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DEVELOPMENT OF CLL IN INDIVIDUALS WITH MILD LYMPHOCYTOSIS, WITHOUT BONE MARROW INFILTRATION, BUT WITH EVIDENCE OF A MONOCLONALLY EXPANDED POPULATION IN PERIPHERAL BLOOD

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ABSTRACT

Mild lymphocytosis (< 10×10^9/L) is a common finding in routine blood tests. When it persists, it raises the question of whether this disorder is an early manifestation of chronic lymphocytic leukemia (CLL). If it is accompanied by bone marrow infiltration, it can be safely considered as a sign of CLL. The aim of this study was to analyze retrospectively the usefulness of immunophenotyping and immunogenotyping for early detection of lymphocyte clonality in ambiguous cases of lymphocytosis without bone marrow infiltration. Twenty-six healthy individuals, 47 to 77 years old, with an absolute lymphocyte count (ALC) at the «onset» of the disorder between 4×10^9/L and 9×10^9/L, without marrow infiltration, were studied and followed for a period of 31 to 51 months. CD19, CD20, CD5, CD2, CD4, CD8 surface markers and amplification of the Ig heavy chain CDR-3 locus were used for immunophenotypic and genotypic analysis, respectively. Our studies indicate that immunophenotyping alone is sufficient and superior to CDR-3 locus amplification for the early detection of lymphocyte clonality in peripheral blood. Furthermore, the high frequency of CLL development in individuals with established monoclonality strongly suggests that patients with mild bordeline lymphocytosis, even without bone marrow infiltration, have to be followed for progression to CLL and its possible complications.

Key words: chronic lymphocytic leukemia, absolute lymphocyte count, bone marrow lymphocytic infiltration, immunophenotyping, PCR, Ig

Mild absolute lymphocytosis (4-10×10^9/L) in elderly asymptomatic patients raises the question of whether this disorder is an early manifestation of chronic lymphocytic leukemia (CLL).

Diagnosis of CLL requires persistent lymphocytosis with an absolute lymphocyte count (ALC) of at least 10×10^9/L and either lymphocyte infiltration in the bone marrow of at least 30% or clonality of blood lymphocytes, as determined by phenotype.[1] It is possible to diagnose CLL with blood lymphocytosis between 5 and 10×10^9/L when the marrow infiltration and clonality criteria are both met.[1]

In many patients with lymphocytosis lower than 10×10^9/L, bone marrow may not show infiltration since blood and marrow lymphocytosis appear to correlate directly.[2] In these individuals a diagnosis of CLL is controversial and a demonstration of clonality remains the only available diagnostic criterion. In clinical practice the clonality of lymphocytes is demonstrated by using immunophenotypic and immunogenotypic analysis.[3-6]

Clonal expansion of B-lymphocytes is observed in 95% of patients with CLL.[1] According to the differentiation antigens on the cell surface, B-CLL exhibit a CD19, CD20 and CD5 phenotype, whereas the normal T/B cell ratio is strongly reversed. The κ/λ light chain ratio can also be significantly altered. Polymerase chain reaction (PCR)-based strategies, more sensitive than Southern blotting, have been developed over the last few years for immunogenotypic analysis of lymphoproliferative diseases.[4,7,8,9]

In this study we tried to evaluate the usefulness of immunophenotypic analysis with a simple panel of monoclonal antibodies and amplification of the Ig heavy chain CDR-3 locus in an attempt to analyze the clonality of lymphocytes before the appearance of CLL in ambiguous cases of lymphocytosis without bone marrow infiltration.

Materials and Methods

Mild persistent lymphocytosis was found in 46 individuals at routine blood testing between February 1992 and October 1993, and the number of lymphocytes at this time ranged from 4×10^9/L to 9×10^9/L. During the course of this study 20 individuals were excluded because marrow infiltration at the time of diagnosis was established or because monitiring was inadequate.

Thus, a total of 26 healthy *patients*, 47 to 77 years old (mean age 61.6±8.6), were fully studied and followed. These individu-

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als were monitored for 31 to 51 months (February 1992 to May 1996), and the lymphocytes in the bone marrow did not exceed than 25% at the presentation of the disease. Trephine biopsy was also negative for non diffuse (interstitial, nodular or mixed) bone marrow infiltration at the time of diagnosis. Biopsy was not performed in seven patients (Tables 1 and 2).

Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation. Part of these were placed on glass films and conserved at –20°C for immunophenotyping, and the rest were used for DNA extraction and PCR.

Immunophenotypic analysis

A standard *alkaline phosphatase-anti alkaline phosphatase* (APAAP) protocol was followed for immunophenotypic analysis. Monoclonal antibodies (DACO, Denmark) were used to detect CD19, CD20, CD5, CD2, CD4, CD8, κ and λ light chains. The results were independently interpreted by two observers and 300 to 400 cells were counted for every CD marker.

Polymerase chain reaction (PCR)

After DNA isolation, 0.5 μg were used for PCR. PCR amplifies the unique CDR-3 sequences using primers homologous to the consensus sequences in the variable (V_H) and joining (J_H) segments that flank the diversity segment in the rearranged heavy-chain immunoglobulin locus.[4,8] The sequence of the primers as well as the PCR conditions were based on literature data.[4,8,9] Amplified DNA was electrophoresed in 2.5% agarose gel and the rearranged bands were detected in the range of 80-140 bp.[8,9]

Results and Comment

The 26 patients were separated into two groups, A and B, according to the results of immunophenotypic analysis, PCR and the course of lymphocytosis. Tables 1 and 2 summarize patient age, ALC in the peripheral blood, the infiltration in the marrow at presentation of the disorder, the follow-up time and the course of the lymphocytosis for every «patient» at the end of the monitoring period. All subjects displayed mature lymphocytes at the appearance of the disorder and the mean range of lymphocytes was 6.46±1.64 and 6.81±1.63×10⁹/L for group A and group B, respectively, with no statistically significant difference between the two groups.

For the 11 individuals classified in group A (Table 1), no monoclonality lymphocyte criteria were found with APAAP imunophenotyping or PCR detection of the CDR-3 locus. No one in this group developed a lymphoproliferative disorder during the 31- 51-month monitoring period, but the ALC remained stable. Patients 3 and 12 were diagnosed as having polymyalgia rheumatica and chronic persistent hepatitis B, respectively, 22 and 9 months after the finding of lymphocytosis. Virus studies were negative for all patients except case 12.

Group B consisted of 15 individuals (Table 2) with positivity for B lineage markers and the presence of CD5 (except in cases #5 and #13) on peripheral blood. CD2-positive cells never exceeded 40% of PBMCs and were significantly fewer than CD19-positive cells. The CD4- and CD8-positive cells in many cases were difficult to estimate due to their very low frequency. The κ/λ ratio was often difficult to estimate with APAAP and thus we did not use this parameter as a monoclonality criterion. PCR amplification of the CDR-3 locus was «positive» and showed a distinct band at 80 to 125 bp after gel electrophoresis in 11 of the 15 patients, whereas in cases 6,7,11, and 15 PCR was «negative». Twelve patients from this group have developed CLL (RAI 0) according to the criteria of the International Workshop on CLL,[1] in 26 to 39 months after presentation of the disorder, and eight of the twelve CLL patients were PCR positive. The four PCR negative CLL patients remained negative for the entire follow-up period. This phenomenon depends on PCR parameters and has been described by other authors who eliminated this false result in about 15%-30% of B cell malignancies.[5,8,9] The failure to amplify the monoclonal CDR-3 sequence reflects a loss of primer annealing due to nucleotide alterations on 3'V_H and/or 5'J_H.[4,5,7,8] One patient from the same group (case #10) devel-

Table 1. Individuals with normal B cell distribution at immunophenotypic analysis and smear (negative) on electrophoresis after PCR of the CDR-3 locus.

Pt/age	Lymphocytes		Immunophenotypic analysis (peripheral blood mononuclear cells)								Course of	Time (mos.) appearance–monitoring	
	Peripheral	marrow	CD19	CD20	CD5	CD2	CD4	CD8	κ/λ	CD3 (PCR)	lymphocytosis	of disease	of lymphocytosis
1/71	4.2×10⁹/L	22%*	15%	17%	54%	81%	49%	24%	2.0	Negative	None	–	49
2/57	5.5×10⁹/L	18%	12%	20%	78%	86%	54%	22%	N.E	Negative	None	–	48
3/68	8.7×10⁹/L	22%	21%	24%	44%	69%	41%	31%	1.8	Negative	Polymyalgia rheumatica	22	51
4/59	8.3×10⁹/L	21%	19%	22%	69%	66%	45%	22%	N.E	Negative	None	–	47
5/52	5.8×10⁹/L	14%	11%	26%	33%	61%	30%	26%	2.0	Negative	None	–	44
6/53	4.9×10⁹/L	16%	22%	27%	42%	59%	44%	26%	N.E	Negative	None	–	36
7/55	5.1×10⁹/L	7%*	6%	9%	61%	84%	51%	29%	2.3	Negative	None	–	37
8/63	5.7×10⁹/L	9%*	9%	14%	63%	64%	33%	25%	2.7	Negative	None	–	34
9/72	7.7×10⁹/L	11%*	12%	11%	79%	88%	56%	32%	2.4	Negative	None	–	33
10/47	6.4×10⁹/L	18%	20%	28%	50%	79%	47%	33%	N.E	Negative	Chronic (persistent) hepatitis B	9	33
11/50	8.8×10⁹/L	13%	17%	23%	82%	77%	40%	28%	2.2	Negative	None	–	31

**Bone marrow biopsy was not performed. N.E. Not estimated; this parameter was difficult to estimate with the APAAP method.*

Table 2. Individuals with B-cell expansion in peripheral blood and single band (positive) on electrophoresis after PCR of the CDR-3 locus.

Pt/age	Lymphocytes	Immunophenotypic analysis (peripheral blood mononuclear cells)								Course of	Time (mos.) appearance–monitoring	
	Peripheral–marrow	CD19	CD20	CD5	CD2	CD4	CD8	κ/λ	CDR-3 (PCR)	lymphocytosis	of disease	of lymphocytosis
1/72	8.9×10⁹/L 23%*	50%	62%	44%	28%	N.E	N.E	4.1	Positive	None	–	51
2/60	6.0×10⁹/L 18%*	48%	54%	49%	27%	10%	16%	2.3	Positive	CLL	30	50
3/53	5.1×10⁹/L 15%	62%	70%	56%	29%	N.E	N.E	N.E	Positive	None	–	48
4/59	5.3×10⁹/L 24%	55%	61%	57%	24%	N.E	N.E	0.9	Positive	CLL	32	45
5/67	8.4×10⁹/L 22%	51%	54%	17%	32%	20%	11%	3.3	Positive	CLL	39	46
6/64	7.5×10⁹/L 19%	52%	58%	44%	29%	N.E	N.E	4.9	Negative	CLL	26	44
7/69	4.8×10⁹/L 21%	47%	51%	41%	33%	N.E	N.E	3.0	Negative	CLL	36	44
8/52	8.3×10⁹/L 23%*	54%	52%	49%	36%	7%	7%	N.E	Positive	CLL	35	42
9/58	4.9×10⁹/L 20%	49%	42%	44%	40%	14%	10%	1.1	Positive	CLL	31	41
10/74	4.2×10⁹/L 14%	51%	54%	48%	35%	N.E	N.E	2.0	Positive	Autoimmune hemolytic anemia	14	39
11/70	7.7×10⁹/L 19%	51%	57%	49%	36%	17%	14%	3.8	Negative	CLL	26	40
12/63	7.1×10⁹/L 25%	47%	59%	43%	33%	13%	12%	0.5	Positive	CLL	34	39
13/77	8.9×10⁹/L 22%	54%	60%	21%	32%	16%	13%	1.9	Positive	CLL	32	38
14/50	6.9×10⁹/L 25%	62%	68%	71%	19%	N.E	N.E	N.E	Positive	CLL	29	35
15/68	8.2×10⁹/L 25%	52%	69%	46%	22%	N.E	N.E	N.E	Negative	CLL	25	34

**Bone marrow biopsy was not performed. In cases #5 and #13 CD5 is considered negative. N.E. Not estimated; these parameters were difficult to estimate with the APAAP method.*

oped autoimmune hemolytic anemia (sign of CLL?) 14 months after the presentation of lymphocytosis, without other evidence of lymphoproliferative disease. Only two individuals in group B (cases 1 and 3) with B cell marker expansion and PCR positivity have remained disease free for up to 4 years. Faguet *et al*.[6] also studied 25 patients with mild lymphocytosis and peripheral B cell expansion according to immunophenotyping, without marrow infiltration, and observed that eight of these patients developed CLL.

Overall, we conclude that immunophenotypic analysis of certain surface markers seems to be more sensitive than amplification of the CDR-3 locus for early detection of lymphocyte clonality in peripheral blood. Although B CLL in early stage does not need treatment, many cases are still heterogeneous with respect to prognosis.[10] Moreover, early diagnosis and monitoring of patients with mild lymphocytosis by means of other specific parameters may have an impact on overall survival and possibly increase our understanding of the natural course of CLL. We believe that if the monoclonality criterion is established through immunophenotyping in individuals who present with mild lymphocytosis in the absence of bone marrow infiltration, even if they cannot be characterized as CLL, they should be followed for progression to CLL and/or its possible complications.[1]

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