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Abnormalities in the expression of the leucocyte–common antigen in chronic lymphocytic leukaemia

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SUMMARY

The surface glycoproteins of lymphocytes isolated from patients suffering from B cell chronic lymphocytic leukaemia (B-CLL) have been studied by radioactive labelling with impermeable probes and with MoAb. Several features not found in normal B cells have been observed. The abnormalities found in the expression of polypeptides of the leucocyte common (L–C) antigen, identified by appropriate MoAb, have been examined in detail. It has been shown by both biochemical analysis and MoAb binding that this group of polypeptides can, within a panel of B-CLL patients, range from a typical B cell pattern to the pattern resembling that normally found in T cells. The T lymphocyte profile is correlated with a poor prognosis (MVA C rating) and in the one patient where a change in the glycoprotein profile towards that of the T cell was observed, the change coincided with a clinical deterioration. The biological significance of the molecular diversity is discussed.

Keywords leukaemia surface labelling leucocyte common antigen lymphocytes

INTRODUCTION

The diagnosis of the two major categories of chronic lymphocytic leukaemia (CLL) was, until recently, relatively straightforward, being based, for B-CLL, on the presence of surface immunoglobulin and, for T-CLL, on the ability of the lymphocytes to rosette with sheep red cells. This distinction might still be acceptable for clinical purposes, although the rosetting would probably now be replaced by the use of a suitable T cell specific monoclonal antibody (MoAb). However, from a cytological view point the distinction has become blurred by reports of the ability of B-CLL lymphocytes to bind what were originally regarded as T cell markers (Foon, Schroff & Gale, 1982: Munker *et al.*, 1983).

The significance of these ambiguities in the phenotype of the B leukaemic cell is not understood although it can be argued that, irrespective of other markers, a cell which is able to synthesize immunoblobulin is by definition a B cell, and that the apparently abnormal features, rather than jeopardising its identity as a B cell, imply that the leukaemic cell represents an early or a specialised sub-type of B cell. Thus, the affinity of B-CLL for *Helix pomatia* lectin (Hellström *et al.*, 1978) initially regarded as a T cell marker, might in fact be a reflection of the immaturity of the leukaemic cells, a conclusion in line with their limited immunoglobulin synthesis and their ability to rosette mouse red cells. On the other hand, the observation that the leukaemic cells bind anti-Y 29/55, which is a marker characteristic of non-recirculating sessile B lymphocytes, might imply that they have certain features in common with sedentary B cells (Forster *et al.*, 1982), while the existence of a small number of cells in normal tonsils which have a sIg^+, $T101^+$ (a MoAb with the same specificity

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as Leu 1), mouse RBC^+ phenotype, i.e. the phenotype of B-CLL (Caligaris-Cappio *et al.*, 1982) implies that the leukaemic cells have more in common with a previously unrecognized minor population of B cell. Whatever the ultimate significance of such characteristics, it would at present be unwise to make categorical statements as to the precise cellular status of the leukaemia.

This ambiguity surrounding the cellular origins of the disease might also be reflected in its clinical heterogeneity which ranges from a relatively benign, non-progressive form to an aggressive type with severe clinical consequences. The starting point for the present investigation was the possibility that the clinical diversity is mirrored by phenotypic differences between leukaemic lymphocytes of different patients and that these differences might be detected by study of the lymphocyte surface glycoproteins (Smith & Maddy, 1984) by surface labelling of the intact cells with chemical probes. A number of abnormalities in the surface glycoproteins of the leukaemic cells have been found and in this communication we report variation in the labelling patterns of the leucocyte-common (L–C) antigen of lymphocytes of a panel of patients suffering from CLL. All members of the panel are characterized by a monoclonal B cell lymphocytosis of cells whose ability to rosette with mouse red cells is increased by neuraminidase treatment of the red cells. In conjunction with the chemical labelling we have examined cells of the same patients for reactivity towards the two types of MoAb against the human L–C antigen, i.e. antibodies against all four polypeptide chains of the antigen complex and one that is restricted to the high molecular weight polypeptide of B cells and some T cells. (Dalchau & Fabre, 1984; Dalchau, Kirkley & Fabre, 1980; Sarmiento *et al.*, 1982; Spickett *et al.*, 1983). These immunological markers confirm the conclusions obtained by the chemical probes.

MATERIALS AND 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. Prior to labelling the cells were washed in a balanced salt solution consisting 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 lymphocytes were prepared from tonsils freshly obtained after tonsilectomy. The soft lymphatic tissue was scraped out and suspended in a balanced salt solution (133 mM NaCl, 4·5 mM KCl, 10 mM HEPES, pH 7·4 = buffer B) containing 500 u/ml penicillin + 500 μg/ml streptomycin and 5 μg/ml amphotericin (10x regular antibiotic levels) and dispersed by repeated passage through a hypodermic syringe without a needle. The large tissue particles were allowed to settle and the cells in the supernatant washed three times in the same medium. Viable mononuclear cells were isolated by the method of Davidson & Parish (1975) and monocytes depleted by incubation on glass Petri dishes at 37°C for 1 h in RPMI 1640 containing 10% of human serum +50 u/ml penicillin + 50 μg/ml streptomycin at 1×10^7 cells/ml. The bulk of the T cells were removed by rosetting with neuraminidase treated sheep erythrocytes and centrifugation over Ficoll-Paque. The remaining B lymphocytes were purified by incubation with a $F(ab')_2$ fragment of FITC (fluorescein isothiocyanate) labelled goat anti-human IgM (Kallestad), which bound to 90% of the cells, the suspension passed through an anti-FITC column (Bio-Rad), and the unbound cells discarded. The FITC positive cells were finally eluted with excess of the antibody and washed in buffer A.

Normal T lymphocytes were collected from buffy coat, from human volunteers, by leukophoresis, mononuclear cells isolated by centrifugation over Ficoll-Paque, monocytes depleted by incubation on glass Petri dishes at 37°C for 1 h in RPMI 1640 containing 10% calf serum + antibiotics. Contaminating B cells were removed using the anti-FITC column as described above. Non-adherent T cells were collected from the column elutate and washed in buffer A.

Tritiation of surface glycoproteins. The methods are based on those of Gahmberg & Hakomori (1973). (a) To 5×10^7 cells in 1 ml buffer A were added 25 μl neuraminidase *Vibrio comma* (Behringwerke AG, Marburg, iu/ml) and 50 μl galactose oxidase (Sigma Type V at 200 u/ml). After 20 min at 37°C the cells were washed twice in buffer A and tritiated for 10 min at 20°C in 1 ml of

buffer A at pH 8·0 with 2 mCi tritiated sodium borohydride. Free tritium was removed by two washes with buffer A (pelleting the cells through a cushion of 20% Percoll made isotonic with 20x concentrated buffer A) and finally one wash with buffer B. The pellet was suspended in 100 μl buffer B and an equal volume of 1% Triton X-100 containing 2 mM EDTA, 1 mM phenylmethyl sulphonyl fluoride (PMSF) and 0·5 TIU/ml aprotinin. After 10 min at 0°C the insoluble material was removed by centrifugation at 1,000**g** for 10 min and the 'Triton soluble extract', which contain all the labelled proteins but is devoid of histones, stored at −20°C. (b) Periodate oxidation, 40 μl of 0·5 M sodium periodate was added to 5×10^7 cells suspended in 1 ml buffer B and the reaction allowed to proceed for 10 min at 0°C in the dark. The cells were centrifuged out, washed once in buffer A, tritiated and solubilized as described above.

Measurement of antibody binding. Lymphocytes (1×10^6 in 20 μl RPMI 1640 containing 2% fetal calf serum) were incubated with 50 μl of the appropriate antibody at saturating dilutions for 45 min at 4°C, washed twice with RPMI 1640 containing 2% fetal calf serum + 0·1% sodium azide and then incubated with 25 μl of rabbit anti-mouse IgG-FITC, again for 45 min at 4°C. After three washes in RPMI with fetal calf serum and azide, the cells were resuspended and fixed in 0·25 ml of this medium containing 1% formaldehyde prior to analysis using a FACS IV. All but viable lymphocytes were excluded by the appropriate scatter gating.

Immunoprecipitation of antigens. The Triton X-100 content of the Triton solubilized extracts of labelled cells was increased to 1·0% and the mixture incubated at 0°C for 10 min then centrifuged for 10 min in an Eppendorf microfuge. Fifty to one hundred microlitres of the supernatant was incubated with 1 ml of antibody solution in buffer A containing 0·5% Triton X-100 and adjusted to pH 8·0 for 2 h at 20°C. One hundred microlitres of a 20% (vol./vol.) suspension of protein A–Sepharose in the same buffer was then added and the whole incubated with mixing for a further 2 h. The resulting antigen–antibody complexes were washed three times in the buffer, once in water, and finally prepared for gel electrophoresis.

Western blotting. The proteins were transferred from polyacrylamide gel by electroblotting onto cellulose nitrate paper (Towbin, Staehelin & Gordon, 1979) and blotted with a solution of the anti-L–C MoAb PD7/26 kindly donated by Dr D. Mason (Warnke *et al.*, 1983). The cellulose nitrate transfer was probed with ^{125}I-F(ab′)$_2$ sheep anti-mouse immunoglobulin (Amersham) and exposed for autoradiography.

Gel electrophoresis. The Triton soluble extract was prepared for electrophoresis by addition of an equal volume of 0·125 M Tris-HCl (pH 6·8) containing 10% (wt/vol.) SDS, 20% glycerol, 0·002% bromophenol blue, 1 mM EDTA and 100 mM dithiothreitol. This mixture was then heated at 100°C for 5 min. The polypeptide constituents were separated by electrophoresis in the presence of sodium dodecyl sulphate in the discontinuous buffer system of Laemmli (1970) with an 8–15% gradient polyacrylamide gel.

Polypeptides were stained with Coomassie brilliant blue R250. Tritiated glycopeptides were detected by fluorography after impregnation of the gels with scintillant (Laskey & Mills, 1975) before drying and exposing them to pre-flashed X-ray film.

RESULTS

The surface glycoprotein profile of lymphocytes

The glycoprotein profiles of the cell surfaces of healthy human B and T lymphocytes are shown in Fig. 1 and they agree well with published data from human (Gahmberg & Anderson, 1982). When comparing the patterns of the two cell types, two regions of the gels are of particular interest. First, the high molecular weight region where the L–C antigen polypeptides are found. In B cells this antigen is represented almost exclusively by one 230 kD polypeptide, while in T cells a band at 200 kD predominates with minor bands at 230 kD, 215 kD and 185 kD. The molecular weights attributed to these polypeptides vary between 260–185 kD (Axelsson, Hammarström & Mellstedt, 1979) and 210—160 kD (Gahmberg & Anderson, 1982) but the precise values are at present only of secondary importance especially when it is remembered that the results may be invalidated by the glycosylation of the polypeptides. We use human spectrin as a high molecular weight marker and

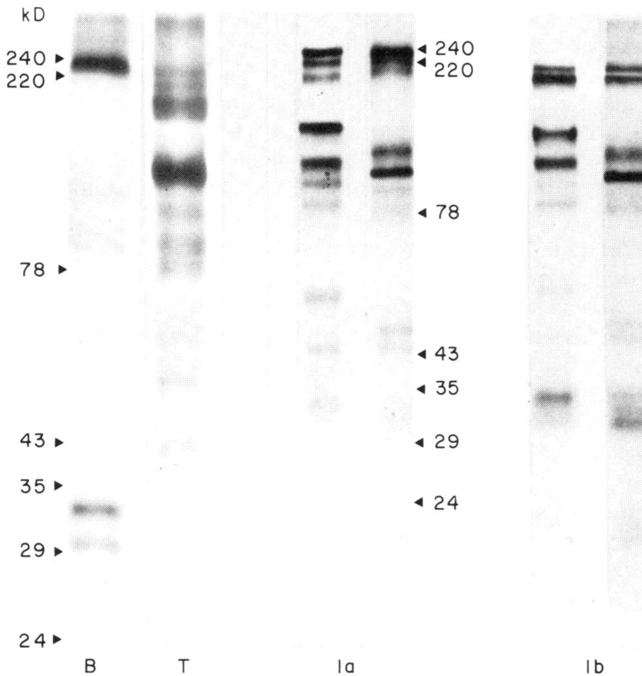

Fig. 1. Fluorograph of the surface glycoproteins of normal and leukaemic human lymphocytes. The normal lymphocytes were labelled by reductive tritiation after galactose oxidase and neuraminidase treatment. With the leukaemic cells the left lane of each pair shows cells also tritiated after galactose oxidase and neuraminidase treatment, while the samples in the right hand lanes were tritiated after periodate oxidation. The solubilised glycoproteins were fractionated by SDS-PAGE using an 8–15% gradient polyacrylamide gel. Human erythrocyte ghost proteins were used as molecular weight markers.

find that the largest L–C polypeptide has a mobility intermediate between the two spectrin polypeptides of 240 kD and 220 kD. Secondly, the intermediate molecular weight region which contains little radioactivity in B cells but contains several components in T cells, particularly a heavily labelled band which has an apparent molecular weight of 120 kD after removal of sialic acid and 100 kD in the intact molecule after periodate oxidation. This we presume to be the protein referred to as a leucocyte sialoglycoprotein (Brown & Williams 1982).

Aberrations in B-CLL lymphocytes are observed in both the high molecular weight and intermediate molecular weight regions (Fig. 1). In the latter several bands around 100 kD are always present so that the region is more akin to the normal T lymphocyte than the B lymphocyte. These results are dealt with in greater detail in a future communication. Returning to the high molecular weight region containing the L–C antigen, the typical B cell radioactive profile has been found in only four patients. Other patients have been ranked in order of the progressive appearance of the lower molecular weight forms of L–C until eventually individuals are found where the pattern is indistinguishable from both normal peripheral T lymphocytes and the one case of T-CLL examined. For the purposes of analysis we have divided patients into three categories, Type 1a where the 230 kD band is clearly predominant, although in only four out of the 12 individuals in this category is it at the 'normal' level, Type 1b (18 individuals) where the pattern approaches the T cell with a major band at 200 kD and minor bands at 230 kD, 215 kD and 185 kD and an intermediate category composed of six patients where all four bands are of approximately equal intensity (Fig. 2). This diversity is not due to variation in the sensitivity of the glycoproteins to neuraminidase as it is also apparent after periodate oxidation. It can also be detected when the cells have simply been treated with galactose oxidase, although under these conditions the low incorporation of tritium requires inconveniently long exposure of the fluorographs. In our hands the 230 kD band is only

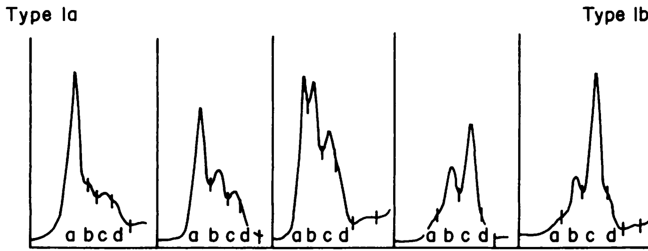

Fig. 2. Densitometer tracing of the high molecular weight regions of fluorographs of lymphocyte surface glycoproteins from four representative patients of a panel of CLL patients. The lymphocytes were tritiated after galactose oxidase and neuraminidase treatment. The peaks represent the four components of the L–C antigen and are seen to vary from the profile similar to that found in normal B cells (Type 1a) through a series of intermediate forms to the profile found in normal T cells (Type 1b).

weakly iodinated by lactoperoxidase catalysed iodination and consequently this technique is not suitable for study of the heterogeneity of L–C antigen.

Identification of the tritiated high molecular weight glycoproteins as L–C antigen

Four MoAb have been used to identify the polypeptides. Three bind to all four chains, F10-89-4 (kindly donated by Dr J.F. Fabre, PD7/26 (kindly donated by Dr D. Mason), EZB 17 (raised in our own laboratory) and F8-11-13 (also donated by Dr Fabre) which binds to the 230 kD band (Dalchau & Fabre, 1981). The results obtained by immunoprecipitation and Western blotting with the first three MoAb identify the four tritiated components as L–C antigens and the differences between patients detected by surface tritiation are conserved in the immunoprecipitates and the blots (Fig. 3).

The presence of other proteins co-migrating with the L–C antigen in the 200 kD region cannot be formally excluded at present, but as the relative labelling of the different bands in the total membrane extracts is retained after both immunoprecipitation and Western blotting such contamination cannot be extensive, at least with respect to labelled proteins.

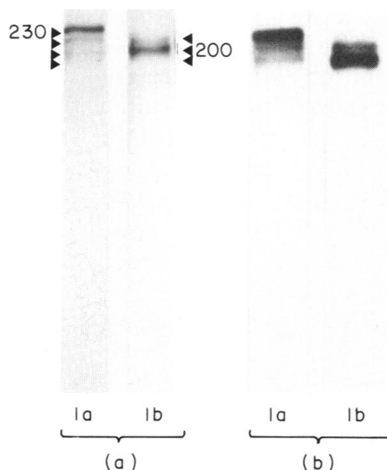

Fig. 3. (a) Fluorographs of immunoprecipitates of the L–C polypeptides from a soluble extract of the tritiated surface glycoproteins of lymphocytes from Type 1a and Type 1b patients. The antigens were precipitated with anti-L–C MoAb F10-89-4 (see text) and separated as in Fig. 1 (b) The L–C antigens illuminated by the MoAb PD7/26 on Western blots taken from electropherograms of the proteins of lymphocytes from Type 1a and Type 1b patients.

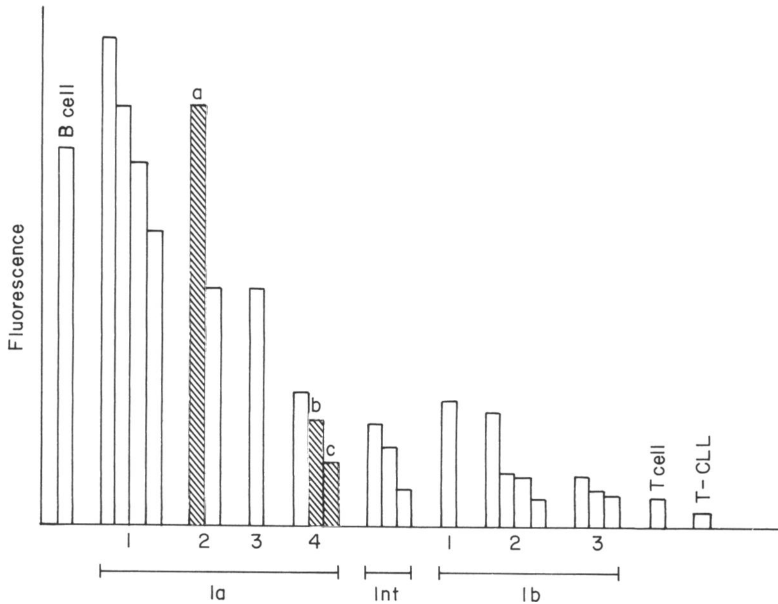

Fig. 4. The relationship between F 8-11-13 binding, as measured by fluorescence intensity in a FACS and the L–C antigen polypeptides of a panel of B-CLL patients. The major subdivisions of the L–C pattern, i.e. Type 1a and Type 1b and subdivided according to the progressive decrease of the 230 kD polypeptide into 1a 1–4 and 1b 1–3. Int = intermediate. The binding to control B and T lymphocytes, and one case of T-CLL, are shown for comparison. The shaded bars were prepared from one patient at different dates over a period when her glycoprotein profile changed (Fig. 5) and her clinical condition deteriorated. a = 28/3/83, b = 27/9/83, c = 2/2/84.

Correlation of L–C antigen profile with antibody binding

The differences revealed by tritiation are further confirmed by reaction with F8-11-13. Type 1a and Type 1b categories were subdivided by inspection of the fluorographs into subgroups according to the progressive loss of the high molecular weight band and the amount of F8-11-13 binding of these patients measured by FACS analysis. As may be seen from Fig. 4 there is a good correlation between the radioactive labelling profile and the antibody binding. The one patient whose pattern has shifted towards a Type 1b has also lost F8-11-13 binding sites. Correlations between L–C profile and other cell surface markers are currently under investigation.

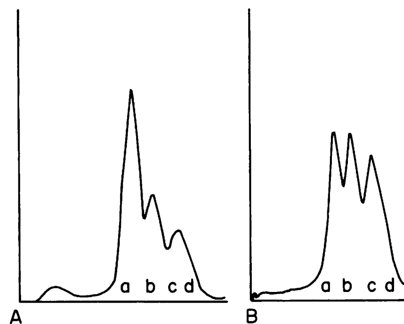

Fig. 5. Densitometer tracing of tritiated L–C antigen polypeptides separated on an 8–15% acrylamide gradient gel to illustrate the change in profile in one patient that coincided with her clinical deterioration A = 28/3/83; B = 27/9/83.

Correlation of the L–C antigen profile with clinical status

The L–C profile can be correlated with the clinical condition of patients as assessed by multivariant analysis (MVA) in two respects. First, correlation of the profile with MVA rating in patients whose clinical condition is relatively stable and, secondly, examination of the glycoproteins of patients whose clinical condition is deteriorating. Both approaches show that the predominance of the lower molecular weight polypeptides is associated with a worse prognosis.

MVA C rating is confined to patients with a T lymphocyte like profile (Type 1b) and sequential examination of the one patient whose clinical condition has deteriorated significantly over the last 9 months reveals a progressive shift from a B cell like towards a T cell like L–C profile (Fig. 5).

DISCUSSION

Differences in the labelling of the L–C antigen can be seen in published profiles of lymphocytes from CLL patients labelled by external chemical probes (Gahmberg & Anderson, 1982, Tötterman *et al.*, 1983) but as far as we are aware there has been no previous systematic investigation of this diversity. The biochemical and immunological results described above show that the L–C antigen of CLL patients can vary from a normal B cell pattern, although this is relatively rare (four of 36 individuals examined), through a series showing progressively increasing quantities of the lower molecular weight polypeptides to a pattern that resembles the typical T lymphocyte profile.

A number of possible explanations may be considered for these observations: (i) they are a trivial consequence of T cell contamination, (ii) they provide another example of a T cell marker on B-CLL lymphocytes, (iii) they reflect a fundamental heterogeneity of the B cell compartment, (iv) they represent a progression of the disease within each individual, (v) leukaemias are a consequence of a defective differentiation of the B cell and the variation between patients results from differences in the precise point of maturation arrest. Taking each possibility in turn. (i) The possibility that the variation is due to increasing levels of T cell contamination in the samples as they change from Type 1a to 1b can be excluded as T cell contamination is always measured. T cells levels, obtained either by rosetting the T cells with SRBC or by reacting the cells with OKT3 MoAb, average at 5% and there is no correlation between the L–C profile and T cell content. (ii) The possibility that the results are a consequence of the B-CLL possessing, to a greater or lesser extent, a certain T lymphocyte characteristic has some precedent. Some instances, e.g. *Helix pomatia* lectin binding might as mentioned previously, indicate the immaturity of the CLL cell rather than any affinity with T cells *per se* (Hellström *et al.*, 1978) but in other instances, e.g. the binding of pan-T MoAb such as Leu 1 or T 101, immaturity does not appear to be the explanation and perhaps indicates one aspect of the aberrant nature of the B-CLL cells. (iii) The discovery of a small fraction of cells with a CLL phenotype in normal tonsils (Caligaris-Cappio *et al.*, 1983) could imply that the B compartment is considerably more complex than has previously been suspected and the differences we observe in the L–C antigen are perhaps another expression of this complexity. However, as the diversity of the antigen in B-CLL appears to represent a continuous spectrum rather than a set of discrete subtypes, any explanation based on a naturally occurring variety of subtypes would have to presume a very large number of subtypes indeed.

(iv) If the third alternative were correct it should be demonstrable by longitudinal studies on individual patients, and if the progression of the disease caused a clinical deterioration the L—C profile would be correlated with the patient's clinical condition. Longitudinal studies are affected by the prolonged course of the disease, especially since it probably exists at a subclinical level for some time prior to diagnosis. One patient who has been monitored over 20 months has shown a change from Type 1a towards 1b which was correlated with a deterioration of her clinical condition. L–C profile is related to clinical status to the extent that MVA C rated members of our panel are all of Type 1b. Some patients are undergoing therapy, others not, but the L–C pattern does not appear to be affected by treatment. We do not confirm the correlation reported by Tötterman *et al.* (1983) between the progression of the disease and glycoproteins of molecular weight 120 kD, 72 kD and 67 kD. Attempts to correlate some feature of the leukaemic phenotype with the progress of the disease have, on the whole, been both disappointing and conflicting (Ligler *et al.*, 1983; Gordon *et al.*, 1983).

(v) The last possibility i.e. that the variations in the L–C antigen reflect different stages in the ontogenic progression of the antigen is currently under investigation. Our work is based upon the observation that phorbol ester treatment alters the expression of the antigen in CLL lymphocytes, bringing about the loss of the 230 kD polypeptide of Type 1a and changing the profile towards Type 1b. As the changes induced by phorbol ester are generally regarded as features associated with the maturation of the B cell (Tötterman, Nilsson & Sundström, 1980: Tötterman *et al.*, 1981: Guy *et al.*, 1983), and as plasma cells (at least those obtained from cases of multiple myeloma) lack both F8-11-13 binding sites and the L–C polypeptides (data not shown), it could be implied that the antigen changes its form during development and that Type 1b represents a more mature state than Type 1a. However, it would be rash to jump to a conclusion on the basis of just one cell marker and no firm decision may yet be made regarding the degree of maturity of CLL lymphocytes, if indeed they represent any stage in the normal differentiation of B lymphocytes.

It is not yet possible to determine which one, or more, of these possible factors is responsible for the diversity of the L–C antigen and further work is in progress. At present attention may be drawn to the potential of CLL patients as a valuable source of material, not only for structural studies on the antigen, but also for evaluation of the variation of its mode of expression and possibly its function. At the present juncture it cannot be ascertained whether the diversity is a valid model of the normal population or a consequence of the leukaemic condition. In either case it merits further study.

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