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Two maturation-associated mouse erythrocyte receptors of human B cells

I. IDENTIFICATION OF FOUR HUMAN B-CELL SUBSETS

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SUMMARY

Using rosetting tests with untreated mouse erythrocytes (M) and pronase-treated M (pro M), four human B cell subsets can be identified. Three of these, possessing the phenotypes $B_{M+\ pro\ M+}$, $B_{M-\ pro\ M+}$ or $B_{M-\ pro\ M-}$, constitute 17%, 61% and 22% of normal blood B cells respectively. The fourth subset, $B_{M+\ pro\ M-}$, does not occur in normal tissues but was found in the pre-B-cell line of Raji cells, indicating that this phenotype may be a marker for early B cells. Some differences in the proportion of each subset were found in cord blood, lymph nodes and tonsils. Surface-immunoglobulin-positive (SIg^+) and -negative (SIg^-) non-T cells were present in each subset. M and pro-M rosetting tests were applied to cells from blood of 27 cases of chronic lymphocytic leukaemia (CLL) and to cells from involved nodes, spleen or marrow in five cases of non-Hodgkin's lymphoma (NHL). In 15 cases of CLL, there was considerable increase in the $B_{M+\ pro\ M+}$ subset ($B_{M+\ pro\ M+}$ type CLL); in seven cases, there was a predominance of $B_{M-\ pro\ M+}$ cells and in another four cases, $B_{M-\ pro\ M-}$ cells predominated. All five cases of NHL were greatly enriched in $B_{M-\ pro\ M-}$ cells. There was no obvious correlation between rosetting and other surface markers but $B_{M-\ pro\ M-}$ clones in CLL or NHL always stained brightly with FITC-anti-Ig. This was not found in $B_{M+\ pro\ M+}$ or $B_{M-\ pro\ M+}$ clones. Rosette formation of neuraminidase-treated B cells with M identifies the same subset as B-pro-M rosetting in normals and CLL. Evidence is presented that two types of receptors are involved in M and pro-M rosetting, designated R_1 and R_2, binding to corresponding M ligands L_1 and L_2. M rosetting is due to R_1–L_1 binding while R_2–L_2 binding mediates B-pro-M rosetting. Shifts between subsets within the same clone in some cases of CLL suggest that the subsets are distinct maturational stages of B-cell development rather than families of B cells of different lineage. The following B-cell maturation sequence is proposed: $R_1^+\ R_2^- \rightarrow R_1^+\ R_2^+ \rightarrow R_1^-\ R_2^+ \rightarrow R_1^-\ R_2^-$.

INTRODUCTION

Recently, using the B-cell-specific marker—rosetting with mouse erythrocytes (M)—it has been possible to classify human B lymphocytes into two subgroups, B_{M+} cells which rosette with M and B_{M-} cells which do not (Forbes & Zalewski, 1976; Gupta, Good & Siegal, 1976). These two subsets are present in different proportions in different normal lymphoid tissues (Forbes & Zalewski, 1976; Gupta *et al.*, 1976; Black *et al.*, 1980; Leong *et al.*, 1979) and either may be expanded monoclonally in chronic lymphocytic leukaemia (CLL) and other B-cell lymphoproliferative disorders (Forbes & Zalewski, 1976; Catovsky *et al.*, 1976; Forbes *et al.*, 1978, 1979). In line with the general concept that

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differentiation is arrested in leukaemia (Sachs, 1978), it is believed that B_M+ cells are less mature than B_M- cells and that CLL is most often associated with an arrest of differentiation of B_M+ cells to the B_M- state (Catovsky *et al.*, 1976; Forbes *et al.*, 1978).

In order to determine the mechanism of loss of rosetting with differentiation it is necessary to understand the molecular interactions that are responsible for rosette formation. Rosetting with protease-treated M (Zola, 1977) and rosetting with M after neuraminidase treatment of the B cells (Gupta *et al.*, 1976; Forbes & Zalewski, 1976) have been used to increase the sensitivity of the M-rosette test. In the first paper of this series we report effects of enzymatic modifications of B cell or mouse erythrocyte membranes on the specificity and properties of the binding. We show that there are four distinct B-cell subtypes according to rosetting phenotype and that rosetting may involve two types of bond. Two mouse erythrocyte receptors of different specificity co-exist in the immature B-cell membrane. The activity of one receptor (R_1) is destroyed by gentle trypsin treatment; isolation and partial purification of a glycoprotein with the specificity of R_1 is described in the second paper of the series.

A B-cell maturation sequence is proposed in which maturation is related to receptor phenotype. Malignant B-cell populations differ according to the predominant maturation block.

TERMINOLOGY

Rosettes

B-M rosettes: formed by untreated B lymphocytes and untreated mouse erythrocytes.

Nase B-M rosettes: formed by neuraminidase-treated B lymphocytes and untreated mouse erythrocytes.

B-pro-M rosettes: formed by untreated B lymphocytes and pronase-treated mouse erythrocytes.

Lymphocyte phenotypes

B_M+: cells which rosette with M.

B_M-: cells which do not rosette with M.

$B_{M+\ pro\ M+}$: cells rosetting with M and pro M.

$B_{M+\ pro\ M-}$: cells rosetting with M but not pro M.

$B_{M-\ pro\ M+}$: cells rosetting with pro M but not M.

$B_{M-\ pro\ M-}$: cells which do not rosette with either M or pro M.

The proportions of each subset in the B cells of blood and lymphoid tissues were calculated by the following equations (for explanation see first section of the Results):

$$\%\ \text{B cells which are } B_{M+\ pro\ M+} = \frac{\%\text{(B-M) RFC}^*}{\%\text{(non-T)}} \times 100$$

$$\%\ \text{B cells which are } B_{M-\ pro\ M+} = \frac{\%\text{(B-pro-M) RFC} - \%\text{(B-M) RFC}}{\%\text{(non-T)}} \times 100$$

$$\%\ \text{B cells which are } B_{M-\ pro\ M-} = \frac{\%\text{(non-T)} - \%\text{(B-pro-M) RFC}}{\%\text{(non-T)}} \times 100$$

MATERIALS AND METHODS

Preparation of cells. Human lymphocytes were obtained from venous blood, lymph nodes and tonsils from normals, from blood of patients with CLL and from involved lymph nodes, spleen or bone marrow from patients with NHL. In addition, thymocytes were obtained from fetuses and from a patient with myasthenia gravis. Peripheral blood lymphocytes were separated by Ficoll–Hypaque sedimentation. Cells were washed with Dulbecco's phosphate-buffered saline, pH 7·4 (PBS). Viability was determined by trypan blue exclusion. Raji cell line cells were a gift from Dr

* RFC = rosette-forming cells.

H. Zola and were subcultured in RPMI medium (Flow Labs) containing 10% fetal calf serum (Flow Labs). Mouse blood was obtained from Swiss white mice (in one experiment a variety of mouse strains was tested) by bleeding from the ophthalmic venous plexus, rat blood from the jugular vein, dog blood from a femoral vein and guinea-pig by cardiac puncture. Blood was collected into saline containing 0·38% sodium citrate. Human blood was collected in heparin and ox and sheep blood were obtained as red cell suspensions in Alsever's solution (Commonwealth Serum Labs). All erythrocytes were washed in PBS. Care was taken to remove the buffy coat in each case. Pronase-treated erythrocytes were prepared by incubation at 37°C for 30 min in the ratio of 4 vol of 2% erythrocytes to 1 vol of pronase (2 mg/ml) from *Streptomyces griseus* (Sigma) followed by washing.

Enzyme treatment of lymphocytes. Lymphocytes (10^7/ml) were incubated with *Vibrio cholerae* neuraminidase (Calbiochem), 50 units/ml, at 37°C for 30 min and then washed three times.

Trypsin- and papain-treated lymphocytes were prepared by incubation of lymphocytes for 10 min at 37°C with varying concentrations of trypsin (Calbiochem) or papain (Merck) in the presence of 100 μg/ml deoxyribonuclease type I (Sigma) to prevent cell clumping. Concentrations of trypsin and papain used are given in the Results.

Surface marker testing. Rosette formation with mouse erythrocytes was performed as published previously (Forbes & Zalewski, 1976). An identical method was used for rosette formation with pronase-treated mouse erythrocytes or with other animal erythrocytes; surface immunoglobulin and Fcγ receptors and T-cell identification by rosetting with AET-treated sheep erythrocytes were assayed as in an earlier report (Forbes *et al.*, 1978).

Isolation of lymphocyte subpopulations. T and non-T cells were prepared from blood and tonsils. A T-cell-rich fraction was prepared by positive selection by rosetting. Non-T cells were collected at the interphase and monocytes removed using lymphocyte separator reagent (Technicon) followed by Ficoll–Hypaque sedimentation. Pro-M^+ and M^+-cell-rich fractions of non-T cells were prepared by removal of rosettes and interphase cells were used as pro-M^- and M^- T-cell-depleted fractions respectively.

Pre-rosetting experiments. CLL cells were rosetted with M and the pellet, after 60 min at 4°C, was lysed by the addition of 1 ml of distilled H_2O for 20 sec followed by restoration of isotonicity by adding a large volume of PBS. Cells were then washed and rosetted with M.

Fluoresceination of mouse erythrocytes. To 4 ml of 2% red cell suspensions in PBS was added 1 ml of 0·2 M Na_2HPO_4 dropwise. One milligram of fluorescein isothiocyanate (Sigma) dissolved in 2 ml of 0·1 M Na_2HPO_4 was then added to the red cell suspension and the reaction allowed to proceed for 30 min at room temperature. Fluoresceinated erythrocytes were then washed extensively with PBS.

RESULTS

B-cell subsets in normals and malignant lymphoproliferative disease

Frequencies of B-M, Nase B-M and B-pro-M rosette-forming cells in normal lymphoid tissues. Table 1 shows the percentages of B-M, Nase B-M, B-pro-M and sheep erythrocyte rosette-forming cells and SIg^+ cells in adult blood, cord blood, lymph nodes and tonsils of normal subjects. Neither thymocytes nor isolated normal T cells formed rosettes with M or pro M, nor with M after neuraminidase treatment of the T cells. Thus these rosettes are formed only by non-T cells. For convenience, we equate non-T cells to B cells, thereby including in this category SIg^+ and SIg^- cells.

When cells rosetting with pro M were removed from the non-T population, the residual cells did not rosette with M indicating that the B_{M+} cells are amongst those rosetting with pro M. It follows that three subsets can be enumerated using rosette tests with M and pro M: $B_{M-\ pro\ M-}$, cells not rosetting with pro M; $B_{M+\ pro\ M+}$, cells rosetting with M; and $B_{M-\ pro\ M+}$, cells rosetting with pro M minus those rosetting with M. The separate existence of cells with these three phenotypes is further substantiated in studies with malignant B lymphocytes (next section).

Normal adult blood B cells contain 17% $B_{M+\ pro\ M+}$, 61% $B_{M-\ pro\ M+}$ and 22% $B_{M-\ pro\ M-}$ cells; other lymphoid tissues showed moderate variations from these proportions (Table 1).

Table 1. Frequencies of rosetting cells and B-cell subsets in blood, lymph nodes and tonsils of normals

Cell source	Number	Per cent T†	Per cent SIg^+‡	Per cent rosetting cells: B-M	Nase B-M	B-pro M	Per cent B cells in subsets*: $B_{M+\ pro\ M+}$	$B_{M-\ pro\ M+}$	$B_{M-\ pro\ M-}$
Adult blood	10	82	8	3	14	14	17	61	22
Cord blood	4	60	28	8	31	26	20	45	35
Lymph nodes	5	69	18	3	22	26	10	74	16
Tonsils	4	57	42	12	34	33	28	49	23

* For derivation of per cent B cells in each subset, see under Terminology.

† Per cent rosette formation with AET-treated sheep erythrocytes.

‡ Total SIg^+ cells determined by fluorescence labelling with polyvalent anti-Ig.

Frequencies of B-pro-M and Nase B-M rosetting cells were similar for each tissue studied indicating that they may be markers for the same subset.

SIg^+ and SIg^- cells were present in each subset. Thus T-cell-depleted pro-M^+ and pro-M^--cell-rich fractions from normal blood contained 73 and 30% SIg^+ cells respectively, and T-cell-depleted M^+ and M^--cell-rich fractions contained 63 and 64% SIg^+ cells respectively.

Frequencies of B-M, Nase B-M and B-pro-M rosette-forming cells in malignant lymphoproliferative disease. Table 2 shows an analysis of malignant B lymphocytes in blood of 27 patients with CLL, and involved lymph nodes, bone marrow or spleens from five cases of NHL. They could be divided into three groups according to the predominant subset. In 15 cases of CLL the subset $B_{M+\ pro\ M+}$ predominated and in seven cases of CLL the predominant subset was $B_{M-\ pro\ M+}$. The subset $B_{M-\ pro\ M-}$ was predominant in all cases of NHL and in four cases of CLL. A case of T-cell CLL is included to illustrate the lack of rosette formation with M and pro M.

As with normal B cells, the frequency of Nase B-M RFC was almost identical to the frequency of B-pro-M RFC in all cases (data not shown). Individual populations of selected cases of B-cell malignancy show the distinct identity of each receptor phenotype, e.g. case 8 with 93% $B_{M+\ pro\ M+}$, case 20 with 92% $B_{M-\ pro\ M+}$ and case 31 with 100% $B_{M-\ pro\ M-}$. $B_{M+\ pro\ M-}$ cells were not found.

Serial studies of the 27 cases of B-type CLL have been made for up to 5 years. Seven cases now recorded as predominantly $B_{M-\ pro\ M+}$ or $B_{M-\ pro\ M-}$ had a predominance of $B_{M+\ pro\ M+}$ cells when first observed. The light chain class did not change in any. Thus in patient 19 in August 1979 there were 71% SIg^+ cells (61% κ, 5% λ), 62% B-M, 86% Nase B-M and 80% B-pro-M rosetting cells, while in September 1980 there were 76% SIg^+ cells (76% κ, 1% λ), 11% B-M, 76% Nase B-M and 73% B-pro-M rosetting cells. The lymphocyte count was 12,200/μl and 11,500/μl respectively. Three of the four cases of $B_{M-\ pro\ M-}$ CLL initially had cells which were B_{M+} and expressed the same light chain type as later in the disease. However, there were very marked increases in intensity of staining of SIg.

Brightly staining SIg^+ cells in normal blood. Cells of malignant $B_{M-\ pro\ M-}$ clones were always found to stain brightly (+3) with FITC-anti-Ig whereas those of B_{M+} and $B_{M-\ pro\ M+}$ clones infrequently had a +3 reaction (Table 2). A small proportion (approximately 0·5%) of normal blood SIg^+ cells have a very intense ring fluorescence with FITC-IgM. These cells were enriched in the pro-M^- cell population isolated by pro-M rosette sedimentation indicating that they lack capacity to rosette with pro M as do malignant $B_{M-\ pro\ M-}$ cells.

Rosetting phenotype of Raji lymphoblastoid cells. Raji lymphoblastoid cells are one of the few B-cell lines forming B-M rosettes (Bertoglio, Laldjim & Dore, 1979). We confirmed this in this study. This property was found 4 or more days after subculture. At days 2 and 4 after subculture the mean percentage of cells rosetting was 0 and 40% respectively. Rosetting with Raji cells was only slightly increased by neuraminidase treatment. The cells differed from normal or malignant B_{M+} cells in failure to rosette with pro M. These cells have the phenotype $B_{M+\ pro\ M-}$.

Table 2. Frequencies of rosetting cells and B-cell subsets in CLL and NHL

Patient	Lymphocyte count/μl	Per cent T	Per cent SIg^+	Int.*	SIg isotype	Per cent rosetting cells			Per cent B cells in each subset†			Predominant subset
						%Fcγ	B-M	B-pro M	$B_{M+\ pro\ M+}$	$B_{M-\ pro\ M+}$	$B_{M-\ pro\ M-}$	
CLL 1	22,600	7	47	1	Mλ	60	75	96	81	19	0	$B_{M+\ pro\ M+}$
CLL 2	8,300	49	56	2	MDκ	50	42	46	82	8	10	
CLL 3	13,800	4	86	1	Mκ	83	79	83	82	4	14	
CLL 4	30,800	11	69	1	MDλ	61	60	88	67	31	2	
CLL 5	620,000	2	35	1	Dκ	88	81	95	83	14	3	
CLL 6	15,300	8	77	2	Mκ	77	60	91	65	34	1	
CLL 7	52,700	3	89	1	MDλ	93	73	98	75	25	0	
CLL 8	34,600	10	74	1	Mκ	94	84	90	93	7	0	
CLL 9	51,200	0	29	1	Mκ	n.d.	67	88	67	21	12	
CLL 10	22,100	9	58	1	λ	92	67	98	74	26	0	
CLL 11	11,700	11	44	1	MDκ	65	60	97	67	33	0	
CLL 12	2,600	12	76	1	Mκ	n.d.	50	88	62	38	0	
CLL 13	51,600	1	83	1	Mκ	n.d.	54	91	55	37	8	
CLL 14	16,000	7	72	1	κ	84	60	96	65	35	0	
CLL 15	n.d.	9	58	1	MDλ	84	67	96	74	26	0	
CLL 16	22,600	7	50	1	Mκ	81	40	83	43	46	11	$B_{M-\ pro\ M+}$
CLL 17	15,200	4	74	1	MDκ	58	7	84	9	80	11	
CLL 18	2,200	42	60	2	Mλ	47	22	75	38	62	0	
CLL 19	11,500	35	76	2	MDκ	83	11	73	23	77	0	
CLL 20	18,700	16	69	1	Dλ	90	5	82	6	92	2	
CLL 21	22,500	21	27	1	Mκ	n.d.	8	51	10	54	36	
CLL 22	3,100	30	48	3	MDλ	43	0	41	0	59	41	
CLL 23	72,000	1	99	3	MADκ	84	0	12	0	12	88	$B_{M-\ pro\ M-}$
CLL 24	75,900	0	100	3	MDκ	n.d.	0	2	0	2	98	
CLL 25	184,300	2	93	3	MAλ	50	10	16	10	6	84	
CLL 26	141,100	2	97	3	GAλ	8	11	26	11	15	74	
NHL 27	Spleen	16	86	3	Mκ	95	0	12	0	14	86	
NHL 28	Spleen	38	93	3	GAMλ	5	1	1	2	0	98	
NHL 29	Bone marrow	6	91	3	MDλ	n.d.	1	4	1	3	96	
NHL 30	Lymph node	48	75	3	Mκ	1	0	3	0	6	94	
NHL 31	Lymph node	0	90	3	Gκ	n.d.	0	0	0	0	100	
CLL 32	11,200	75	2	—	—	5	1	4	—	—	—	T

Properties of the B-M bond and B-pro-M bond

Species specificity of B-M, Nase B-M and B-pro-M binding. In tests with mouse, rat, dog, sheep, ox, guinea-pig and human erythrocytes only M formed substantial percentages of rosettes with B cells (Table 3). Rat and human erythrocytes were capable of rosetting with neuraminidase-treated B cells but these properties varied greatly among different populations of CLL cells. With pronase-treated erythrocytes only mouse red cells formed substantial percentages of rosettes with B cells.

Strain non-specificity of B-M and B-pro-M rosetting. Lymphocytes from two B_M+ CLL patients having approximately the same percentage of M-rosetting cells were tested against erythrocytes from 17 strains of mice: NZX, AKR, Swiss white, NZC, BALB/c, A/J, C57, C3H/He, NZY, NZW, NZO, AQR, SJL/J, NZB, 129/J, DBA/1 and CBA.

The percentage of rosettes ranged from 44 to 58% suggesting that there was no marked difference in this property in the mouse strains tested; none formed rosettes with lymphocytes from a case of B_M− CLL. Similar results were obtained using neuraminidase-treated B cells and pro M.

Effect of proteases on M-rosette formation. B_M+ CLL cells were treated with proteases (papain and trypsin) for 10 min at room temperature and then assayed for M-rosetting. Deoxyribonuclease, added to prevent protease-induced clumping of cells, did not affect rosetting. With cells from the majority of cases tested, B-M, B-pro-M and Nase B-M rosetting were abolished by each of the proteases. Cells from patients 1 and 5 required more severe protease treatment for inhibition of B-M, Nase B-M or B-pro-M rosetting.

Treatment of B_M+ cells for 10 min with low concentrations of papain (50 μg/ml) or trypsin (50 μg/ml) selectively abolished the capacity to form B-M rosettes without abolishing B-pro-M or Nase B-M rosettes. For example, with trypsin (50 μg/ml for 10 min), B-M rosettes fell from 69 to 6% while B-pro M fell from 86 to 69% and Nase B-M from 86 to 76%.

Inhibition of M-rosetting. B_M+ cells pre-rosetted with M and then freed of bound M by agitation or hypotonic lysis were inhibited 50–80% in their capacity to reform M rosettes. Contact with sheep erythrocytes did not inhibit this capacity. In order to test whether pieces of erythrocyte membrane were left on B cells after lysis, FITC-labelled M were used for pre-rosetting. Following lysis there was no detectable fluorescence on the B cells but they were strongly inhibited in rosetting with M.

Table 3. Rosetting of B_M+ CLL cells with erythrocytes of different species*

	Per cent rosetting cells		
	B-E†	Nase B-E	B-pro E
Mouse	60	96	92
Rat	0	26	10
Dog	6	13	n.d.
Sheep	4	10	6
Ox	0	0	0
Guinea-pig	0	0	10
Human (group O)	0	0	0

* Cells of case 6 used.

† Rosette formation of untreated B cells with untreated erythrocytes (E).

Nase B-E = rosette formation of neuraminidase-treated B cells with untreated E, B-pro E = rosette formation of untreated B cells with pronase-treated E.

DISCUSSION

Previously, two subsets of human B cells were distinguished by the M-rosette test (Forbes & Zalewski, 1976; Gupta *et al.*, 1976). We now report that by the addition of tests with pro M, four human B-cell subtypes can be identified. Rosetting of neuraminidase-treated B cells with M apparently identifies the same subset as rosetting of untreated B cells with pro M.

B cells from adult blood, cord blood, lymph nodes and tonsils consist of three subsets: $B_{M+\ pro\ M+}$, $B_{M-\ pro\ M+}$ and $B_{M-\ pro\ M-}$. Most normal B cells were of the $B_{M-\ pro\ M+}$ type. Some differences were found in the proportions of each subset in the different lymphoid tissues. In CLL, the $B_{M+\ pro\ M+}$ subset predominated in 15 cases, $B_{M-\ pro\ M+}$ in seven and $B_{M-\ pro\ M-}$ in four. The latter subset also predominated in tissues from five cases of NHL.

No features, including labelling with anti-Ig, SIg isotype, per cent T cells, per cent Fcγ-bearing cells and white cell count distinguished these subsets other than the consistent finding of brightly staining SIg in CLL of the $B_{M-\ pro\ M-}$ type and weakly or moderately staining SIg in the $B_{M+\ pro\ M+}$ and $B_{M-\ pro\ M+}$ types. A small proportion of normal blood $B_{M-\ pro\ M-}$ cells had brightly staining SIg. These cells may be normal counterparts of the malignant $B_{M-\ pro\ M-}$ cells.

The effect of therapy on each subset in CLL is not known. However, it was observed in serial studies that seven of 10 cases with low proportions of B_{M+} cells first presented with high proportions of B_{M+} cells. Each case expressed the same membrane light chain throughout the change, indicating that the same clone was involved. In four of these, B-pro-M rosetting was retained, while the other three lost both B-M and B-pro-M-rosetting capacities. These serial studies indicate that CLL may originate as a clone of B_{M+} cells that gradually loses its M-rosetting capacity, and that the $B_{M+\ pro\ M+}$, $B_{M-\ pro\ M+}$ and $B_{M-\ pro\ M-}$ phenotypes are distinct stages of clonal maturation rather than markers of distinct families of B cells.

The maturation sequence appears to be $B_{M+\ pro\ M-} \rightarrow B_{M+\ pro\ M+} \rightarrow B_{M-\ pro\ M+} \rightarrow B_{M-\ pro\ M-}$ (Fig. 1) for the following reasons:

(1) B_{M+} cells occur early in ontogeny (Gupta *et al.*, 1976).
(2) Plasma cells are B_{M-} (Catovsky *et al.*, 1976).
(3) $B_{M+\ pro\ M+}$ and $B_{M-\ pro\ M+}$ clones express sparse SIg while $B_{M-\ pro\ M-}$ clones express dense SIg. Density of SIg is believed to increase during maturation of malignant B cells (Salmon & Seligmann, 1974).
(4) Raji cells ($B_{M+\ pro\ M-}$) are considered by some to have pre-B-cell properties (Preud'homme *et al.*, 1978) and this cell line is one of the few which is capable of forming B-M rosettes (Bertoglio *et al.*, 1979). Normal pre-B cells have not yet been tested for rosette formation with M or pro M.

Maturation arrest is believed to be a feature of leukaemia and, indeed, of malignancy in general (Sachs, 1978). Previously it has been suggested that the clone of B cells in CLL is frozen at a particular stage (Salmon & Seligmann, 1974). Our observations support this concept and show that the sites of these maturation blocks can now be defined by differentiation markers. It would appear that the average maturity may advance during the course of the disease. Common CLL is partially inhibited at the $B_{M+\ pro\ M+} \rightarrow B_{M-\ pro\ M+}$ step, resulting in an accumulation of $B_{M+\ pro\ M+}$ cells. $B_{M-\ pro\ M+}$ CLL is mainly blocked at the next step, $B_{M-\ pro\ M+} \rightarrow B_{M-\ pro\ M-}$. The $B_{M-\ pro\ M-}$ subtype of

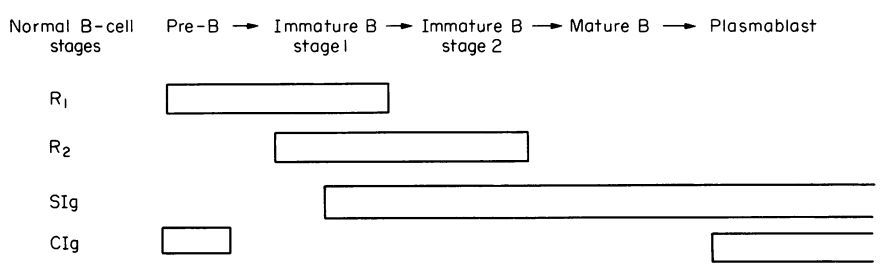

Fig. 1. Suggested human B-cell maturation sequence.

CLL is blocked at the B_{M-} $_{pro\ M-}$→plasmablast step. All cases of NHL so far tested have been blocked at this stage.

Rosetting results from a specific interaction of molecules on the B-cell and erythrocyte surfaces. Rosetting showed species specificity (and strain non-specificity), was abolished by protease treatment of the B cell and was specifically inhibited by pre-rosetting with M and lysis of bound M. The mechanism of this inhibition is unclear but is apparently not due to receptor blockade by pieces of lysed membrane as reported with EA rosetting (Cordier, Samarut & Revillard, 1977), as fluorescence-labelling studies show. It may be due to an irreversible change in the membrane itself on formation of rosettes, e.g. by membrane protein rearrangement (Zalewski & Forbes, 1979).

From these studies of rosetting by enzyme-treated B cells and M it can be deduced that two types of receptor are involved in rosette formation, R_1 and R_2. Firstly, there are subpopulations which bind M, but not pro M, and vice versa. Secondly, M binding is abolished by gentle trypsin treatment of B cells which spares the capacity to rosette with pro M. Studies with isolated receptor fragments (reported in the following paper) support this concept. It follows that there are corresponding ligands on M, L_1 and L_2. B-M rosetting is due to the interaction of R_1 and L_1, and B-pro-M rosetting involves the R_2–L_2 bond. The effect of pronase treatment of M is two-fold; it destroys L_1 and it modifies L_2 or its environment so that R_2–L_2 binding is facilitated. Nase B-M rosetting has properties similar to B-pro-M rosetting including the requirement of stronger trypsinization of B cells to abolish rosetting and labelling of the same B-cell subset. The simplest explanation of the augmenting effect of neuraminidase on rosetting is that by abolishing steric hindrance or negative-charge repulsion by sialic acid on receptors or neighbouring molecules, R_2–L_2 binding is strengthened and can mediate rosetting in the absence of R_1–L_1 binding.

The four human B-cell subsets can be described in terms of their receptor phenotype. In the order of the proposed maturation sequence they are $R_1^+ R_2^- \rightarrow R_1^+ R_2^+ \rightarrow R_1^- R_2^+ \rightarrow R_1^- R_2^-$.

The existence of these receptors and their corresponding ligands is further substantiated in the next paper of this series, in studies of the isolated fragment of R_1. Studies to be reported elsewhere have confirmed the maturation sequence using a culture system for human B-cell maturation.

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REFERENCES

BERTOGLIO, J., LALDJIM, B. & DORE, J.F. (1979) Mouse erythrocyte rosette formation by human lymphoid cell lines. *Scand. J. Immunol.* **10,** 153.

BLACK, R.B., LEONG, A.S.-Y., COWLED, P.A. & FORBES, I.J. (1980) Lymphocyte subpopulations in human lymph nodes: a normal range. *Lymphology*, **13,** 86.

CATOVSKY, D., CHERCHI, M., OKOS, A., HEDGE, V. & GALTON, D. (1976) Mouse red cell rosettes in B lymphoproliferative disorders. *Br. J. Haematol.* **33,** 173.

CORDIER, G., SAMARUT, C. & REVILLARD, J.P. (1977) Changes of Fcγ receptor-related properties induced by interaction of human lymphocytes with insoluble immune complexes. *J. Immunol.* **119,** 1943.

FORBES, I.J. & ZALEWSKI, P.D. (1976) A subpopulation of B lymphocytes that rosette with mouse erythrocytes. *Clin. exp. Immunol.* **26,** 99.

FORBES, I.J., ZALEWSKI, P.D., COWLED, P.A. & SAGE, R.E. (1979) Maturation in B lymphocyte leukaemia. *J. clin. Lab. Immunol.* **1,** 329.

FORBES, I.J., ZALEWSKI, P.D., LEONG, A.S.-Y., SAGE, R.E., DALE, B. & COWLED, P.A. (1978) B cell leukaemia distinguished from chronic lymphocytic leukaemia by surface markers. *Aust. N.Z. J. Med.* **8,** 532.

GUPTA, S., GOOD, R.A. & SIEGAL, F.P. (1976) Rosette formation with mouse erythrocytes. II. A marker for human B and non-T cell lymphocytes. *Clin. exp. Immunol.* **25,** 319.

GUPTA, S., PAHWA, R., O'REILLY, R., GOOD, R. & SIEGAL, F. (1976) Ontogeny of lymphocyte subpopulations in human fetal liver. *Proc. Natl. Acad. Sci. (USA)*, **73,** 919.

LEONG, A.S.-Y., FORBES, I.J., COWLED, P.A., SAGE, R.E., BLACK, R.B., DALE, B. & ZALEWSKI, P.D. (1979) Surface marker studies in chronic lymphocytic leukaemia and non-Hodgkin's lymphoma. *Pathology*, **11,** 461.

PREUD'HOMME, J.L., GOURDIN, M.F., REYES, F. & FELLOUS, M. (1978) Human lymphoid cell lines with pre-B cell characteristics. In *Human Lymphocyte Differentiation. Its Application to Cancer* (Inserm Symposium No. 8) (ed. by B. Serrou and C. Rosefield), pp. 345–352. Elsevier/North-Holland, Amsterdam.

SACHS, L. (1978) Control of normal cell differentiation in leukaemic white blood cells. In *Cell Differen-*

tiation and Neoplasia (ed. by G. F. Saunders), pp. 223–237. Raven Press, New York.

SALMON, S. & SELIGMANN, M. (1974) B cell neoplasia in man. *Lancet*, **ii,** 1230.

ZALEWSKI, P.D. & FORBES, I.J. (1979) Studies of the human lymphocyte mouse erythrocyte bond. *Clin. exp. Immunol.* **36,** 536.

ZOLA, H. (1977) Fractionation of human lymphocytes using rosette formation with papain-treated mouse erythrocytes. *J. Immunol. Methods*, **18,** 387.