Development of B Cell Subpopulations in Humans and its Relevance to Malignancy

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A. Introduction

B cell lineages are influenced by antigen-independent development and by immune activation when antigens stimulate the development of both memory B and plasma cell clones. It is difficult to investigate the origin of B cell malignancies without knowing the details of these physiological events. In 1974, Salmon and Seligmann [1] suggested that the various types of B lymphoid malignancies may derive from normal cells representing different levels of maturation. In addition, the degree of differentiation which takes place within the malignant clone also seems to be variable: some malignant cells, e.g. chronic lymphocytic leukaemia (CLL), are apparently "fixed" and unable to differentiate further, while in another disease, e.g. myeloma, the lymphoid or lymphoplasmacytoid "target cells" of malignancy differentiate into plasma cells. In our study we have, therefore, decided to approach the origin of B lymphoid disorders with a simple approach by asking the following questions:

1. What are the phenotypic features of B lymphocytes as they emerge in the normal bone marrow (BM) during childhood? The main aim here has been to find an answer to the question whether B cell populations in the BM versus peripheral lymphoid organs have mutually distinctive features.

2. How do B cells develop in the normal fetus? Do B cells at different sites show distinct phenotypic features? In this part of the study samples of fetal BM, para-aortic lymph nodes and spleen have been studied.

3. Can B cell types (and the corresponding B cell malignancies) change their features when stimulated in vitro?

There have been many previous clues about the early stages of B cell differentiation both in humans and animals (reviewed in [2, 3]). Small to medium-sized lymphoid cells with terminal transferase (TdT) enzyme activity are the likely precursors of larger pre-B cells which synthesize small amounts of cytoplasmic (Cy) μ heavy chain first, but no light chain [4, 5]. These pre-B cells, in turn, give rise to B cells with membrane IgM, but no IgD expression, and probably also to a proportion of IgM$^+$, IgD$^+$ B cells during the next stage of development [2, 6]. But these latter cells constitute a mixture: some of them (particularly during secondary responses) appear to return to the BM from the peripheral lymphoid organs [7]. Our aim has been to establish the reactivity of these cell types using a wide range of monoclonal antibodies (MoAb) against various non-Ig membrane antigens in combination with the analysis of Ig isotype expression. Thus, the purpose of the study is to endow the hypothetical differentiation scheme with further discriminating reagents. Clearly, Ig isotype expression alone is not sufficient for clear discrimination; e.g. mature B cells lose IgD [6]; IgM$^+$, IgD$^-$ cells, without

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further markers, cannot be affiliated to a given (early or late) maturation stage.

B. Methods

"Double-marker" methods were used with green fluorescein (FITC)- and red rhodamine (TRITC)-labelled antibodies in various combinations [4, 8]. For example, the reactivity of MoAb was studied on TdT$^+$ cells (labelled with rabbit anti-TdT) and μ^+ as well as δ^+ B cells (labelled with an anti-IgM and anti-IgD antisera, heavy chain specific, respectively). The overall proportion of μ^+, δ^- and μ^+, δ^+ cells was also determined in each sample. In histology the same double-immunofluorescence technique [8] and immunoperoxidase was used together with haematoxylin counterstaining on sections of frozen biopsies.

The induction of differentiation changes using 12-O-tetradecanoylphorbol-13-acetate (phorbol ester: TPA) was an empirical method to assess links between cells expressing different phenotypes. The concentration of TPA in experiments with cell lines was 10^{-7} M and with leukaemic cells was 1.6×10^{-8} M [9, 10].

The samples used were from normal child BM or regenerating samples taken from cessation of chemotherapy for AML or ALL. The fetal tissues were from medically approved abortions. All suspensions, including samples from patients with chronic lymphocytic (CLL), hairy cell leukaemia (HCL) and blood samples from normal donors, were separated on Ficoll–Hypaque gradients.

C. Results

I. Phenotypic Differences Between B Cells in the BM and Peripheral Lymphoid Organs

The MoAb used are shown in Table 1. The important common feature of B1, To15, RFB-4, RFB-6 and Y29/55 is that, when tested in double-marker combination with IgM in suspensions of blood and tonsil, these reagents react with >92% of IgM$^+$ B cells. These are, therefore, pan-B reagents by these criteria. Still, when these anti-

bodies were tested on BM B cells, a heterogeneity emerged which was related to the cells' Ig isotype. While the IgM$^+$, IgD$^+$ cells showed the characteristic pan-B staining (>92% IgD$^+$ cells positive) with all these antibodies, the IgM$^+$, IgD$^-$ population was, in contrast, essentially negative with To15, RFB-4 and RFB-6 and had only about 50% reactivity with Y29/55. In addition, pre-B cells and the TdT$^+$ BM precursors were also negative with this set of B reagents (Table 2). These results can be interpreted to indicate that membrane antigens appear during B cell development in an orderly fashion. B1 is an "early marker" positive on the whole lineage starting from TdT$^+$ cells [11]. Other markers such as Y29/55 appear halfway through the generation of IgM$^+$ cells, and additional markers To15, RFB-4 and RFB-6 are expressed when IgM$^+$ cells also acquire IgD$^+$ and become functionally mature B cells. (This confirms previous results with RFA-2 and RFA-3; [12].)

There are two additional facts to support this scheme. First, another antibody, AL-1, showed a complementary pattern: strongly positive on TdT$^+$ and pre-B cells, weaker on IgM$^+$, IgD$^-$ cells, but negative on the mature IgD$^+$ B cells of peripheral lymphoid tissue (Table 2). Second, the antigens To15 and RFB-4, albeit absent from the cell membrane of pre-B and IgM$^+$, IgD$^-$ cells, can be found in the cytoplasm of the same cells. As Campana et al. [9] have recently shown, blast cells in fresh cases of common acute lymphoblastic leukaemia and permanent cell lines of non-T, non-B and pre-B type (KM3, REH and NALM-6) also show a cytoplasmic, but no membrane, expression of To15 and RFB-4. The interesting additional result is that these lines start expressing membrane To15 and RFB-4 when incubated with TPA for 48–72 h. It seems therefore that not only cytoplasmic μ can change to membrane IgM during B cell development, but some other molecules also demonstrate a similar sequence of events [9].

II. Investigations of Fetal BM

Around the 17th gestational week the BM contains large numbers (40%) of lymphoid-

Table 1. Antibodies and heterologous antisera used in this study

Name	Source	Reactivity	Species and class	Molecular weight
Monoclonal Ab-s				
AL-1	A. M. Lebacq	Pre-B, B	Rat IgG$_2$	120 k
BA-1	T. LeBien and Hybritech (Cat. No. 0452)	Pre-B, B, polymorph	Mouse IgM	30 k
B1	Coulter (Cat. No. 6602140)	Few pre-B, B	Mouse IgG$_2$	32 k
To15	D.Y. Mason and Dakopatt (Cat. No. M708)	B	Mouse IgG	140 k
RFB4	Royal Free Hospital	B	Mouse IgG$_1$	140 k
RFB6	Royal Free Hospital	B	Mouse IgG$_1$	180 k
Y29/55	H.K. Forster	B	Mouse IgG$_2$	
Anti-Tac	Drs. Uchiyama & Waldmann	IL-2 receptor	Mouse IgG	
SHCL-3	Dr. G. Schwarting	Hairy cell leukaemia, monocytes	Mouse IgG	
Heterologous antisera				
Anti-Hu IgM – F(ab′)$_2$	Kallestad (Cat. No. 140)		Goat	900 k
Anti-Hu IgD – F(ab′)$_2$	Kallestad (Cat. No. 138–148)		Goat	190 k
Anti-Ia	Royal Free Hospital		Chicken	28 + 33 k
Anti-TdT	F. J. Bollum and Supertech (Cat. No. 004)		Rabbit	67 k

Table 2. The reactivity of pre-B and B cells in the infant (4 samples) and regenerating bone marrow (4 samples) with B cell-specific Ab[a]

Ab	TdT$^+$	Pre-B	sIgm$^+$ B cells Total	sIgD^{+b}
Heterologous antisera				
sIgD	<1	<1	33±7	100
Monoclonal antibodies				
AL-1	70±5	80±9	83 ±5	53±2
BA-1	80±6	98±1	98 ±1	92±3
B1	10±2	98±1	97 ±3	98±1
To15	<1	<1	24 ±10[c]	98±1
RFB-4	<1	<1	25 ±4[c]	94±3
RFB-6	<1	<1	23 ±6[c]	92±4
Y29/55	<1	<1	71.3±3[c]	98±0.5

[a] Results are expressed as percentage of cells membrane-labelled with MoAb within the given population. The ranges of these populations in the samples are: TdT$^+$ cells 0.5%–12%; pre-B cells (cyμ$^+$) 0.1%–1.5%; sIgM$^+$ B cells 5%–10% of Ficoll-Triosil-separated cells

[b] These cells also express IgM (sIgM$^+$, IgD$^+$) and represent a subset (33% ±7%) of all sIgM$^+$ cells

[c] Within the To15$^+$, RFB-4$^+$, RFB-6$^+$ and Y29/55$^+$ populations the following percentages are sIgD$^+$: 91%, 85%, 92% and 57%

[d] The sIgM$^+$ B cells in the fetal bone marrow at 17–20th gestational week are largely (>90%) sIgD$^-$, AL1$^+$, BA1$^+$, B1$^+$, and To15$^-$, RFB4$^-$, RFB6$^-$ (see [13])

Table 3. B cell subpopulations in the developing human fetus[a]

Phenotype of relevant cells	Gestational week			
	Bone marrow 16–22	Spleen 16–22	Lymph node 16	17–22
Precursor TdT$^+$, IgM$^-$	5–20 (scattered)	<5	NT	NT
TdT$^-$, IgM$^+$, IgD$^-$, T1$^-$	70–80	10–20 ⎫	95	<10
B IgM$^+$, IgD$^+$, T1$^-$	2– 5	80–90 ⎭		In diffuse areas >90
IgM$^+$, IgD$^+$, T1$^+$	0.1–0.9	1–2	<5	In clusters >90 (Fig. 1)[b]
T cells IgM$^-$, T1$^+$ (T3$^+$, T11$^+$)	1%–2.5% of mono-nuclear cells	In separate T areas	In diffuse lymphoid zone (Fig. 1)	

[a] The results shown refer to the percentage of cells within the B lineage (unless otherwise indicated), determined by the following labelling combinations: TdT/IgM; IgM/IgD; IgM/RFT1 (T1 antigen; 67 k); IgM/UCHT1 (T3 antigen; 19 k); IgM/RFT11 (T11 antigen; 50 k). In the 16-week-old LN samples only IgM/RFT1 and IgM/UCHT1 combinations were tested

[b] The clusters are formed around follicular dendritic reticulum cells

looking cells. Of these 15%–25% were TdT$^+$ and a further 5%–10% were cytoplasmic IgM$^+$ pre-B cells; the majority of B lineage cells were membrane sIgM$^+$, sIgD$^-$ B lymphocytes. It was confirmed in tissue section that these populations were indeed predominantly negative for To15, RFB-4 and RFB-6 antigens [13]. Thus, the BM B cells in the fetus represent "early" types of B cells, as expected on the basis of findings in the infant BM (Table 2).

Further studies were performed to identify the T-lineage cells in the fetal BM. Two antibodies, OKT3-like (UCH-T1) and OKT1-like (RFT1) were used, the latter in double combination with anti-IgM. T cells (T3$^+$, T1$^+$) were rare (0.5%–1%) in 17-week-old fetal BM, and these were IgM$^-$ (Table 3). The proportion of RFT1$^+$, IgM$^+$ double-labelled cells was very low: 0.1%–0.9% amongst the IgM$^+$ population (0.02% –0.15%) of all BM cells. This contrasts with the findings observed in fetal lymph nodes (see Sect. C.IV).

III. Analysis of Fetal Spleen

In the samples of fetal spleen taken at the 18th gestational week 4–5 times more B cells were found than T cells. Of all cells, 4%–6% were IgM$^+$ and another 1%–2% T3$^+$. The T cell population was clustered around small blood vessels, and the B cells formed a loose band of cells at the edge of the splenic red pulp. No follicular dendritic reticulum cells were seen.

There were two important findings about the splenic B cells. First, these splenic B cells have clearly shown a "peripheral B cell" phenotype: 80%–90% were IgM$^+$, IgD$^+$ and virtually all (>95%) B cells clearly reacted with MoAb RFB-4, To15 and RFB-6. On the other hand, we could not observe significant numbers of IgM$^+$, IgD$^-$ B cells. This is interesting because in the adult human (and rat) spleen a clearly visible population of IgM$^+$, IgD$^-$ cells is present in the marginal zone [14], but these are probably mature cells in a relatively late stage of development. Second, immunohistological techniques were used to identify B cells expressing T cell-associated markers such as T1 and only a few (1%–2% of IgM$^+$ cells) appeared to express the T1 antigen (Table 3) up to the 23rd week when more T1$^+$, IgM$^+$ cells appeared [13].

IV. Analysis of Fetal Lymph Nodes

In the earliest lymph nodes found (at 16th gestational week) T and B cell areas were
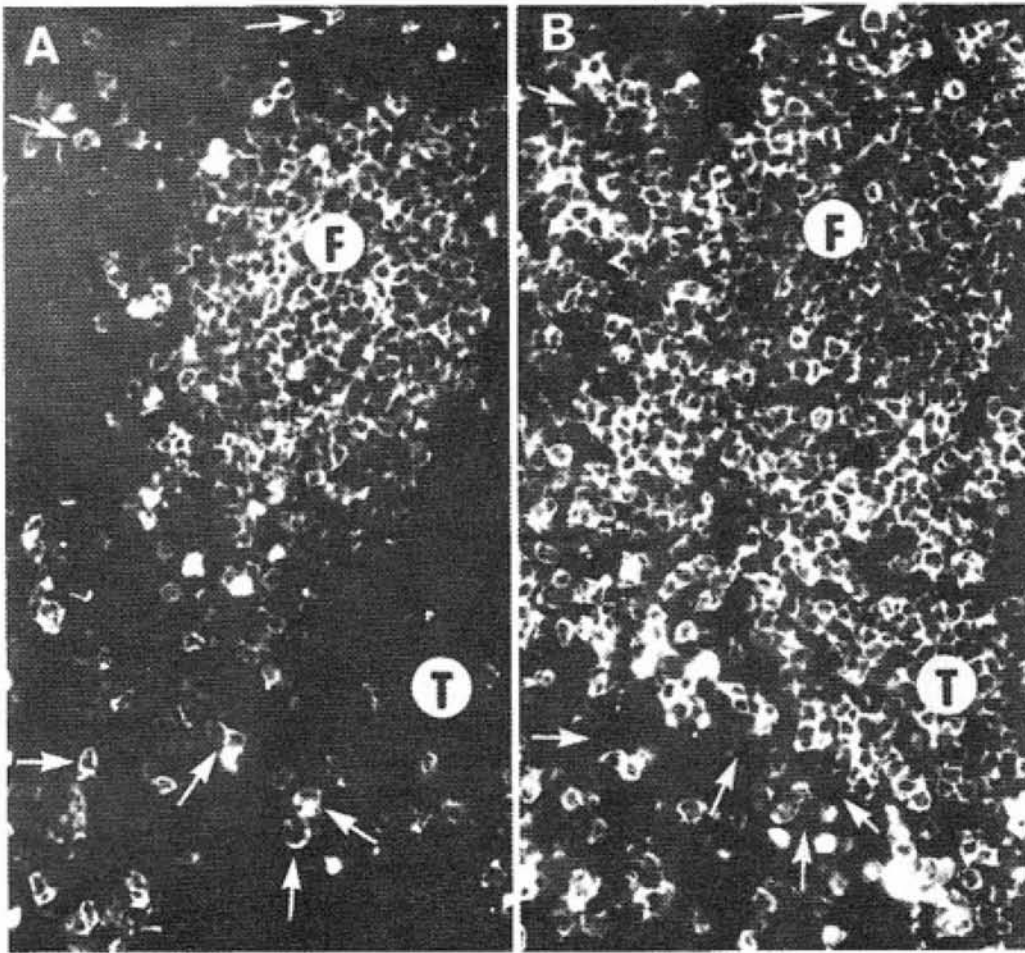
Fig. 1 A, B. B lymphocytes within the primary lymphoid nodules of fetal lymph nodes (20th gestational week) show double staining for IgM (**A**) and the T cell-associated antigen Tl (**B**). These B cells are accumulating around dendritic reticulum cells [13]. The rare B cells diffusely distributed in the paracortical T area are strongly IgM$^+$ and Tl$^-$ (*arrows*). This B cell population is dominant in the fetal spleen (Table 2; [13])

not yet separated. T lymphocytes (Tl$^+$, T3$^+$) as well as the IgM$^+$ B cells (30% of lymphocytes) were diffusely mixed. No Tl$^+$, IgM$^+$ double-labelled cells were seen. In the lymph node samples taken at 18th week tight clusters of lymphocytes appeared accumulating 100–200 cells around follicular dendritic cells (FDR cells; identified by RFD-3 antibody) and uniformly showed a peculiar phenotype. All these cells were IgM$^+$, IgD$^+$ (moderately strongly), expressed BA-1 positivity and reacted with RFB-4, To15 as well as RFB-6 (B cells of peripheral type). In addition, the B cells in the clusters were invariably Tl$^+$ (moderately strongly; Fig. 1) but HLA-DR

positive and T3 negative. The T lymphocytes (T3$^+$, Tl$^+$, HLA-DR$^-$, IgM$^-$) were found in the diffuse lymphoid area and showed a much stronger Tl$^+$ staining. Finally, it is important to point out that within the small B lymphoid follicles no large germinal centre blasts were seen, and the BA-1$^-$, weak IgM$^+$, IgD$^-$ central population seen in the germinal centres of adult lymph nodes were absent.

V. Malignant B Cells

TdT$^+$ cells are present in large proportions amongst the lymphoid cells of fetal liver [11, 15] and fetal BM [13, 15]. These cells lack a number of other B cell markers such as Y29/55 [16], RFB-6, or show only cytoplasmic, but no membrane expression of RFB-4 or To15. The phenotypic features of these TdT$^+$ cells are similar to the blast cells seen in common acute lymphoblastic (cALL) and pre-B ALL [9, 11, 15–17].

As we recently emphasized [13], the Tl$^+$, IgM$^+$, IgD$^+$ cells with peripheral B cell features (Y29/55$^+$, RFB-6$^+$, RFB-4$^+$,

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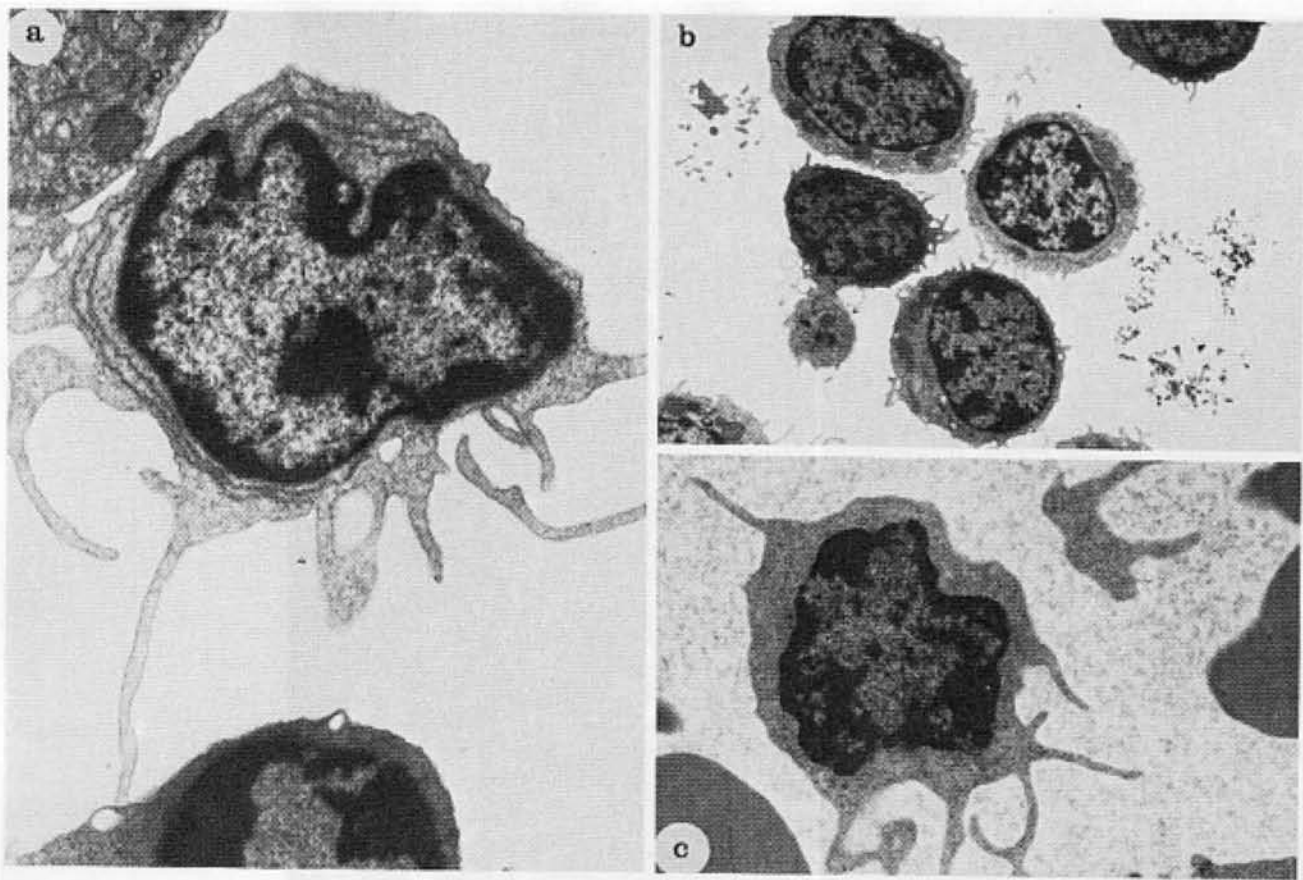
Fig. 2a–c. B-CLL cells after 72 h TPA stimulation develop "hairs" (**a** × 10 000). Cells in the control unstimulated B-CLL culture have smoother surface (**b** × 6000) and normal B cells in the presence of TPA also remain smooth (not shown; but see [23]). For comparison hairy cell leukaemia is shown (**c** × 8000). From [10] with permission

To15[+]) which cluster around follicular dendritic reticulum cells in fetal lymph nodes (see Sect. C.IV) are very similar, by phenotypic criteria, to two B cell malignancies: centrocytic lymphoma [18] and B-type chronic lymphocytic leukaemia (B-CLL; [19]). The further distinctive phenotypic features of these malignancies are the reactivity of Tü-33 Mo-Ab with centrocytic lymphoma and that of Tü-1 Ab with B-CLL [18]. Indeed, the normal equivalent T1[+], IgM^{+} cells in the primary nodules of normal fetal LN samples are also heterogeneous in respect of Tü-33 and Tü-1 expression [13]. Thus, centrocytic lymphoma and B-CLL might originate from the Tü-33[+] and Tü-1^{+} B cells of primary nodules, respectively.

The exact origin of hairy cell leukaemia (HCL) is nevertheless still unknown. This disease, in its typical form, is a B cell malignancy with the following features: (a) SmIg expression and Ig gene rearrangements [20]; (b) characteristic membrane protrusions and veils; (c) strong cytoplasmic tartrate-resistant acid phosphatase activity (TRAP; [21]); (d) reactivity with MoAb to interleukin-2 receptor (anti-Tac; [20]); and (e) reactivity with MoAb to SHCL3 antigen, a membrane moiety not expressed by B-CLL [22]. As the "normal equivalent" cell of HCL has so far been elusive, we have investigated whether any known malignant B cells can be transformed into HCL. The details will be published elsewhere [23] and the salient observations are as follows.

First, it has been observed that the expression of TRAP enzyme activity can be readily induced by TPA in *normal* tonsil B cells and their mouse rosetting subset [23] during a 72-h culture period (60% and 18% positivity, respectively). A few SHCL3-positive B cells were also generated in the same cultures (25% and 7%, respectively). Only very few Tac^{+} B cells were seen, however, and these were best generated in 6- or 7-day cultures stimulated by PWM (Ig[+] blasts; 5% Tac[+]). Thus, with the possible

Table 4. TPA-induced changes in B-chronic lymphocytic leukaemia (B-CLL) and hairy cell leukaemia (HCL)[c]

Phenotype	B-CLL (16 cases), incubated for		HCL (4 cases), incubated for	
	0 h	72 h with TPA[b]	0 h	72 h with TPA
A. Morphology	Small ly	Hairy cells[c]	Hairy cells	More adherent
B. HCL-associated features				
SHCL3[+d]	3.3±2.1[e]	(3) 5 ±3[f]	87±7	44±4
		(13) 74 ±11		
Anti-Tac[+]	(15) 4.0±2	(7) 5.1±2.1	84±4	47±8
	(1) 80	(9) 64 ±21		
Tartrate-resistant acid phosphatase (TRAP)	< 5	65–85	60±5	50–70
C. B-CLL-associated features				
M-rbc[+] (rosettes)	61±8	< 5	33±4	
T1 (p67) antigen	87±6	79±11	1.5, 1, 3, 65	1, 2, 3, 60
sIg[+]	80±3 (weak)	<10 (very weak)	22± ¨	< 5
Cytoplasmic Ig[+]	<10	(12) >50	15±5	>40
		(4) <10		

[a] 16 cases of B-CLL and 4 cases of HCL were studied. There has been no change in the features of B-CLL and HCL cultures for 72 h without TPA (except a slight decrease in M-rbc rosetting). For further details see [23]

[b] TPA (12-*O*-tetradecanoylphorbol-13-acetate) was used for 72 h at 1.6×10^{-8} M concentration

[c] See Fig. 2

[d] A MoAb from Dr. Schwarting [22]

[e] Percentage (±standard error) of cells reacting with each marker

[f] Numbers in parentheses refer to numbers of cases

exception of membrane irregularities, the features of HCL are not fully leukaemia specific. Next, various *leukaemias* were "induced" by TPA. These included 16 cases of B-CLL (T1[+], sIgM[+], weak), 4 cases of prolymphocytic leukaemia and "mantle zone" lymphoma (strongly Ig[+] positive; T1 mostly negative) and 2 cases of common ALL (TdT[+], sIg[−]).

We have reported previously that TPA-induced B-CLL cells developed membrane perturbations characteristic of hairy protrusions (Fig. 2; [10]). Clearly, other "HCL-associated" features could also be observed in the majority of cases. Of 16 cases, 13 had >50% SHCL3^{+} cells, and 9 showed strong Tac positivity in 64%±21% of activated B-CLL cells; 60%–85% of cells were TRAP[+] (Table 4). These "HCL-like" features on activated B-CLL cells were much stronger

than on any of the TPA-activated normal B cell populations, including the mouse rosetting subpopulation. Similar HCL-like features (e.g. membrane irregularities and SHCL3 positivity) have not been recorded in the other leukaemias, although cases of PLL and mantle zone lymphoma did develop Tac positivity (45%–80%; results not shown, but see [23]). Finally, cases of HCL (four patients) when activated with TPA, did not show major phenotypic changes, and have not developed CLL-like features [23].

D. Discussion

The two main findings about emerging B cells during fetal development are that: (a) the "early" B cells (pre-B and IgM[+], IgD[−] B)
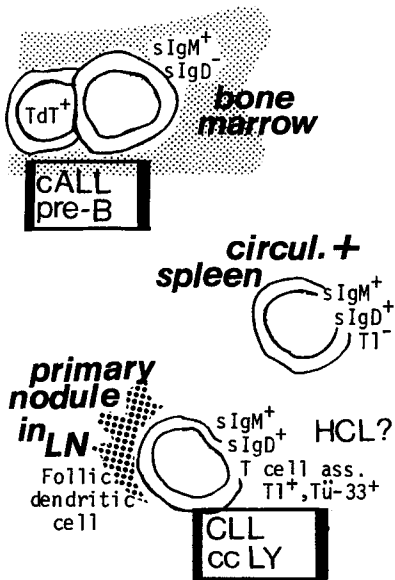
Fig. 3. The three main B cell types observed in the fetus. In the bone marrow pre-B and B cells are IgM$^+$, IgD$^-$, AL-1$^+$ and do not express membrane RFB-4, To15, RFB-6. On the periphery strongly Ig$^+$ (IgM$^+$, IgD$^+$) cells circulate in blood and are seen in the spleen while B cells in the primary nodules in the lymph node show weaker IgM$^+$ and IgD$^+$ and express T cell-associated antigen such as T1 and Tü-33. For further details see also [9, 13]

in fetal bone marrow are different, by a number of criteria, from peripheral B cells [9]; and (b) there is a dichotomy amongst the peripheral B cell populations [13]. This leads to emphasizing the existence of three major B cell types in the fetus: one in the BM, and two in the periphery (Fig. 3). To this heterogeneity further forms (e.g. germinal centre blasts, and plasma cells) are added when the individual is exposed to extrinsic antigens during later life. This scheme is similar to those presented by Galton and MacLennan [14] and also indicated by Stein et al. [18] on the basis of lymphoma heterogeneity. Indeed, the new findings here support the existence of normal B cells (most probably virgin peripheral B lymphocytes of primary lymphoid nodules) which express some T cell-associated markers such as T1 and Tü-33. These B cells are also present in adults [19], but only in smaller numbers, perhaps because they are "diluted out" by other newly emerging cell types.

The finding of IgM$^+$, T1$^+$ normal B cells in the primary nodules may explain two unrelated puzzling phenomena. First, certain B cell malignancies express T cell-associated features with some regularity. The T1 antigen on B-CLL, T1 plus Tü-33 on centrocytic lymphoma (Kiel classification; [18]) are the obvious examples (see Sect. C.), but T3 as well as T11 molecules were also seen in a few cases of B-CLL and B lymphomas, in spite of the fact that these showed monoclonal ($\varkappa$ or λ) light chain, IgM as well as HLA-DR [24–27]. Similarly, Cawley et al. [28] have emphasized that HCL were capable of expressing T cell features (sheep erythrocyte rosetting) during certain stages of disease progression. The derepression of T cell genes may be more frequent in the recently identified T1$^+$, IgM$^+$ cells or their malignant counterparts than in the other B cell subsets.

At least a subgroup of HCL might also be related to the B-CLL group. This is indicated by the observations of "inducing" HCL-like features in B-CLL cells with TPA (Table 4). Taken at face value, these results and those described by Caligaris-Cappio [10, 23] show that TPA-induced B-CLL and HCL both have abnormal membrane characteristics in terms of "hair" formation, which is not demonstrated in the equivalent normal cells. In other respects, HCL appears to be an activated variant of B-CLL or that of a closely related cell type. An alternative explanation is that the HCL-associated markers (Tac expression, TRAP and SHCL3 positivity) are nonspecific features which appear on unrelated B cell types. Clearly, more work is needed in this area, but it is already clear that other B cell malignancies such as prolymphocytic leukaemia, mantle zone lymphoma and pre-B ALL (and the corresponding cell lines) do not develop HCL-like features when induced by TPA [23]. Thus, the CLL–HCL link has so far appeared to be a rather special one. The possibility of the CLL–HCL link is also supported by the existence of leukaemias showing circulating HCL together with B-CLL-like histological pattern [29, 30].

Second, it has been demonstrated in the mouse that Ly-1$^+$, IgM$^+$ B cells are also present and, in the NZB strain, contribute to autoantibody synthesis [31]. The observation of frequent autoantibody forma-

tion in B-CLL [32] raises the possibility that in these patients the whole T1$^+$, IgM$^+$ lineage is hyperactive and the monoclonal malignancy might be just one of the later, although dramatic, consequences of this dysregulation. This is an interesting possibility from the point of view of preventing a malignant disease. Similarly, further studies are necessary to investigate this B cell population in autoimmune disorders. It will be interesting to see whether the increased numbers of circulating mouse rosetting B cells in rheumatoid arthritis [33] also show the T1$^+$, IgM$^+$ phenotype.

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