Brief Definitive Report

INFREQUENT NORMAL B LYMPHOCYTES EXPRESS FEATURES OF B-CHRONIC LYMPHOCYTIC LEUKEMIA

By FEDERICO CALIGARIS-CAPPIO,* MARCO GOBBI, MARGARITA BOFILL,‡ AND GEORGE JANOSSY§

From the Department of Immunology, Royal Free Hospital School of Medicine, London NW3, United Kingdom

The cellular origin of chronic lymphocytic leukemia of B cell type (B-CLL) is unresolved (1). Most cases of B-CLL express detectable surface immunoglobulin (SmIg) (albeit weakly), HLA-DR, and B cell-associated membrane molecules as identified by monoclonal antibodies (MAb) (2-7). T lymphocyte-associated antigens are also observed on these malignant B cells when analyzed by both heterologous antisera (8) and MAb (9-13). In these studies no obvious normal counterparts of the malignant B-CLL cells have been found in the human bone marrow (BM), blood, and lymph nodes (LN) (9-11), which either suggests a surprisingly regular disturbance of gene expression in B-CLL or indicates that the "normal equivalent" cells in this disease are rare.

In this study we have used the fact that both B-CLL and a subset of normal B lymphocytes form rosettes with mouse erythrocytes (MRBC) (14, 15) and can therefore be separated on Ficoll-Hypaque gradient from other, nonrosetting cells. The analysis of $MRBC^+$ B cells shows that a small population of normal B cells (2-5%) in human lymph nodes (but virtually none in the BM and blood) expresses the phenotypic characteristics of B-CLL. On the basis of sensitive double-staining methods used in tissue sections, we suggest that at least a proportion of these normal B cells are situated within the germinal centers.

Materials and Methods

Conventional Antisera. Goat (G) antisera to human Ig isotypes (μ, δ, κ, λ and mixed $\mu + \delta + \kappa + \lambda$) were labeled with tetraethyl-rhodamine isothiocyanate (TRITC) and used in direct immunofluorescence (IF) test. Chicken (C) antiserum to HLA-DR (Ia-like) antigens was a specific reagent used in indirect IF test labeled with sheep-anti-C-Ig-TRITC (16).

MAb. OKT1 (12) and OKT3 (17) were from Ortho Pharmaceutical, Raritan, N. J. Leu-1 (11, 13, 18) was from Becton, Dickinson & Co., Orangeburg, N. Y. (BD 5300). Additional MAb were prepared at the Royal Free Hospital, London (19). RFA-1 (selected from a fusion using spleen cells of mice immunized with B-CLL) reacted with B-CLL, T lymphocytes, and thymocytes in a pattern identical to OKT1 and Leu-1. These three MAb recognize epitopes on the same (family of) membrane molecules (65,000-67,000 mol wt) (17, 19); OKT1 and RFA-1 show competitive inhibition of binding. The hybrids RFA-2 and -3 were obtained from fusions using spleen cells of mice immunized with the peripheral B cells from a patient with non-Hodgkin lymphoma in leukemic phase (19). The common feature of these two MAb is

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‡ Supported by Jaume Bofill Foundation, Barcelona, Spain.

§ To whom correspondence should be addressed at the Department of Immunology, Royal Free Hospital, Pond Street, London NW3 2QG, U. K.

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their lack of reactivity on terminal transferase positive lymphocyte precursors, pre-B cells, $SmIg^+$, μ^+, δ^- lymphocytes, and plasma cells in the BM, contrasting their strong positivity with various B cell subsets and plasma cells in the peripheral lymphoid organs (for more details see 19).

MAb were labeled with G-anti-M-Ig-FITC (green) second layers and used in various double-labeling combinations with conventional antisera tagged with TRITC (red) as described previously (16, 20). SmIg staining was routinely used with RFA-2 and -3 antibodies. In addition, MAb were coupled to arsenilic acid (RFA-1-ARS) or to biotin (OKT3-Biotin) and used with appropriate second layers in combinations (G-anti-ARS-TRITC, and avidin-FITC) (21).

Preparations of Cell Suspensions and MRBC Rosettes. Leukocytes from blood and BM were passed through a Ficoll-Hypaque gradient; cells from tonsils and LN were teased with blunt forceps and washed three times (16). Cells (10^7/ml) were incubated with antisera (1:20–1:40 dilution) and/or MAb (10–100 μg/ml for 10 min at 4°C), washed three times, and stained with second layers (1:40 dilution, 10 min at 4°C), followed by washing and mounting on slides (16).

MRBC were treated with neuraminidase (0.1 U/ml, 30 min at 37°C). The leukocytes were not treated with the enzyme because this step may decrease specificity (22). Leukocytes (5×10^6/ml) were incubated with MRBC (10^8/ml) as a pellet for 5 min at 37°C, followed by incubation for 30 min at 4°C, gently resuspended, and counted as a wet preparation on slides. In five experiments cells were transferred onto Ficoll-Hypaque after MRBC rosetting and spun (400 *g* for 25 min). Cells in the pellet (>70% $MRBC^+$) were treated with 0.1% Tris-NH_4Cl (15 min, 20°C) to lyse RBC, and passed through fetal calf serum to obtain enriched $MRBC^+$ leukocytes (23). In parallel experiments the suspensions have been stained for membrane antigen and incubated with MRBC as described above. Preparations were studied on a Zeiss microscope using IV/Z attachment and phase contrast (Carl Zeiss Inc., London).

Study of Tissue Sections. Sections (5 μm) of frozen tonsil (five samples) and mesenteric LN (two samples from patients with no malignancy) were cut on a cryostat and dried for 10 min at 20°C. This was followed by fixation in equal mixtures of acetone and chloroform, lyophilization, and rehydration in buffered saline (24). Combinations of MAb (1–5 μg/10 μl saline, each) were studied with fluorescent second layers (1:40 dilutions; see above) using both the TRITC and FITC channels.

Results

Phenotypes of MRBC-Rosetting Normal B Cells. In the five samples of blood, 6.8 ± 1.4% (SD) of all lymphocytes were $MRBC^+$. In tonsil and LN, 2.3 ± 0.4% $MRBC^+$ cells were seen. No $MRBC^+$ could be identified in the normal BM; only a few leukocyte clumps ("pseudorosettes") were formed. The percentages of $SmIg^+$ B cells were higher (12 ± 5% in blood, 42 ± 11% in LN, and 7 ± 3% in BM) than the proportions of $MRBC^+$ cells.

The direct observation of membrane staining in MRBC rosettes and the investigation of MRBC-enriched populations (70–75% purity) yielded similar conclusions (Table I). $MRBC^+$ cells observed in both peripheral blood and LN were $OKT3^-$ and stained for both SmIg and HLA-DR antigens. The staining for SmIg appeared to be weaker than that of additional $MRBC^-$ B cells also present in the suspension. The $MRBC^+$ cells were strongly positive with both RFA-2 and RFA-3, confirming that they were different from the μ^+, δ^- B cell population in the BM (which are $RFA\text{-}2^-$, $\text{-}3^-$; Table I). A further finding was that $MRBC^+$ cells in the tonsil and LN, but not in the blood, reacted with all three MAb recognizing the OKT1-type antigen ($OKT1^+$,$Leu\text{-}1^+$,$RFA\text{-}1^+$; see Fig. 1). Thus the dominant phenotype of normal $MRBC^+$ cells in lymphoid organs is $SmIg^+$ (weak), $HLA\text{-}DR^+$,$OKT3^-$,$RFA\text{-}2^+$,$RFA\text{-}3^+$,$OKT1^+$ ($Leu\text{-}1^+$,$RFA\text{-}1^+$). This phenotype is identical to the features of typical B-CLL cases but different from the features of prolymphocytic leukemia (PLL; Table I).

TABLE I
*Subset Characterization of Normal and Malignant B cells**

	Normal cells					Malignant cells	
		LN and tonsil		Peripheral blood		Peripheral blood	
	BM‡	All B cells‡	MRBC⁺§	All B cells‡	MRBC⁺§	CLL (n = 11)	PLL (n = 5)
SmIg	(100)	(100)	70 (weak) ‖	(100)	70 (weak)	70 (weak)	>90 (strong)
MRBC	<1	5–6	>75	50	>80	>70	<2
HLA-DR	>90	>90	>75§	>90	>75	>90	>90
OKT3	<5	<1	<10	<2	<10	<5	<5
RFA-2	<10 (μ^+, δ^-)	>90	>75§	>90	>75	>90	>90
RFA-3	<10 (μ^+, δ^-)	>90	>75§	>90	>75	>90	>90
OKT1**	<5	5‡‡	61§	<2	<5‡‡	>80	<5
RFA-1**	<5	6‡‡	59§	<2	<5‡‡	>80	<5
Leu-1**	<5	5	60§	<2	<5	>80	<5

* The B cells were recognized by SmIg staining. MRBC⁺ were studied after separation or in double marker studies.

‡ SmIg⁺ cells represent 7% of BM cells, 42% of LN and tonsil, and 12% of blood mononuclear cells. BM cells do not form MRBC rosettes, and only very few MRBC⁻, SmIg⁺, OKT1⁺ cells can be seen (0.3% of SmIg⁺ cells).

§ MRBC⁺ were separated with 70–75% purity. The corresponding values in double marker test cells were higher (80–90%).

‖ 80% of these cells were double SmIg⁺, OKT1⁺. These represent a mixture of κ^+ and λ^+ B cells.

** These three MAb recognize the same molecule (65–67,000 mol wt) (18).

‡‡ Double marker experiments with OKT1 (or RFA-1) and SmIg revealed that 5–6% of LN and tonsil B cells were double labeled with both reagents (Fig. 2). These cells were OKT3⁻. The OKT1⁺, SmIg⁺ cells were not seen in the blood, even when the MRBC⁺ population was studied.

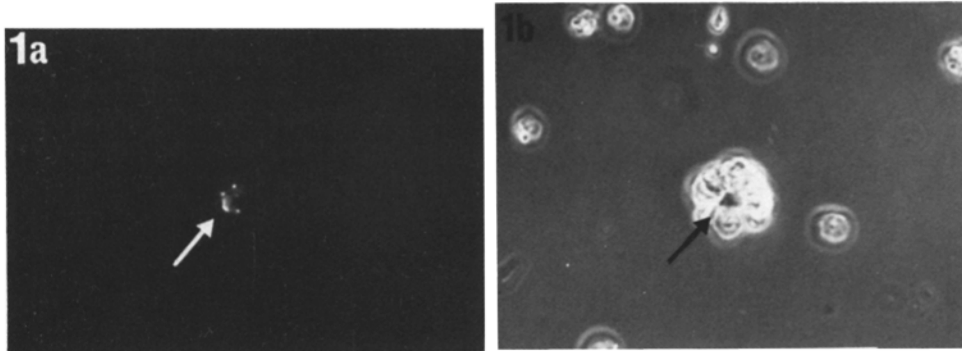

FIG. 1. MRBC rosetting cells in the human tonsil express OKT1 (a) (phase b).

Combination Staining for OKT1, SmIg, and OKT3. The observations above have suggested that in tonsils MRBC⁺ (2–2.5% of all cells) carry both OKT1 and SmIg. This finding was directly confirmed by the staining combination (6% OKT1⁺,SmIg⁺ doubles in Table I; Fig. 2). These doubles expressed both antigens weakly. A similar proportion of OKT1⁺ cells failed to express OKT3 when analyzed with hapten-conjugated MAb in suspension (3.5% OKT1⁺,OKT3⁻ tonsil cells).

Localization of OKT1⁺, OKT3⁻ Cells. MAb to MRBC receptors are not yet available. For this reason, the equivalent OKT1⁺,OKT3⁻ cells were studied in tissue sections. These cells have been observed in the germinal centers (GC) of tonsil and LN amidst the rich deposition of antigen-antibody complexes. The OKT3⁻ cells represented only 10–15% of all OKT1⁺ cells present (Fig. 3). These observations do not exclude the possibility that additional OKT1⁺,OKT3⁻ non-T cells may also be present in the T cell areas where they are more difficult to detect.

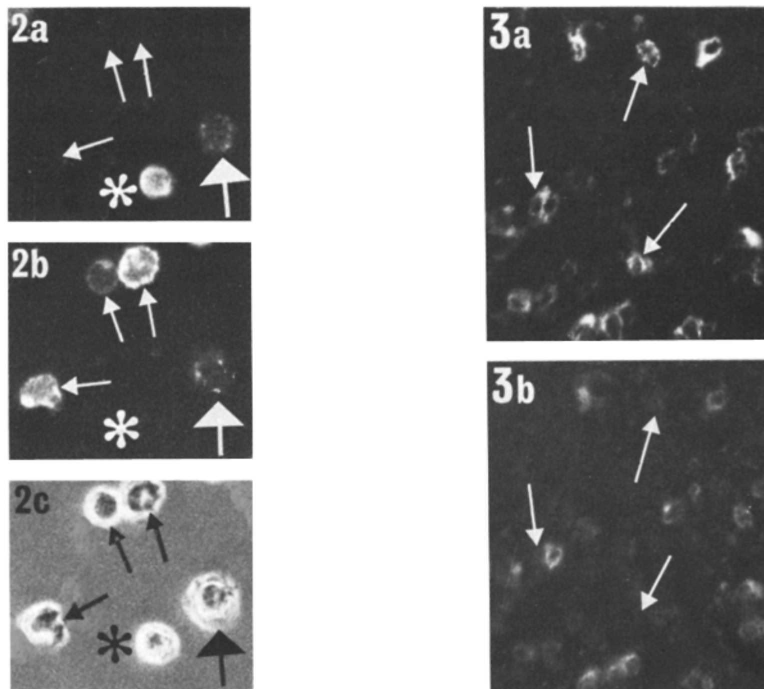

FIG. 2. A small proportion (5–6%) of lymphocytes (⟶) in the human tonsil simultaneously and weakly express OKT1 (a) and SmIg (b) (phase c). These cells are selectively enriched when the MRBC$^+$ population is isolated (Table I). Note that T cells show strong OKT1 staining (SmIg$^-$; [*]) and the dominant B cell population shows SmIg staining (OKT3$^-$; [→]).

FIG. 3. Sections of human tonsil were stained with RFA-1 (OKT1-like) antibody (TRITC; a) and OKT3-biotinated antibody (FITC; b). A few RFA-1$^+$ cells (10% of all RFA-1$^+$ cells) are OKT3 negative in the germinal center area shown (→).

Discussion

This study identifies a small population of B lymphocytes that appear to be the normal equivalent cells of B-CLL. These express SmIg weakly, are HLA-DR positive, form MRBC rosettes, and react with MAb recognizing OKT1-like (P65) antigen(s). This small subset is unlikely to be a (pre-)malignant population for three reasons. First, it was seen in all tonsil and lymph node samples studied. Second, MRBC$^+$ cells consisted of a normal mixture of κ^+ and λ^+ lymphocytes. Finally, a similar subpopulation of Thy-1$^-$,Lyt-1$^+$ (homolog of Leu-1 in mouse) non-T cells have been identified in the GC of murine LN (24). Thus this population is not unique for man.

Two pieces of evidence support the possibility that the normal equivalent cells of B-CLL are peripheral B cells. First, only tonsils and LN contain cells expressing both MRBC and OKT1 markers together with the other B characteristics (SmIg$^+$,HLA-DR$^+$), whereas in the BM these MRBC$^+$, OKT1$^+$, SmIg$^+$ cells are absent. Second, two recently standardized MAb, RFA-2 and -3, strongly react with virtually all peripheral B cells including B-CLL. These reagents are nevertheless unreactive with μ^+, δ^- BM cells.

It has been puzzling that cases of pre-B acute lymphoblastic leukemia as well as the corresponding pre-B cells in normal BM synthesize heavy (μ) chain only (25, 26), whereas B-CLL and the normal equivalent cells (putative early B cell precursors? See

27) synthesize excess light chains. The most likely simple explanation is that these two types of putative early B cell precursors represent two distinctly different stages of B lymphocyte development (in the BM and GC, respectively). The normal equivalent cells of B-CLL, putative early memory cells (1), are now available for cell separation (using MRBC rosetting) and for further functional analysis.

Summary

An infrequent (2–3%) B lymphocyte subpopulation was found in the normal human tonsil and lymph nodes that shows the phenotypic characteristics of B-chronic lymphocytic leukemia (B-CLL) (rosette formation with mouse erythrocytes, weak expression of membrane Ig, staining for HLA-DR, and OKT1 or Leu-1 detecting a T cell-associated p65 antigen). Preliminary evidence suggests that at least a subpopulation of these cells is found, in small proportions, within the germinal centers. These cells were not observed in the human bone marrow. B-CLL may involve this peripheral B lymphocyte subset.

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