Smith *et al.*, 1985) we described anomalies in the expression of one set of glycoproteins, the leucocyte-common antigen (L-CA), which was found to vary from a B cell-like expression in some patients (Type 1a) through a series of intermediate forms to a T cell-like expression in other patients (Type 1b). We here describe anomalies in other surface glycoproteins of the CLL lymphocyte which, with a minor exception, are found in the leukaemic cells of all patients.

METHODS

Cell preparation. Leukaemic lymphocytes were isolated from freshly collected leukaemic blood by centrifugation on Ficoll-Paque for 15 min at 400 *g* and washed free of plasma in RPMI 1640 medium. Before labelling the cells were washed in a solution of 133 mM NaCl, 4·5 mM KCl, 5 mM $MgCl_2$ and 2 mM $CaCl_2$ buffered to pH 7·4 with 10 mM Hepes (Buffer A). The viability, monitored by acridine orange/ethidium bromide staining (Parks *et al.*, 1979), was routinely found to be >95%. Normal B and T lymphocytes were prepared from tonsils and peripheral blood respectively, as previously described (Smith *et al.*, 1985).

Tritiation of surface glycoproteins. The surface glycoproteins were tritiated after sialidase and galactose oxidase or periodate treatment and solubilized by Triton X100 as previously described (Smith *et al.*, 1985).

Gel electrophoresis. An equal volume of 0·125 M Tris-HCl (pH 6·8) containing 10% (weight/vol) sodium dodecyl sulphate (SDS), 20% glycerol, 0·002% bromophenol blue, 1 mM EDTA and 100 mM dithiothreitol was added to the Triton soluble extract and the mixture heated at 100°C for 5 min.

The polypeptides were separated in one-dimension by electrophoresis in the discontinuous buffer system of Laemmli (1970) with an 8–15% gradient polyacrylamide gel. Two-dimensional separations were carried out by the technique of Thompson, Rennie & Maddy, (1980). In this method the first dimension involves electrophoresis in 4% polyacrylamide gel using the buffer system of Fairbanks, Steck & Wallach (1971) and the second carried out in a 12% gel with the Laemmli (1970) buffer.

Polypeptides were stained with Coomassie brilliant blue R250. Tritiated glycopeptides were detected by fluorography after impregnation of the gels with scintillant (Bonner & Laskey, 1974) or Amplify (Amersham Co, Amersham, UK) before drying and exposing them to pre-flashed X-ray film.

Antibody binding was measured by flow cytometry as described previously (Smith *et al.*, 1985). The antibodies used are listed in Table 1.

Immunoprecipitation of antigens. The Triton X100 content of the Triton solubilized extracts of labelled cells was adjusted to 1·0%, the mixture incubated at 0°C for 10 min and centrifuged for 10

Table 1.

Designation	Specificity	Source
gpL 115	Leucocyte sialoglycoprotein	Kindly donated by Professor F. S. Rosen & Dr Remold-O'Donnel
EZB 52	105 Kd gp.	Edinburgh Zoology Department
F10-44-2	80 Kd gp.	Kindly donated by Professor J. Fabre
W6 32	MHC Class I	Sera Laboratories
DA6 32	MHC Class II	Kindly donated by Dr K. Guy
FMC 7	—	Sera Laboratories

min in an Eppendorf microfuge. 50 μl of the supernatant was incubated for 2 h at 20°C with 0·5 ml of antibody solution in Buffer A, adjusted to pH 8·0, containing 0·5% Triton X100 + 0·5% sodium deoxycholate + 0·05% SDS (Omary & Trowbridge, 1980). 75 μl of a 20% (vol) suspension of Protein A-Sepharose in the same buffer was then added and the whole incubated with gentle mixing for 16 h at 4°C. The resulting antigen-antibody complexes were washed three times in the buffer, once in water, and finally prepared for gel electrophoresis, as previously described (Smith *et al.*, 1985).

Lectin binding. 30 μl of Triton extract was added to 30 μg lectin bound to Sepharose 4B beads suspended in 0·6 ml Buffer A, adjusted to pH 8·0, containing 0·5% Triton X100 + 0·5% sodium deoxycholate + 0·05% SDS and the mixture gently rotated end over end for 16 h at 4°C. The beads were then washed four times in the same buffer and finally resuspended in the buffer used for the preparation of samples for gel electrophoresis.

RESULTS

The leukaemic samples were all obtained from patients diagnosed as B CLL by the presence of a monoclonal expansion of lymphocytes expressing a single immunoglobulin light chain and which rosetted with mouse red cells in high frequency: this frequency was increased by sialidase treatment of the red cells. The cells expressed Class II MHC and B1 binding (Stashenko *et al.*, 1980). T cell contamination was monitored in all samples by sheep red cell rosetting or pan T cell MoAb binding and shown to be < 5%.

Electrophoretic analysis

One-dimensional gel electrophoresis of the surface glycoproteins of CLL lymphocytes revealed the presence of several components absent in normal B cells but present in normal T cells (Fig. 1). This similarity between the CLL cells and normal T cells was confirmed by two-dimensional gel electrophoresis, where again several T cell glycoproteins were observed along with B cell glycoproteins in the B CLL cells (Fig. 2). The two-dimensional technique used involves SDS-PAGE in both dimensions (Thompson *et al.*, 1980). As the mobility of glycoproteins depends upon the particular electrophoretic conditions employed and is not a simple function of molecular weight, in

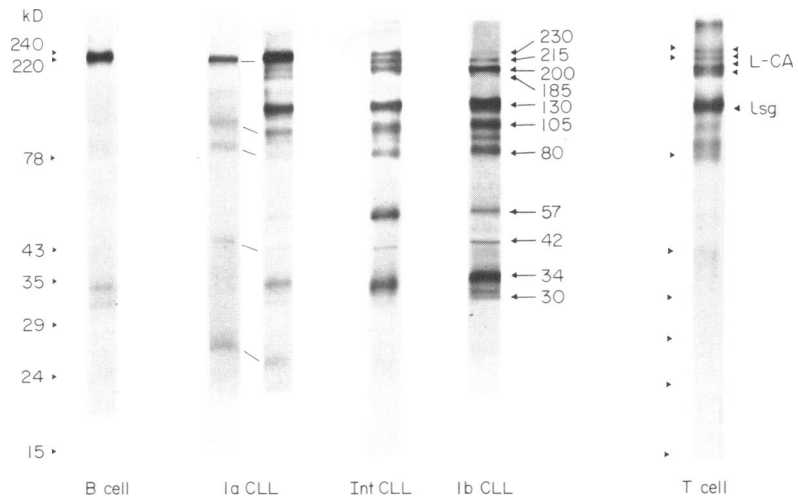

Fig. 1. Fluorographs of tritiated surface glycoproteins of lymphocytes after separation by SDS-PAGE on an 8–15% polyacrylamide gradient gel. The left-hand lane of the *Type Ia* CLL cells illustrates the absence of the leucocyte sialoglycoprotein in a small minority of patients in this category. Human erythrocyte membrane proteins are used as molecular weight markers. L-CA = leucocyte common antigen; l.s.g. = leucocyte sialoglycoprotein.

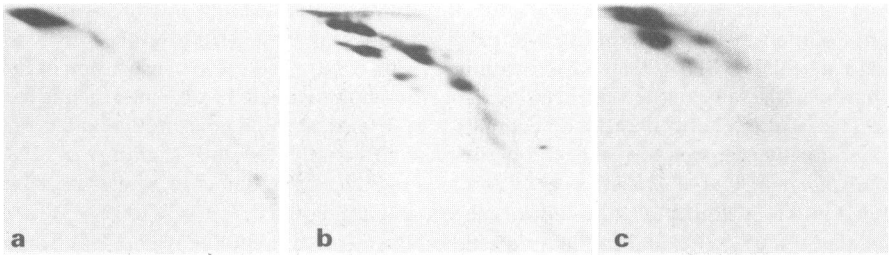

Fig. 2. Two-dimensional electrophoresis of tritiated surface glycoproteins by the method of Thompson *et al.* (1980). The first dimension was run on a 4% gel with a Fairbanks buffer, the second on a 12% gel with a Laemmli buffer. (a) normal B cells; (b) CLL cells; (c) normal T cells.

this method they are displaced from the arc described by the non-glycosylated proteins and distributed over a wide area of the gel. The technique is advantageous for the resolution of high molecular weight glycoproteins which are difficult to focus into sharp spots using isoelectric focusing.

Identification of the glycoproteins resolved by electrophoresis

MoAb binding confirms the identities of the components in the different types of cell that are suggested by electrophoresis. Analysis of the MoAb binding by flow cytometry further shows that in each case the aberrant glycoprotein is expressed by virtually all (>90%) of the leukaemic lymphocytes in each sample.

The 200 Kd region. These high molecular weight glycoproteins were identified as the leucocyte common antigen (L-CA) by immunoprecipitation with anti-L-CA MoAbs (Smith *et al.*, 1985). The expression of L-CA in CLL varies from a B lymphocyte-like pattern in some patients, with a single band at 230 Kd (Type 1a), through a series of intermediate forms, showing a progressive increase of the lower molecular weight chains, to a pattern with a T cell-like profile with bands at 230, 215, 200 and 185 Kd (Type 1b patients). This variation in the expression of L-CA and its correlation with the clinical assessment of CLL is described in detail elsewhere (Smith *et al.*, 1985; Brown, Smith & Maddy, 1985).

The 130 Kd component. This glycoprotein was present in T cells and CLL lymphocytes in the majority of patients (see below), but absent in B cells (Fig. 1). It was identified as the glycoprotein called leucocyte sialoglycoprotein (l.s.g.) by Brown & Williams (1982) on the following grounds. (i) Its apparent molecular weight was changed from 100 Kd to 130 Kd by desialylation with sialidase (Smith *et al.*, 1985). (ii) After sialidase treatment the molecule bound peanut and *Helix pomatia* (HP) lectins. (iii) The glycoprotein was immunoprecipitated by the anti-l.s.g. MoAb gpL 115 (Remold-O'Donnell *et al.*, 1984) (iv) Cells possessing this component were shown by flow cytometry to bind the gpL 115, but less was bound by CLL cells than normal T cells (Fig. 3). Expression of antibody binding in terms of fluorescence in a flow cytometer shows that CLL cells from 20 patients had an average value of 235 ± 168 units, while the average value for eight normal T cell samples was 409 ± 138 units (t-test $P < 0{\cdot}01$).

A small minority of patients (5/46) were distinguished by the absence of l.s.g. (Fig. 1), a feature also displayed by prolymphocytic leukaemia (PLL) cells. PLL cells also share with these five patients a B cell-like L-CA profile, a high level of surface immunoglobulin and an aggressive disease (PLL patients were distinguished diagnostically from CLL by their large cell size and the inability of sialidase to enhance mouse red cell rosette formation). This sub-group of CLL patients possess a leukaemia intermediate between CLL and PLL, and in this respect are reminiscent of the prolymphocytoid variant described by Catovsky *et al.* (1981) yet differ in lacking both a prolymphocytoid morphology and an affinity for the MoAb FMC 7.

The 105 Kd glycoprotein. A component of this size was readily detected by surface tritiation of CLL lymphocytes and normal T cells. Its presence in normal B cells was apparent only after prolonged exposure of the fluorographs. The molecule was immunoprecipitated from Triton X100

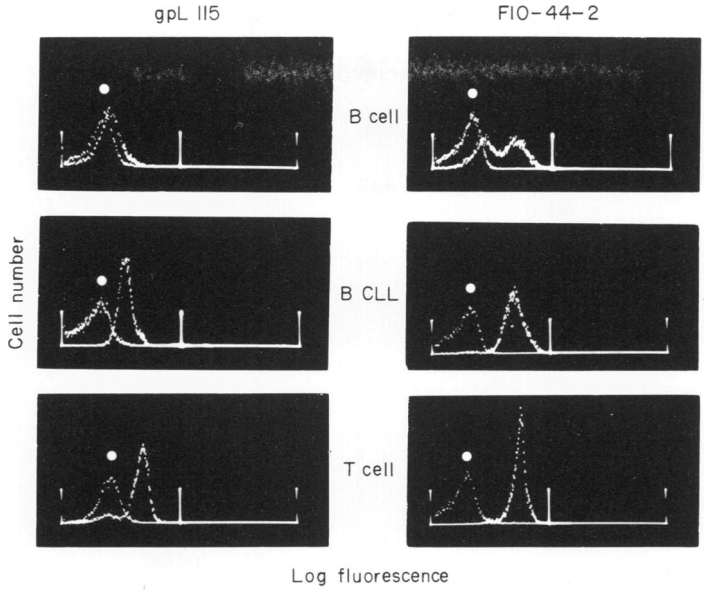

Fig. 3. The binding of monoclonal antibodies gpL 115 and F10-44-2 by flow cytometry. gpL 115 is bound by B CLL cells and normal T cells but is not bound to normal tonsil B lymphocytes, less antibody is bound by the CLL than the T cells. F10-44-2 is also strongly bound by both B CLL and normal T cells, but a comparable level of binding is found only in a sub-population of tonsilar B cells O = control fluorescence of second antibody.

extracts of CLL cells by a new MoAb—EZB 52—developed in our laboratory (Brown *et al.*, 1985) (Fig. 4). Flow cytometry revealed its presence on the surface of all three types of cell: the fluorescence was uniformly bright in CLL and normal T cells but in the tonsil B cell samples two fractions were detected both with slightly less bright fluorescence than the CLL cells. The heterogeneity of the B cell samples was correlated with their heterogeneous size differences as described below for the MoAb F10-44-2.

The 100 Kd glycoprotein. This molecule is present in T cells, CLL cells and possibly in small quantities in B cells. No further information on this molecule is available.

The 80 Kd glycoprotein. As with the 105 Kd glycoprotein, a molecule of 80 Kd was readily detected by surface tritiation of CLL lymphocytes and normal T cells but only after long exposure of fluorographs of tonsil B cells. The glycoprotein was shown by immunoprecipitation to bear the epitope for the MoAb F10-44-2 (Dalchau, Kirkley & Fabre, 1980) (Fig. 4). The CLL and T cell samples were homogeneous in flow cytometry with a bright fluorescence, but the tonsil B cell samples were, as with EZB 52, heterogeneous, showing a bimodal fluorescence with faint and bright fractions (Fig. 3). Preliminary studies have shown that the bright fluorescence was obtained from large cells which could correspond with activated B cells of the tonsils. The weakly fluorescent cells were smaller in size and thought to correspond with peripheral B cells (Dalchau *et al.*, 1980). (The original identification of F10-44-2 as anti-leucocytesialoglycoprotein has subsequently been modified: McKenzie, Dalchau & Fabre, 1982.)

The 57 Kd component is detected in all lymphocytes and it has been characterized previously by its unusual property of being tritiated without prior oxidation (Andersson, Wasastjerna & Gahmberg, 1976).

The 42 Kd component was identified as the MHC Class I heavy chain by precipitation with the anti-Class I MoAb W6 32 (not shown). It is expressed on T, B and CLL cells.

34 and 30 Kd bands were identified as the α and β chains of MHC Class II by their molecular weights and immunoprecipitation by the anti-Class II MoAb DA 6 231 (Guy *et al.*, 1982) (not shown). They are expressed in B and CLL cells but not the T cells we have isolated from peripheral blood.

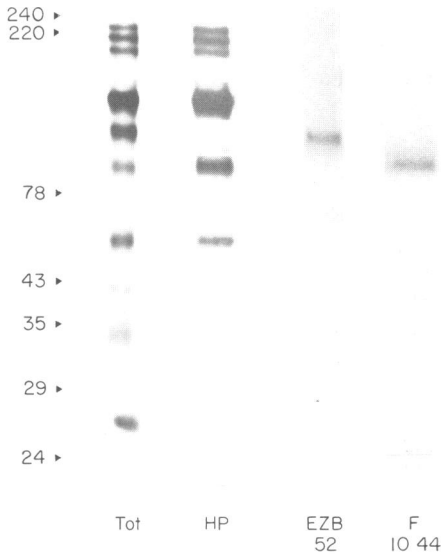

Fig. 4. Identification of the aberrant surface glycoproteins of B CLL lymphocytes by lectin and monoclonal antibody binding. Electrophoretic conditions as in Fig. 1. Tot = total Triton soluble extract of tritiated lymphocytes. HP = glycoproteins bound by *Helix pomatia* coated Sepharose beads. EZB 52 and F10 44 = immunoprecipitates obtained from the Triton soluble extract by precipitation with the MoAbs EZB 52 and F10-44-2 respectively.

Helix pomatia binding studies

This lectin was originally believed to bind to only a 150 Kd glycoprotein (Axelsson *et al.*, 1978)—presumably the l.s.g.—but subsequently other binding sites have been reported (Axelsson *et al.*, 1979). We found that in B CLL and normal T cells the lectin binds to the L-CA glycoproteins and the bands at 130 Kd (l.s.g.) 80 Kd and 57 Kd (Fig. 4). The L-CA, 80 and 57 Kd components of normal B cells do not bind the lectin. A small fraction of normal B cells has been reported to bind the lectin, and as this proportion increases in cord blood it has been concluded that HP binding is a characteristic of immature B cells (Hellström *et al.*, 1978) and, consequently, the binding by CLL cells has been interpreted as evidence for their immaturity. Since CLL lymphocytes possess several different binding sites for HP, this conclusion cannot be upheld until it has been shown that the binding sites of CLL cells are identical with these of immature cells.

In summary, it has been shown that B CLL lymphocytes express several surface glycoproteins which are absent or weakly expressed on normal B lymphocytes yet strongly expressed on normal T lymphocytes.

DISCUSSION

When the entire range of leukaemias of the B cell lineage is examined the different types can be arranged into a sequence which parallels the normal differentiation sequence of the B lymphocyte (Foon, Schroff & Gale, 1982; Anderson *et al.*, 1984). This ranking depends upon the expression by the leukaemic cells of features which characterize immature normal cells and by the effects of certain treatments, e.g. mitogens and phorbol esters, which can induce the expression of more mature characteristics in the leukaemic cells. Nevertheless it remains to be established how closely the leukaemic cells resemble normal immature cells or how reliable they are as model substitutes for the ephemeral immature stages. As the finer details of the phenotypes of the various leukaemias are described the danger of spurious comparisons based on inadequate data receeds, while at the same time it becomes progressively more difficult to identify the 'true' normal counterpart of the

neoplastic cells. The question is further complicated in CLL by the heterogeneity of the condition, both in terms of its clinical progression and the expression of surface markers such as the leucocyte-common antigen (Smith *et al.*, 1985) and immunoglobulin (Gordon *et al.*, 1983).

The designation of the B CLL cell as a small immature B lymphocyte (Foon, *et al.*, 1982; Anderson *et al.*, 1984) originally depended upon its restricted repertoire of Ig isotype, its excessive production of light chain, and its ability to bind *Helix pomatia* lectin and mouse erythrocytes (Johnstone, 1982). More recently the incomplete expression of the MHC Class II genes products has been reported (Guy *et al.*, 1983). The significance of the immunoglobulin expression and light chain export as markers of immaturity has been questioned (Kumararatne & Ling, 1983; Stevenson & Smith, 1984) and, also, we have shown that the binding of *Helix* lectin is complex, involving several binding sites. However, present knowledge of the phenotypes of the different stages of B cell development is limited, and the discovery of a small fraction of B cells in normal tonsils, lymph nodes and blood (Caligaris-Cappio *et al.*, 1982; Anderson *et al.*, 1983) with some of the features of the CLL cell, i.e. sIg^+, $HLA\text{-}DR^+$ and, particularly, mouse red cell rosetting activity and $Leu\text{-}1^+$, supports the hypothesis that the leukaemic cell corresponds fairly closely, if perhaps not precisely, with a stage in normal B cell maturation. The additional features of the CLL surface that we have described constitute further criteria for the assessment of this putative progenitor as the normal counterpart of the CLL lymphocyte.

In the meantime, the possibility that the CLL cell is of unique phenotype without normal counterpart should not be discounted. It is possible that the cell has not arisen by an abnormal proliferation of some near normal developmental stage, and the presence of the aberrant surface molecules is not due to the mere temporary cessation of normal ontogeny. Instead, it may be interpreted as a consequence of a temporal disruption of the co-ordinated genetic activity of the healthy cell. If differentiation is regarded as the co-ordinated parallel progression of several processes, rather than a simple single dependent sequence, interruption of any one process could lead to the eventual cessation of development, i.e. maturation arrest, yet in the interim the other aspects of development may continue to varying degrees, producing a cell of unique phenotype without any normal counterpart. Some facets of differentiation could have failed to run to completion, hence the apparent immaturity of the cells (these defects might be rectified by phorbol esters). But, other facets could have proceeded beyond their normally prescribed course or be overactive, and some genes which are never expressed in the corresponding normal development pathway might have been activated.

It should be possible to demonstrate a disordered asynchronous expression of the genome by analysis of a panel of patients with a wide range of markers. The effect would be that individual patients would have attained one level of maturity with respect to one marker and another level when challenged with a different marker. The conclusion of Gordon *et al.* (1983) that no correlation could be found between the expression of a battery of markers within their panel of CLL patients is consistent with this prediction. These workers found that in some patients although the leukaemic cells had the typical small lymphocyte morphology the expression of cytoplasmic immunoglobulin was reminiscent of the pre-B stage of normal lymphopoiesis. This could imply that the CLL cell represents the transitional stage between pre-B and small B lymphocyte stage, but many CLL cells express antigens normally found in late stages of B cell differentiation, i.e. activation antigens of the p45 or CD23 cluster as defined by the Second Workshop on Human Leucocyte Differentiation Antigens—Boston, 1984. (Our observations on EZB 52 and F10-44-2 could fall into this category). The anomalous expression of early and late markers in CLL is again demonstrated by the BL MoAb series (Wang *et al.*, 1984). BL1 is normally expressed by B cells at an early stage in development and BL3 at a late stage. Many CLL patients express both markers at the same time. An example of asynchronous development has also been reported for T-ALL where the cells express both MHC Class I and TdT (terminal deoxynucleotidyl transferase), while in normal maturation MHC Class I polypeptides are not acquired until TdT has been lost (Greaves *et al.*, 1981). It is possible that the appearance of apparent 'T' cell features in B CLL, both those we have described and a number of others (Gupta & Good, 1980; Bournsell *et al.*, 1980; Martin *et al.*, 1981; Kamoun *et al.*, 1981; Willard-Gallo, Humblet & Symann, 1984) are further examples of asynchronous gene expression rather than evidence for the existence within the healthy haematopoietic system of cells with an

ambiguous B/T status or that the target cells for the neoplastic transformation precede the normal B/T divergence.

In addition to these examples of asynchronous expression, the proposition that CLL is the result of a temporal disruption of genetic activity may be supported by the heterogeneity of the disease which could be interpreted as a consequence of minor differences in the ultimate outcome of the disturbance. The heterogeneity of the leukaemia is less readily accommodated within the concept of leukaemia as an abnormal proliferation, or perhaps accumulation, of some normally occurring cells without further postulating a similar level of heterogeneity among the progenitors.

Two general observations emerge as details of the surface architecture of the CLL cell are uncovered. First, the divergence of the cell surface glycoprotein profile from that of the normal B cell, or at least the most prevalent type of B cell, and, secondly, the heterogeneity of the disease. Both these features suggest that the quest for any normal counterpart of the diseased lymphocyte could be a fruitless exercise, but on a more positive note the more abnormal the surface, the greater the feasibility of exploiting a combination of defects for the development of specific therapeutic agents or regimens. The significance of the presence of these glycoproteins in the leukaemic surface on cell behaviour remains obscure until their functions in normal cells have been elucidated.

We are indebted to the Cancer Research Campaign for financial support and to Drs G. Besley, D. Kilpatrick and A. Sutherland for tonsils, leucocytes and sheep erythrocytes respectively and Mr A. Sanderson for assistance with the FACS analysis. V.A.B. holds an M.R.C. Research Studentship.

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