Antigen modulation followed by quantitative flow cytometry of B-chronic lymphocytic leukemia cells after treatment*

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Presented study analyzed the immunophenotypic characteristics and antigen density of peripheral blood (PB) and bone marrow (BM) cells of 23 patients with B-chronic lymphocytic leukemia (B-CLL) and 10 control subjects using flow cytometry. The patients were subclassified into two groups. Group I formed 13 patients with B-CLL at the time of diagnosis and group II 10 patients with B-CLL after the therapy but not in remission. For definition of B-CLL cells we used immunological marker analysis of surface markers characteristic for B-CLL pattern: CD5, CD19, CD20, CD23 and HLA DR and enumeration of fluorescence intensity of these markers given by molecular equivalent of soluble fluorochrome – MESF. In group II of B-CLL patients, who were already treated, in PB and BM somehow lower MESF values for CD19, CD20 and CD23 markers and higher MESF values for CD5 marker (in PB and BM) than in group I patients have been detected. The MESF level of HLA DR marker was little higher in group II than in group I B-CLL patients. However in PB and BM the percentage expression of HLA DR and CD19 markers in both patients groups was approximately the same. The values of HLA DR, CD19, CD20, CD23 and CD5 markers (% expression and MESF values) in both patients groups with B-CLL were significantly higher versus controls ($p<0.001$ resp. $p<0.01$) even in PB and BM. In conclusion, in our study we observed that the patients with B-CLL after therapy (group II) had similar or a little smaller (except CD5) but nonsignificantly decreased expression level of markers characteristic for B-CLL, but the MESF values of some of them (CD19, CD23) were significantly ($p<0.05$) decreased when compared with untreated B-CLL patients (group I). The determination of antigen density (MESF values) may be an important marker to characterize the leukemic cells. Our results showed that chemotherapy did not influence in a significant level the antigen modulation of B-CLL cells, however, could influence MESF values of some chracteristic markers. Quantitative analysis of some markers in B-CLL cells seems to offer valuable information concerning possible influence of some chemotherapeutics on antigen equipment of leukemic cells.

Key words: B-cell chronic lymphocytic leukemia, immunophenotyping, molecular equivalent of soluble fluorochrome, flow cytometry

B-cell chronic lymphocytic leukemia (B-CLL) is one of chronic lymphocytic disorders which is characterized by the accumulation in the blood, bone marrow, lymph nodes and spleen of a clonal population of non-dividing, usually CD5 positive B-lymphocytes that weakly express surface immunoglobulins, HLA-DR and surface markers of B-cell differentiation: CD19, CD20, CD23 and CD40. CD10, CD22 and FMC7 markers are either weakly or not expressed [12, 20]. CD38 marker is expressed in approximately 10 – 15% of B-CLL cases [23], and his prognostic value is supposed.

Immunophenotyping is an essential and mandatory test for the diagnosis and classification of B-CLL and other chronic lymphocytic disorders. Various combinations of markers have been applied in order to distinguish B-CLL from other chronic lymphoproliferative disorders.

Longevity of B-CLL cells is caused mainly by cell arrest in G0/G1 phase of cell cycle and by the inhibition of programmed cell death, resulting in cell survival [14]. The predominant cell is a small lymphocyte [12]. Although B-CLL cells show a decreased ability to proliferate compared with

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normal B-cells, they have a longer lifespan *in vivo*, which serves to maintain the tumor cell population [21, 24].

Conventional chemotherapy has been used for B-CLL treatment and it improved in recent years. Several modalities have been used as first line therapy in B-CLL patients, including chlorambucil, which has been most extensively used, purine analogues (fludarabine, 2-chlorodeoxyadenosine), monoclonal antibodies (rituximab) and combination chemotherapy programmes [23, 29]. Nevertheless, all patients with B-CLL relapse after initial response [13].

Flow cytometry allows in addition to the determination of positive cells, to establish even the intensity of fluorescent staining, that can be converted into antigen density. The concept of antigen density appears to improve the efficiency of immune techniques in the monitoring of hematopoietic malignancies [19, 22, 27].

Quantitative techniques provide data contributing to a more precise definition of cell differentiation [3, 18, 28] and improve scoring system for the differential diagnosis among the different chronic B-cell malignancies [15, 16, 20]. Quantitative immunophenotyping is thus suitable for the diagnosis of malignancy, contributes to prognosis [7, 16, 17] and could provide new relevant pathophysiological informations.

The present paper will focus on the exact immunohenotyping of B-CLL which enabled to distinguish B-CLL from other chronic B-lymphocytic disorders. Furthermore, the MESF values were used to compare their numbers in B-CLL cells before treatment and after treatment in patients having not complete remission and thus to define the effect of antigen modulation by chemotherapy treatment.

Material and methods

Patients and leukemia samples. Heparin anticoagulated bone marrow (BM) or peripheral blood (PB) samples from 23 adults patients were studied for B-CLL associated phenotype. Leukemia blasts were classified according to Rai criteria. Ten B-CLL patients were treated with chemotherapy before our investigation, the rest of patients (13) were examined before starting of any treatment. In the frame of this study quantitative immunofluorescence using calibration microbeads was also performed. Peripheral blood and bone marrow lymphocytes of 10 healthy donors were simultaneously studied for quantitative immunophenotyping.

Immunological marker analysis. The membrane immune phenotype was analyzed in all B-CLL patients and healthy donors by direct immunofluorescence technique. Briefly: anticoagulated blood or bone marrow (100 μl) was incubated for 15 min with 10 μl antibody, followed by standardized lysis of red cells with Optilyse (Immunotech, Marseille, France) and immediate flow cytometry analysis.

Monoclonal antibodies (MoAbs). MoAbs against CD19, CD20, CD22, CD23, CD5, CD45, CD38 and anti-HLA DR were purchased from Immunotech (Marseille, France). MoAbs used were labeled with fluorescein-isothiocyanate (FITC). As negative controls appropriate isotype controls Ig FITC MoAbs (Immunotech, Marseille, France) were used.

Flow cytometric analysis. Flow cytometric analysis was performed on EPICS ALTRA Flow Cytometer (Beckman Coulter), which is equipped with 15 mW argon laser operating at 488 nm. Data were analyzed using WinMDI, Vers. 2.8 (Scripps Institute, La Jolla, USA). At least 10,000 cells were analyzed in each sample. Gating of cells was performed using forward light scatter (FSC) and side light scatter (SSC), or orthogonal SSC and CD45 parameters. Data were expressed as percentage of labeled cells or fluorescence histograms (dot plots or contour ones).

Evaluation of marker density. For evaluation of marker density, expressed in flow cytometry by mean of fluorescence intensity, it is necessary to calibrate flow cytometer by means of fluorescent calibration microbeads. Photomultiplier tube (PMT)-sensitivity in response to changes in the physical environment of the machine and PMT was calibrated in absolute fluorescence units, called molecules of equivalent soluble fluorescein (MESF) and fluorescence histograms of these particles were measured on logarithmic scale. Immunobrite (Beckman Coulter, USA) calibration particles were used with a blank and four different fluorescence intensities [25]. Using mean values of histogram peaks and MESF values linear regression was calculated. Calibration curves and subsequent calculations were performed with standard software MS Excel (Microsoft, USA). It was necessary to repeat the cytometer calibration on every day of measurement. The day-to-day variation in terms of MESF was below 2% (data not shown).

Statistical analysis. Student's t-test for equal and unequal variance was used to analyze the statistical significance of the results. Values of $p<0.001$, $p<0.01$ and $p<0.05$ were considered to indicate a statistically significant difference.

Results

For definition of B-CLL cells we used immunological surface markers characteristic B-CLL pattern: CD5, CD19, CD20, CD23 and HLA DR and enumeration of fluorescence intensity of these markers given by number of MESF. Patients were arbitrarely classified according to their therapy status into two groups:

– group I (patients at diagnosis) and
– group II (patients after therapy – not being in remission).

Patients in group I (13 patients) display the following characteristics: age (66 median, 39–88 range), sex (9 male and 4 female) and white blood cells count in PB ($21x10^9$/l

Table 1. Flow cytometric expression in % and MESF values of HLA DR, CD19, CD20, CD23 and CD5 markers in PB and BM cells of B-CLL patients at diagnosis (group I)

Patient No	HLA DR		CD19		CD20		CD23		CD5	
	%	MESF	%	MESF	%	MESF	%	MESF	%	MESF
1. PB	82	5590	81	4280	71	2140	81	3430	91	3130
2. PB	40	610	41	180	28	290	18	130	88	6000
BM	71	2830	64	1710	65	1550	58	920	92	6250
3. PB	88	9220	85	2580	84	3950	82	1890	95	4180
4. PB	82	11770	80	3260	77	2660	81	2970	97	6230
BM	89	17380	87	4330	88	4070	79	2310	96	5810
5. PB	51	2080	51	1620	15	220	40	510	88	5020
6. PB	88	5340	81	4780	43	580	81	2980	90	2580
BM	74	2520	92	7380	41	600	91	3780	88	1980
7. PB	99	12360	97	6240	19	140	93	5260	87	2220
8. PB	86	11930	82	5730	79	2880	78	3070	82	2580
BM	93	11600	91	7790	59	1130	86	3600	76	1830
9. PB	94	8170	91	10820	69	1390	90	5670	64	990
BM	96	7710	96	12240	68	1260	93	5940	74	1220
10. PB	85	7220	82	2440	79	2320	77	1470	89	3360
11. PB	88	6510	80	2230	62	1800	nt	nt	59	1610
12. PB	nt	nt	nt	nt	71	2400	53	1560	90	3220
BM	nt	nt	nt	nt	66	1930	56	1560	87	3040
13. PB	nt	nt	93	1570	95	5830	72	1080	55	830
PB mean	84[a]	8019[a]	82[a]	4140[a]	73[a]	2595[a]	79[a]	2938[a]	87[b]	3648[b]
PB ± SD	13	3349	12	2733	14	1447	11	1554	10	1529
BM mean	85[a]	8408[a]	86[a]	6690[a]	65[a]	1757[a]	77[a]	3018[a]	86[b]	3355[b]
BM ± SD	11	6264	13	3964	15	1217	16	1816	9	2158
Controls of 10										
PB mean	17	280	6	40	6	80	8	70	62	2230
BM mean	28	720	10	110	12	170	8	110	55	920

[a]p<0.001 vs controls, [b]p<0.01 vs controls, nt – not tested, SD – standard deviation.

Table 2. Flow cytometric expression in % and MESF values of HLA DR, CD19, CD20, CD23 and CD5 markers in PB and BM cells of B-CLL patients after therapy (group II)

Patient No	HLA DR		CD19		CD20		CD23		CD5	
	%	MESF	%	MESF	%	MESF	%	MESF	%	MESF
1. PB	61	2180	34	290	32	160	36	400	89	5600
BM	83	9580	85	4370	66	990	77	2290	95	3370
2. PB	98	14780	95	3570	31	460	91	3110	99	5040
3. PB	80	5920	57	1240	37	410	52	760	96	6980
4. PB	97	15170	91	4320	33	760	86	3400	92	2920
BM	97	12740	94	4430	36	970	81	3190	90	3080
5. PB	69	6370	70	2280	73	3520	63	2570	nt	nt
6. PB	96	10040	nt	nt	71	1300	35	470	98	3540
BM	95	5720	nt	nt	41	470	34	470	98	3540
7. PB	80	8370	79	2290	51	1090	50	1040	88	2760
BM	82	5930	81	2440	44	830	42	940	89	2600
8. BM	96	15650	nt	nt	48	1380	65	2240	93	4820
9. PB	91	10740	86	2700	86	5400	80	3060	nt	nt
10. BM	nt	nt	nt	nt	33	140	85	1430	88	1240
PB mean	87[a]	10199[a]	80[a]	2733[a]	55[a]	1849[a]	65[a]	2059[a]	94[b]	4473[b]
PB ± SD	11	3704	14	1082	22	1891	21	1253	5	1679
BM mean	91[a]	9924[a]	87[a]	3747[a]	47[a]	928[a]	70[a]	2018[a]	93[b]	3482[b]
BM ± SD	7	4315	7	1132	11	328	17	867	4	829
Controls of 10										
PB mean	17	280	6	40	6	80	8	70	62	2230
BM mean	28	720	10	110	12	170	8	110	55	920

[a]p<0.001 vs controls, [b]p<0.01 vs controls, nt – not tested, SD – standard deviation.

median, 8–87x10^9/l range) and white cells count in BM (22x10^9/l median, 15–67x10^9/l range).

Patients in group II (10 patients) display the following characteristics: age (66 median, 40–75 range), sex (7 male and 3 female) and white blood cells count in PB (30x10^9/l median, 4–170x10^9/l range) and white blood cells count in BM (50x10^9/l median, 10–102x10^9/l range).

PB and BM lymphocytes from group I and group II patiens were analysed for their HLA DR, CD19, CD20, CD23 and CD5 expression. Lymphocytes of all patients were simultaneously quantified using MESF values. PB and BM lymphocytes of 10 healthy donors were simultaneously studied for both parameters.

The results of CD markers expression (in %) and quantification (in MESF) of lymphocytes in PB and BM from both groups with means, standard deviations and statistical significances of differences are presented in Table 1 and Table 2.

The data of HLA DR marker expression showed the similar levels in PB and BM of this marker in group I (mean 84%, range 40–99% and mean 85%, range 71–96%, for PB and BM, respectively) and in group II (mean 87%, range 61–98% and mean 91%, range 82–97%, for PB and BM, respectively).

The MESF values of HLA DR were higher in group II (mean 10199, range 2180–15170 and mean 9924, range 5720–15650, for PB and BM, respectively) than in group I (mean 8019, range 610–12360 and mean 8019, range 2520–17380, for PB and BM, respectively). Levels of HLA DR expression and MESF values in control healthy donors were very low.

We observed that CD19 antigen expression was in the mean 82%, range of 41 to 97% in PB and in the mean 86%, range of 64 to 96% in BM cells of group I patients and similarly in PB in the mean 80%, range of 34 to 86% and in BM in the mean 87%, range of 81 to 94% cells of group II patients.

In PB and in BM of group II patients significantly lower MESF values of CD19 antigen (mean 2733, range 290–4320 and mean 3747, range 2240–4430, for PB and BM, respectively) comparing to the MESF values of group I patients (mean 4140, range 180–10820 and mean 6690, range 1710–2240, for PB and BM, respectively) have been detected. Levels of CD19 expression and MESF values were in controls very low, similar to HLA DR.

The data of CD20 antigen expression showed higher levels in PB of this antigen in group I (mean 73%, range 15–95%) and in BM too (mean 65%, range 41–88%) than in PB (mean 55%, range 31–86%) and BM (mean 47%, range 33–66%) in group II patients.

MESF values of CD20 antigen in the group I were higher in PB (mean 2595, range 140–5830) and BM (mean 1757, range 600–4070) than in group II patients (mean 1849, range 160–5400 and mean 928, range 140–1380, for PB and BM,

Table 3. Comparison of % expression and MESF values of five markers in PB and BM cells of group I and group II B-CLL patients

	HLA DR		CD19		CD20		CD23		CD5	
	%	MESF	%	MESF	%	MESF	%	MESF	%	MESF
Group I										
PB mean	84[b]	8019[b]	82[b]	4140[a]	73[a]	2595[b]	79[b]	2938[a]	87[b]	3648[b]
BM mean	85[b]	8408[b]	86[b]	6690[a]	65[a]	1757[b]	77[b]	3018[a]	86[b]	3355[b]
Group II										
PB mean	87	10199	80	2733	55	1849	65	2059	94	4473
BM mean	91	9924	87	3747	47	928	70	2018	93	3482

[a]$p<0.05$ vs group II, [b]not significant.

respectively). The CD20 expression and MESF values were also very low in controls.

In PB and BM of group I patients we detected significantly higher levels of CD23 antigen expression than in the group II patients. In PB the values of mean 79% (18–93% range) and in BM mean 77% (56–93% range) in the group I and in PB mean 65% (35–91% range) and in BM mean 70% (34–85% range) in the group II patients were observed.

The MESF values of CD23 were higher in the group I (mean 2938, range 130–5670 and mean 3018, range 920–5940, for PB and BM, respectively) than in the group II (mean 2059, range 400–3400 and mean 2018, range 470–3190, for PB and BM, respectively). The data for controls were similar to HLA DR, CD19 and CD20 antigens expression.

CD5 antigen had slightly higher levels of expression in group II (mean 94%, range 88–96% and mean 93%, range 88–98%, for PB and BM, respectively) than in group I (mean 87%, range 55–97% and mean 86%, range 74–96%, for PB and BM, respectively).

The MESF values of CD5 were higher in group II in PB (mean 4473, range 2760–6980) in comparing to MESF levels in PB (mean 3648, range 830-6230) in group I. The MESF values were also higher in the group II in BM (mean 3482, range 1240–4820) than in BM (mean 3355, range 1220–6250) in group I. However the difference in MESF values in CD5 marker was very small, statistically insignificant.

The data for all markers (% expresion and MESF values) in both B-CLL patients groups were statistically tested and were significantly higher ($p<0.001$ resp. $p<0.01$) versus controls.

The means of CD markers expression and quantification of lymphocytes in PB and BM of B-CLL patients in both group I and group II were compared and statistical significance of differences (group I versus group II) is presented in Table 3.

Comparison of CD19, CD23, CD5 and HLA DR markers expression showed the similar data in PB and BM in group I and group II B-CLL patients. CD20 marker expression was significantly higher in group I than in group II. The means of MESF values of CD19 and CD23 markers were significantly higher in group I (4140 and 6690 and 2938 and 3018 in PB and BM, for CD19 and CD23, respectively) than in group II (2733 and 3747 and 2059 and 2018 in PB and BM, for CD19 and CD23, respectively). The means of MESF values for CD20 were slightly higher in group I (2595 and 1757, for PB and BM, respectively) than in group II (1849 and 928, for PB and BM, respectively). The means of MESF values for HLA DR and CD5 were slightly lower in group I (8019 and 8408 and 3648 and 3355 in PB and BM, for HLA DR and CD5, respectively) than in group II (10199 and 9924 and 4473 and 3482 in PB and BM, for HLA DR and CD5, respectively).

Discussion

B-CLL cells have a distinct marker profile characterized especially by the expression of CD5, which is at a higher level than that of normal B lymphocytes and therefore used as a diagnostic criterion for B-CLL and even by high expression of most pan-B cell markers, such as CD19, CD20 and HLA DR. CD23 activation antigen is expressed in the majority of cases. CD3 marker (T cell marker) is low. CD22 is weakly expressed or not expressed [12].

Antigen expression of CD5, CD19, CD20 and HLA DR markers in patients presented in our study represented characteristic levels of these antigens in B-CLL. However, we observed higher expression of CD5 antigen in PB and in BM group II B-CLL patients and lower expression of CD20 and CD23 markers in PB and BM group II of B-CLL patients when comparing with group I patients.

CD23 is a functionally relevant molecule in B-CLL; however, studies dealing with its role have yielded inconclusive results [2, 29]. The membrane instability of CD23, which is rapidly cleaved from the cell surface into a stable form, has important prognostic implication [29]. According to some authors CD23 is a reliable and disease-specific marker that provides information on overall survival and complete remission in early stage patients [8, 26].

There is an evidence that CD23 may replace other well-recognized diagnostic parameters in B-CLL. The CD23 antigen is expressed in the majority of B-CLL and is absent in majority of other chronic B-cell malignancies. In this paper CD23 expression of two groups of B-CLL patients was compared. The CD23 percentage expression was very similar in both groups of patients with only small difference. However the mean MESF value of CD23 was significantly lower in group II than in group I B-CLL patients.

It is well known that B-CLL is a disease characterized by a highly variable clinical expression as well as clinical course [6, 20]. Some patients may have relatively stable disease and a survival comparable to that of the age- and gender-matched general population, whilst others have a progres-

sive course and a significantly shorter survival [21, 22].

More recently in order to improve the diagnostic assessment of B-CLL cases a number of additional clinical and biological parameters have been introduced. However even if biological parameters, like CD23, CD38, p53, p27, Bcl-2/Bax ratio, 13q and 11q deletion and trisomy 12 demonstrate a strong relationship with prognosis, so far, are not in the routine use for definition of different prognostic categories [11].

Advances in understanding of the cell biology and molecular defects in B-CLL [12, 23] may contribute significantly to the changing concept of therapy in this disease.

In recent years some well-characterized prognostic factors have appeared. CD38 marker involved in interactions between cells of the immune system was considered as one of them [23]. DAMLE et al [4] and HAMBLIN et al [9] reported that CD38 expression may vary during the course of the disease and that CD38 levels on leukemic B-cell clones might change with time. Althought the levels of CD38 expression are commonly observed in leukemic cells, understanding the precise role this molecule plays in B-CLL is not known [5]. CD38 expression was noted in only 2 of 23 patients (data not shown) tested in our study. This could confirm the findings of others, concerning the limited prognostic significance of its expression in B-CLL cells [2].

In our study there were no significant differences in age and sex of group I and group II B-CLL patients. The white blood counts in PB and BM B-CLL patients were slightly higher in group II B-CLL patients. These similar parameters permitted to make a comparison of quantitative values of important markers for B-CLL diagnosis.

It was stated that leukemia cells could express some antigens of different density than normal cells, which might be used to detect antigen modulations after chemotherapy and to increase the accuracy of diagnosis by comparison with values in normal or by chemotherapy influenced counterparts [10].

Measuring different marker density in PB and BM cells of two B-CLL patient groups was performed. In group II B-CLL patients we detected lower MESF values for CD19, CD20 and CD23 markers (in PB and BM) and higher MESF values for CD5 markers (in PB and BM) than in the patients group I. The MESF level of HLA DR marker was little higher in group II than in group I B-CLL patients.

However % expression of HLA DR and CD19 markers (in PB and BM) of both patients groups was approximately the same. The expression of CD20 and CD23 markers was lower in group II than in group I B-CLL patients and expression of CD5 marker was higher in group II than in group I B-CLL patients (in both, PB and BM). In our study we observed in B-CLL patients of group II similar or a little lower expression level of markers characteristic for B-CLL, but the MESF values, i.e. the density of these markers was lower than in those B-CLL patients of group I. The data for HLA DR, CD19, CD20, CD23 and CD5 markers (% expression and MESF) in both B-CLL patients groups were significantly higher comparing to values of controls.

MATUTES and POLLIACK [20] described significantly higher antigen density of CD5 marker in majority of B-CLL cases comparing to that of control subjects. These quantitative studies could also help to monitor the numbers of residual malignant cells during and after therapy [1]. We suppose that quantitative immunofluorescence may be important for more precise and reliable leukemia diagnosis as well as for therapy strategy of this disease.

Our results showed that chemotherapy treatment in B-CLL cells did not influence in significant level the antigen modulation of B-CLL cells in sense of their antigen expression. However, we showed that this treatment could modulate MESF values of some characteristic markers (CD5, CD19, CD20 and CD23) in B-CLL patients.

In conclusion, the results described in this paper showed the significance of the antigen density measurement in leukemic cells at diagnosis and after therapy too. The determination of antigen density (MESF values) is an important diagnostic marker of leukemic cells, suitable for study of antigen modulation analysis of some markers of leukemia cells in common and could be even a good model for the study of immunophenotype after treatment. Quantitative analysis of some markers of leukemic cells could be a good model for the study of antigen modulation caused by chemotherapeutic agens.

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