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The role of cell maturation in the generation of phenotypic heterogeneity in B-cell chronic lymphocytic leukaemia

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SUMMARY

B-chronic lymphocytic leukaemia (B-CLL) patients can be ranked along a progression of phenotypes characterized by a decreasing surface expression of CD20, CD21, CD22 and membrane immunoglobulin and a gradual replacement of the high molecular weight (MW) glycoproteins of the leucocyte-common antigen (LC) CD45RA by the lower MW components, including the CD45RO determinant. As CD20, CD21, CD22 and membrane immunoglobulin change during or after B-cell activation, and the CD45RA/CD45RO inversion is implicated in T-cell maturation, the possibility that the phenotypic differences are generated by a maturational diversity of the CLL clones has been investigated by testing the effects of TPA treatment of the leukaemic cells. TPA reduces the level of expression of CD20, CD21, mIg and CD45RA and increases CD45RO binding, thereby minimizing the phenotypic heterogeneity of the CLL clones and causing them to converge towards one end of the natural range. We propose that the phenotypic diversity in CLL is, at least in part, a consequence of maturational diversity where lymphocyte development is disrupted at different stages in different patients.

INTRODUCTION

B-chronic lymphocytic leukaemia (B-CLL) is diagnosed as a monoclonal proliferation of small B lymphocytes which deviate phenotypically from the majority of peripheral B cells by their ability to rosette mouse erythrocytes and by the surface expression of CD5 antigen. Although the condition is treated as a single pathological syndrome, both the clinical variability and phenotypic diversity reveal a significant heterogeneity within the disease. Cytologically perhaps the most striking evidence for this diversity is the presence of variable numbers of large cells (Melo *et al.*, 1986), but it is also apparent from detailed analysis of surface markers—even the CD5 antigen cannot be detected in a small minority of patients (Caligaris-Cappio *et al.*, 1987).

In previous communications (Brown *et al.*, 1985; Smith *et al.*, 1985) we examined the diversity of the expression of the leucocyte-common antigen (LC) on the leukaemic lymphocytes of different patients. Its expression varies from one extreme, where the antigen is expressed on a single 220,000 MW glycoprotein, through a series of intermediate forms, which arise by the progressive loss of 220,000 MW and the acquisition of the 190,000 MW components, to the other extreme where the LC profile is dominated by a 190,000 MW glycoprotein with little of the 220,000 MW chain. Although the variation between patients appears continuous across the panel, for the purposes of analysis we subdivided the patient panel into Type 1a patients where the high molecular mass glycoproteins predominate and Type 1b patients where the 190,000 MW glycoprotein is predominant. These differences were demonstrated by vectorial labelling of the lymphocyte surface and by flow cytometry with the CD45RA monoclonal antibody (mAb) F8-11-13. Subsequently we showed tht the variation in LC is correlated with mIg expression, Type la patients having a higher expression of mIg (Brown *et al.*, 1987).

The variation in LC expression has now been confirmed (Roxburgh & Cooper, 1987), and recently the expression of mIg has been correlated with CD21 and CD22 expression (Merson & Brochier, 1988). We now wish to report that the differences between the LC of individuals are correlated with differences in their expression of CD20, CD21 and CD22 as well as mIg and that the loss of the high MW components of LC, detected by CD45RA binding, is accompanied by the reciprocal appearance on the cell surface of the 180,000 MW component, as detected by binding of the mAb UCHL1 (CD45RO) (Smith *et al.*, 1986; Terry, Brown & Beverley, 1988) together with the 190,000 MW glycoprotein. Thus our Type 1a phenotype may now be characterized by a trend towards a relatively high expression of mIg, CD20, 21 and 22 along with the high MW components of LC. Furthermore, since a fall in CD20, 21, 22 and mIg and the

Abbreviations: CLL, chronic lymphocytic leukaemia; FITC, fluoresceine-labelled; HCL, hairy cell leukaemia; LC, leucocyte common antigen; mAb, monoclonal antibody; mIg, membrane immunoglobulin; PLL, prolymphocytic leukaemia; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

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Table 1. Antibodies used in this study

		Source	
CD3	UCHT1	Unipath Ltd	IgG1
CD5	UCHT2	Unipath Ltd	IgG1
CD20	B1	Coulter Ltd	IgG2a
CD21	RFB6	Scottish Antibody Production Unit	IgG1
CD22	RFB4	Scottish Antibody Production Unit	IgG1
CD23	MHM6	Presented by Dr A. J. McMichael	IgG1
CD25	ACT-1	Dako Ltd	IgG1
CD45	EZB17	Edinburgh University Zoology Dept	IgG2a
CD45RA	F8-11-13	Presented by Drs Dalchau & Fabre	IgG1
	WR16	Cymbus Bioscience Ltd	IgG1
CD45RO	UCHL1	Presented by Dr P. C. Beverley	IgG2a

switch in LC expression have been reported to occur during lymphocyte development (Akbar *et al.*, 1988; Freedman *et al.*, 1987a, b), we suggest that the phenotypic diversity of CLL reflects a variation in the maturity of the cells of different patients. Changes that can be induced by TPA treatment of the leukaemic cells are consistent with this proposal and imply that the 1a phenotype represents a less mature stage than the 1b phenotype.

MATERIALS AND METHODS

Chemicals

Reagents were supplied by Sigma Chemical Co. (Poole, Dorset) unless otherwise indicated.

Patients

The panel consisted of patients attending the Haematology Department of the Western General Hospital, Edinburgh.

Diagnosis of B-CLL was made on the basis of a monoclonal lymphocytosis of B cells whose ability to rosette with mouse erythrocytes was enhanced by sialidase treatment of the red cells (Catovsky *et al.*, 1976).

Cell preparation

Leukaemic lymphocytes were isolated from freshly collected blood by centrifugation on Ficoll–Paque (Pharmacia, Uppsala, Sweden) for 15 min at 400 *g* and washed in RPMI 1640 medium (Flow Lab., Irvine, Ayrshire). The viability, monitored by acridine orange/ethidium bromide staining, was routinely found to be >95%.

Measurement of antibody binding

Lymphocytes (1×10^6 in 20 μl RPMI-1640 containing 2% fetal calf serum (FCS) (Sera Laboratories, Oxford) and 0·1% sodium azide were incubated with 50 μl of the appropriate antibody (Table 1) at saturating dilutions for 45 min on ice, washed twice with the same medium and then incubated with 25 μl sheep anti-mouse IgG-FITC $F(ab')_2$ fragment for 45 min on ice. After three washes the cells were fixed in 0·25 ml of this medium containing 1% formaldehyde. The amount of antibody bound was finally measured by flow cytometry in a Becton-Dickinson FACS IV, which was calibrated with fluorescent monodisperse carboxylated microspheres (Polysciences Inc., Northampton) immediately before use. The reproducibility of this method has been discussed previously (Brown *et al.*, 1987). Antibody binding was expressed as the difference between the peak fluorescence channel number of cells treated with antibody + FITC second-step and that of cells treated with only the FITC second-step $F(ab')_2$ fragment. TPA effects were estimated by increase or decrease in these values.

Phorbol ester treatment

Cells were incubated at 1×10^6/ml at 37° for up to 6 days in RPMI-1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 1 mM pyruvate, 50 U/ml penicillin 50 μg/ml streptomycin, 2·5 μg/ml amphotericin B. The effect of phorbol ester was tested by the addition of TPA at $1{\cdot}6 \times 10^{-7}$ M or TPA at $1{\cdot}6 \times 10^{-7}$ plus A231877 at 7×10^{-7} M.

Surface labelling

The vectorial tritiation of the lymphocyte surface by tritiated borohydride after neuramindase and galactose oxidase treatment, gel electrophoresis and fluorography of the tritiated proteins was carried out as described by Smith *et al.* (1985).

RESULTS

The variation in the expression of the LCA

On the basis of the variation of LC between individuals observed by surface labelling and flow cytometry by Smith *et al.* (1985), for the convenience of analysis we separated the two

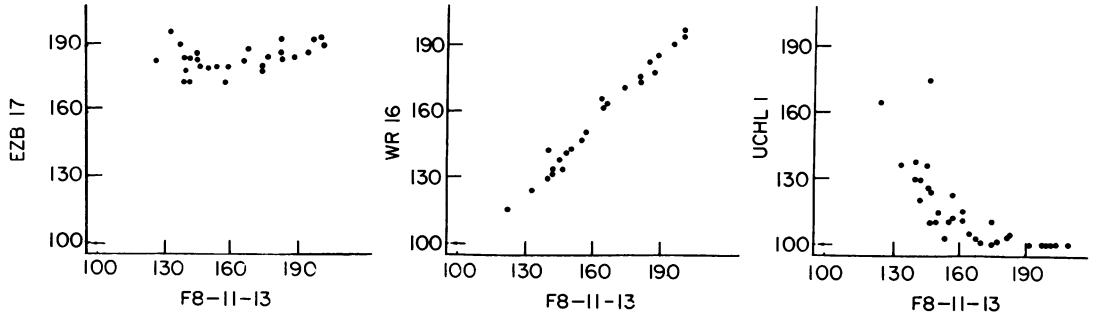

Figure 1. The relationship between the expression of CD45 antigens on the surface of the leukaemic cells of individual B-CLL patients. The binding of EZB17 (CD45), WR16 (CD45R) and UCHL1 (CD45RO) is compared with the binding of F8-11-13 (CD45RA) for each patient. Binding of each antibody is expressed by its peak fluorescence channel number. The arrow indicates the fluorescence of negative control cells.

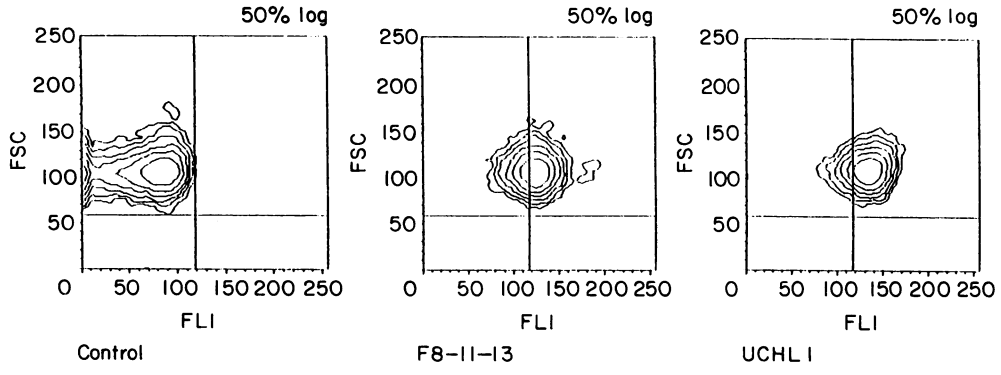

Figure 2. The binding of F8-11-13 and UCHL1 to a Type 1b CLL lymphocyte. Over 90% of the cells are present in the single positive population with both antibodies.

halves of the distribution into two groups, Type 1a patients where the higher MW forms of LC predominated and Type 1b where the 190,000 MW form was dominant. Figure 1 confirms and extends this earlier work; it shows that the overall expression of LC measured by CD45 binding remains relatively constant across the panel, although the binding of CD45RA falls. CD45RA is detected by the mAb F8-11-13 and a similar mAb WR16. These two reagents bind to different epitopes, F8-11-13 binds to the 220,000 and 205,000 MW chains (Dalchau, Flanagan & Fabre, 1986) but WR16 binds almost exclusively to the 220,000 MW component (K. Moore, personal communication). The relative constancy of CD45 is due to the replacement of the higher MW components of LC by the lower MW counterparts. The expression of the 190,000 and 180,000 MW chains has previously been demonstrated by surface tritiation (Smith *et al.*, 1985). The acquisition of the 180,000 MW chain can now also be detected by CD45RO (UCHL1) binding in Type 1b patients; no binding is detectable to Type 1a cells. In Type 1b patients over 90% of the cells belong to a single positive population for both F8-11-13 and UCHL1 (Fig. 2). Repeated analyses of LC in several patients over a period of years has shown that, with only two exceptions, the LC phenotype of an individual remains constant throughout the course of the disease.

Table 2. The Edinburgh B-CLL patient panel at presentation

	Type 1a	Type 1b
No. of patients	46	44
Male/female (%)	68:32	67:33
Average age (years)		
Male	65·8 ± 10·6	62·8 ± 10·4
Female	70·2 ± 9·3	73·3 ± 7·4
M.V.A. (% of panel)		
A	51	67
B	18	12
C	31	21
$CD5^+$ (% of panel)	100	100
Mouse red cell receptor (% of panel)	100	100

The age and sex ratios of the two groups are not significantly different. M.V.A. ratings at diagnosis indicate a slightly poorer prognosis for Type 1a and this is confirmed by a survival study which is in progress (Table 2). Asymptomatic individuals are observed in clinics without treatment: treatment, usually with chlorambucil, is initiated as appropriate when indicated by symptomatic changes in lymphadenopathy, oraganomegaly or lymphocytosis. Treatment has no observable effect on LC expression.

The relationship between LC expression and other surface markers

The expression of LC has been compared with a number of other antigens of the B-cell surface by the binding of the appropriate mAb: CD20 (B1), CD21 (RFB6) and CD22 (RFB4), three antigens lost from the surface at various stages during cell activation; CD23 (MHM6), an antigen expressed during the activation of normal cells; and CD5 (UCHT2), whose determinant is characteristically expressed by B CLL cells but is restricted to a minor B-cell population in healthy adults. CD20, CD21 and CD22 binding is positively correlated with the increase in CD45RA, the correlation coefficients are all significant at the 0·001 level. (CD20, $n=56$, $r=0·543$; CD21, $n=46$, $r=0·626$; CD22, $n=46$, $r=0·564$.) Figure 3 shows the positive slopes obtained by linear regression of CD20, 21 and 22 against CD45RA. CD22 expression is never strong; in 30% of cases the treated cells are less than five channels brighter than the untreated control cells. The level of binding of CD5 and CD23 varies considerably between patients but shows no significant correlation with CD45RA. Absence of correlation has also been found for gpL115 (anti-sialophorin), EZB 52 and CD44 (F10-44-2) (Brown *et al.*, 1985). CD25 (IL-2R) is not detected in 80% of patients prior to TPA treatment. The LC phenotype with the exception of two patients remains stable throughout the course of the disease (above). Systematic longitudinal studies of the other antigens have not been attempted, but in those patients which have been retested the results remain constant.

Immunoglobulin phenotypes

The surface immunoglobulins of Type 1a and 1b patients have been described previously (Brown *et al.*,1987). The majority of patients are IgM^+ IgD^+, but the total surface immunoglobulin

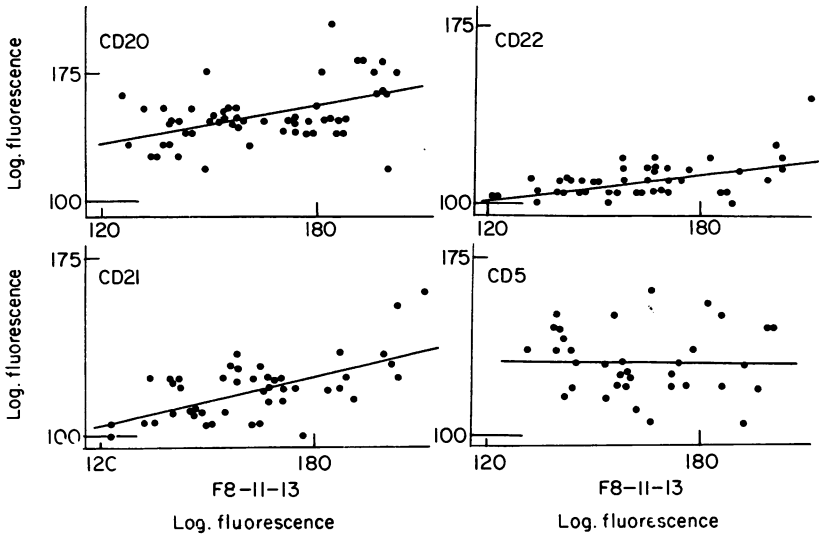

Figure 3. The relationship between the expression of CD20, CD21, CD22 and CD5—abcissas—surface antigens of CLL patients and their expression of CD45R (F8-11-13)—ordinates. Correlation coefficients of CD20, CD21, CD22 against CD45RA are all significant at the 0·001 level. The curves are obtained by linear regression. The results obtained with antibodies that are not correlated are illustrated by the data obtained with CD5 mAb. The negative control cells have a peak fluorescence channel value of 100.

expression is greater in 1a than 1b. Our more extended analyses have confirmed the difference in light chain expression between the two groups. In Type 1a, the kappa-lambda ratio is 38:8, while in Type 1b it is 22:22.

The Type 1a phenotype is therefore characterized by a strong expression of the CD45RA components of LC, correlated with the relatively strong expression of the CD20, CD21, CD22 and surface immunoglobulin, while in the Type 1b phenotype all these features are weakly expressed, with a concomitant increase in the expression of the CD45RO determinant. The traits represent a progressive trend within the panel of patients as the 1a phenotype merges into the 1b phenotype. All facets of the phenotype may not be present in each patient, e.g. it may be seen that some F8-11-13 bright (1a) patients have very little CD20 and one patient is CD21$^-$ (Fig. 3).

The effects of TPA treatment

The treatment was monitored by flow cytometry at 2, 4 and 6 days. Any non-viable cells (circa 20% after 6 days) were gated

Table 3. The effect of TPA on antibody binding

Patient	CD45	CD45RA	CD45RO	CD20	CD21	CD22	CD23	CD25	CD5	K/Δ
1	+	−−	+++	−−−	−−	0	+++	+++	0	+
			++	−−				++		
2	−	−−	+++	−−−	−−−	+	+++	+++	0	−−
			++	−	−		++	+		
3	−−−	−−−	+++	−−−	−	0	+++	+++	−−	−−
	−			−−						
4	0	0	+	+++	−−	+++	+++	++	0	0
5	0	−−	++	−	0	+++	++	+++	++	0
6	−	0	0	−−	0	+	+++	0	−−	−−
							++			
7	−	−−	++	ND	0	++	ND	ND	ND	−−
8	0	0	+++	0	−−−	+++	+++	0	−	−−−
9	−−	−−	0	−−−	−−−	0	+++	−−	−	++
				−−	−−		++			
10	−−−	−−−	++	−−	−−	+	ND	+++	ND	+
11	0	−−	+++	−−	−	+	ND	+++	+	−−−
										−
12	−−−	−−−	+++	−−−	−	++	ND	+++	−−	0
	−			−						

The increase or decrease caused by TPA treatment is expressed as the change in peak fluorescence channel number. 0 < 5 channels difference; ± 5–9 channels; ± ± 10–19 channels; ± ± ±20–29 channels; ± ± ± ± 30–39 channels; ± ± ± ± ± >40 channels; ND, not determined.

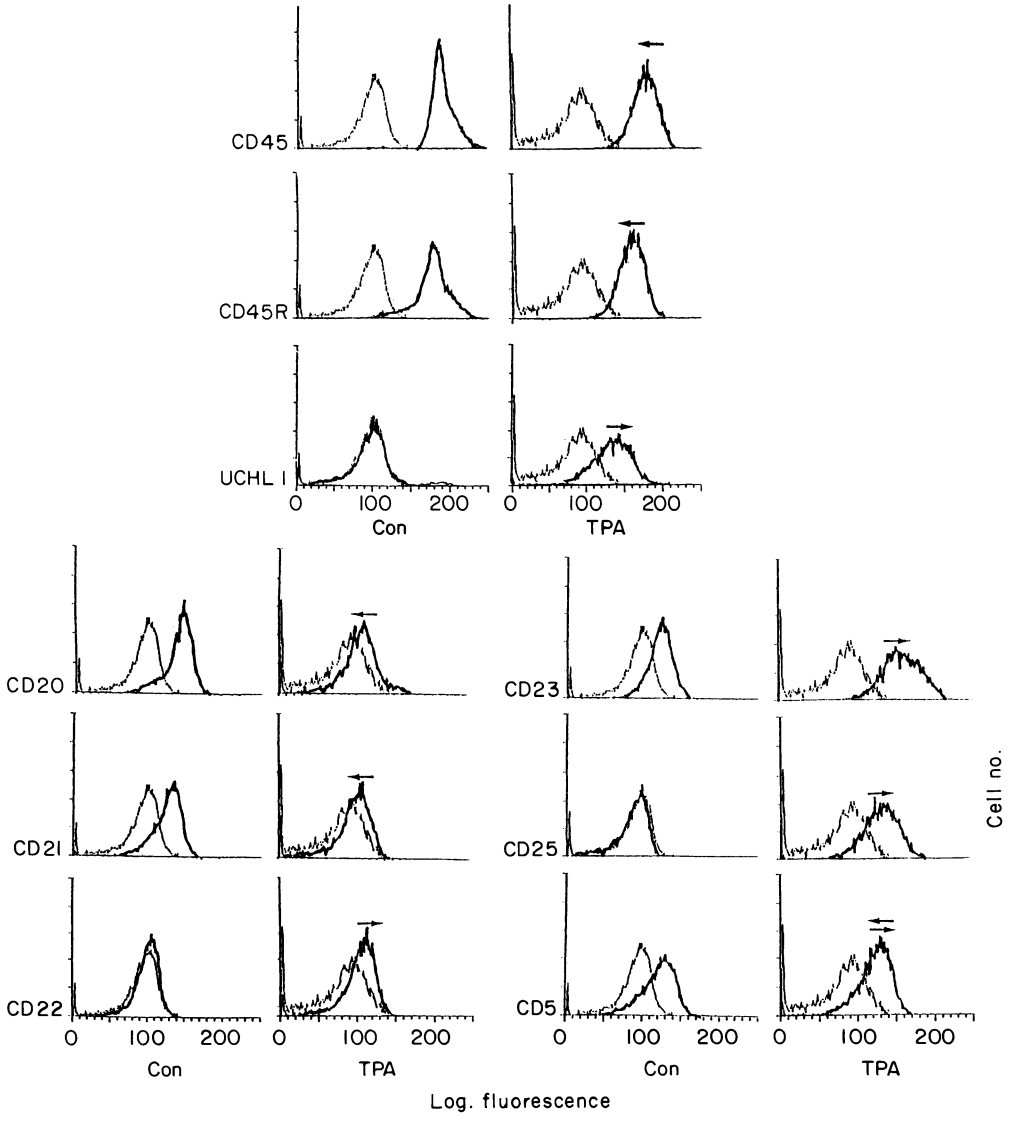

Figure 4. The effect of TPA on the surface expression of antigens on the CLL surface. The fluorescence of cells treated with the second-step reagent alone is indicated by the light trace and the antibody treated cells by the heavier trace. The effect of TPA on the expression of each antigen is indicated by the heavy arrows. Con = cells at day 0. TPA = cells incubated 4 days.

out before analysis. In TPA the proportion of large cells increased over the first 4 days and the changes in antibody binding reached their maxima after 4 days. Addition of A23187 had no significant effect on the antigen changes observed with TPA alone. [The ionophore is stated to have a minimal effect on proliferation at the concentration used (Drexler *et al.*, 1987).] Although, as has been previously reported (Okamura, Gelfaud & Letarte, 1982), cells from different patients vary in the extent of their response to TPA, a clear pattern of changes in surface antigens is obtained from a study of a group of patients: the binding of some antibodies increases, the binding of others decreases and others show no particular trend (Table 3, Fig. 4). The changes in surface antigens mirror the differences observed between untreated patients. The Type 1a phenotype is converted to Type 1b with respect of LC, CD20 and 21 but not CD22. Surface Ig falls, but there is no preferential effect on IgD. Changes in CD5 are small and irreproducible between patients, confirming Hermann *et al.* (1985) rather than Miller & Gralou (1984). The replacement of the high MW components of LC by the smaller components can also be demonstrated directly by vectorial labelling of the cells (Fig. 5). This interchange is consistent with the smaller downward trend in CD45 compared with CD45RA, revealed by flow cytometry. The average T-cell content of the samples measured by CD3 binding fell from 6% to zero during TPA treatment.

The effect of incubation in the absence of TPA or ionophore varies with the batch of FCS used. In some serum samples the viability is low after 48 hr, but in others the cells survive well,

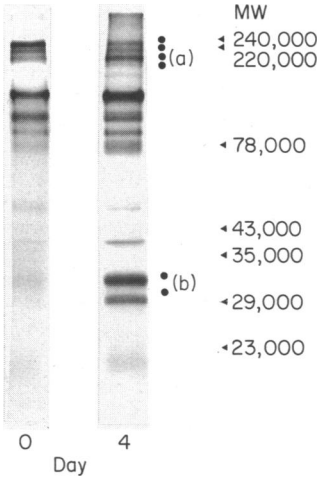

Figure 5. Fluorographs of tritiated surface glycoproteins of the B-CLL lymphocyte to show the changes induced by TPA in 4 days, particularly the change in the relative proportions of the polypeptides of (a) the leucocyte common complex, (b) MHC class II. The identity of the various components of the profiles have been established previously (Smith *et al.*, 1985).

without any apparent morphological change. Most of the surface antigens studied do not change, but there is a consistent decrease in CD20 and increase in CD22 and CD25.

DISCUSSION

The diversity of B-CLL can be expressed by variation in the surface antigens of the leukaemic cells. The above results reveal correlated changes in the surface expression of several surface components. Such phenotypic differences between the leukaemic cells of individual patients might arise from several sources. One such cause could be variation in the level of maturation of the leukaemic cells in different patients. If maturational diversity plays a significant role, the differences between patients would be a reflection, perhaps distorted by pathology, of stages that occur during normal B-cell development. Comparison of patient phenotypes with (i) the stages of normal lymphocyte development, (ii) the immunoglobulin light chain expression of the leukaemic cells, and (iii) the effects of TPA on the phenotypes have suggested to us that maturational diversity is operative and that the Type 1b CLL phenotype represents a more mature cell than the 1a phenotype.

Comparison of the CLL cell phenotypes with stages of normal lymphocyte development

At various times during or after activation normal lymphocytes lose mIgD, CD20, CD21 (Stashenko *et al.*, 1981; Gordon *et al.*, 1984) and CD22 (Dorken *et al.*, 1986; Freedman *et al.*, 1987a, b). If the loss of these markers therefore indicates the stage of development of a B cell, the change of CLL phenotype as it progresses from Type 1a to 1b may be interpreted as an ontogenic process, and the phenotypic heterogeneity between individuals as an expression of maturational diversity. A similar interpretation is possible for the presence of CD45RO in 1b patients, although in this case the connection between CD45RO binding and B-cell development is tenuous. UCHL1 does not bind to the majority of B-cell lines, nor can binding be induced in lymphoblastoid B cell lines (Smith *et al.*, 1986), nor is it detectable in tonsil cells. However, presence of the determinant on myeloma lines could imply an involvement in later stages of B-cell maturation (Smith *et al.*, 1986). In T lymphocytes, activation of $CD45RA^+$ $CD45RO^-$ T cells induces the loss of CD45RA and the acquisition of CD45RO, a process interpreted as a stage in the maturation of primed T cells (Akbar *et al.*, 1988; Byrne, Butler & Cooper, 1988). This apparent similarity between the B-CLL cells and a T-cell subset could be a pathological feature or a property of a minor B subset that is not at present recognized except as its leukaemic product (B-CLL).

Light chain expression

The difference in kappa/lambda ratio between 1a and 1b may also be interpreted in an ontogenic context. Since kappa expression usually leaves the lambda genes in their germ lines configuration, kappa leukaemias have been regarded as more immature cells than lambda leukaemias (Korsmeyer *et al.*, 1981; Martinez *et al.*, 1988), a proposal which again points to 1a leukaemias being less mature than the 1b variants.

TPA treatment

The changes induced by TPA further support the proposal that some of the variations between the patients' cells are attributable to differences in their maturity. TPA, by inducing activation, may be expected to modulate those aspects of phenotypic heterogeneity which have been generated by maturational diversity, namely CD20, CD21, CD22, mIg expression and differences in LC. The loss of CD20, CD21 and mIg, which reproduces the differences between untreated cells, have previously been reported for other cells (Gordon *et al.*, 1984; Stashenko *et al.*, 1981). Although the increase of CD22 caused by TPA (confirming Hermann *et al.*, 1985; Zeigler-Heitbrook *et al.*, 1986) might seem to be the exception, changes in CD22 during activation are complex, increasing initially but then declining (Dorken *et al.*, 1986). An increase of CD25 expression accompanied by a decrease in CD45RA has previously been observed during T-cell maturation (Ledbetter *et al.*, 1985). TPA induces similar changes in both Type 1a and 1b patients, but the overall effect is to shift the 1a phenotype towards the 1b phenotype and the phenotypic diversity of the panel is constricted towards one end (1b) of its natural range.

Although this body of evidence might indicate maturational differences between CLL clones, comparison of leukaemic phenotypes with normal phenotypes is fraught with difficulty. Not only is defective maturation a hallmark of leukaemias, little is known of alternative differentiation pathways in B-cell subsets, particularly that of the 'normal counterpart' of CLL (Freedman *et al.*, 1987a, b). Temporal derangement of CLL maturation is apparent from the simultaneous expression of early and late markers of normal development, such as mouse erythrocyte rosette receptors (Melo *et al.*, 1986) and CD23 (Freedman *et al.*, 1987a, b). Against such a background apparent anomalies as the loss of IgM as well as IgD and changes in the LC, which have previously been seen only in T cells, are not too surprising. Similar ambiguities arise when CLL diversity is considered in relation to PLL and HCL. In view of

the similarity between Type 1a and PLL and HCL phenotypes, which some workers believe to represent relatively mature leukaemias (Gale & Foon, 1987), we previously suggested that Type 1a might represent a more mature phenotype than Type 1b. We now see that more extensive data on the CLL phenotype and comparison with normal lymphocyte maturation leads to the opposite conclusion.

The data we present suggests that the phenotypic diversity in CLL is a consequence of a disordered and truncated maturation of the leukaemic cell. Nevertheless, several anomalies stress the danger of a simplistic application of the concept of maturation arrest and may also indicate the importance of other forces in the generation of phenotypic diversity. Disruption of a single element of a multicomponent process such as differentiation could lead to a variety of end points which may depend not only on the neoplastic clone but on other cells involved in development. While a primary neoplastic event may occur in the leukaemic clone, its penetration into the maturation of the clone could be influenced by the haematopoietic environment in which the clone develops. The effect of incubation in the absence of TPA suggests that the CLL cells are capable of further differentiation. Whether these changes are induced by signals in the FCS or a consequence of removal of the cells from inhibitory forces in the patient's own blood is not clear. The importance of extrinsic factors in the generation of the diversity of CLL lymphocytes is currently under investigation.

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