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ORIGINAL CONTRIBUTIONS

USEFULNESS OF CD45 DENSITY IN THE DIAGNOSIS OF B-CELL CHRONIC LYMPHOPROLIFERATIVE DISORDERS

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ABSTRACT

BACKGROUND: *Although many B-cell chronic lymphoproliferative disorders (BCLPDs) including B-cell chronic lymphocytic leukemia (B-CLL) have characteristic clinical and biological features, the overlapping morphologic and immunophenotypic profiles of various BCLPDs, is still the main problem.* ***Aim:*** *Our aim was to evaluate the usefulness of CD45 expression in the immunological classification of BCLPDs.* ***SETTING AND DESIGN:*** *A prospective study was set in a university hospital to investigate the CD45 intensity, particularly in B-CLL.* ***MATERIALS AND METHODS:*** *The expression of CD45 in 37 patients with BCLPD including typical B-CLL (Group I), atypical B-CLL and CLL/PLL (II), and hairy cell leukemia (HCL), B-prolymphocytic leukemia (B-PLL), and B-non Hodgkin's lymphoma (B-NHL) as non-CLL BCLPDs (III) and in eight healthy age matched controls (IV) was quantitatively compared by flow cytometric CD45/RALS gating strategy.* ***STATISTICAL ANALYSIS:*** *The mean, median, and peak channel scores of CD45 obtained for the four groups were compared using one-way analysis of variance test. A P value < 0.05 was to be considered statistically significant.* ***RESULTS:*** *Lower CD45 density is associated highly with typical CLL and differences between typical CLL and other groups were significant (P<0.001, 0.001, and 0.001). Non-CLL cases had significantly brighter CD45 expression than atypical CLL (P=0.014). No differences were found between normal lymphocytes and non-CLL BCLPD cases.* ***Conclusions:*** *CD45 is a useful marker, to discriminate the typical CLL from the non-CLL BCLPD and from atypical CLL.*

KEY WORDS: *CD45, lymphoproliferative, B-cell chronic lymphocytic leukemia*

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INTRODUCTION

B-cell chronic lymphoproliferative disorders (BCLPDs) can be classified based on morphology and immunological markers defined by the French-American-British (FAB) group[1] as follows: B-cell chronic lymphocytic leukemia (B-CLL), B-prolymphocytic leukemia (B-PLL), CLL/PLL, hairy cell leukemia (HCL), follicular lymphoma (FL) and mantle zone lymphoma (MCL) in leukemic phase. Although, B-CLL has heterogeneous biological and clinical features, many BCLPDs including B-CLL have characteristic immunological markers.[1,2] However, based on systematic use of monoclonal antibodies (MoAb), it has became evident that the overlapping morphologic and immunophenotypic features of various BCLPDs can make diagnosis difficult.[2-7] The intensity of the expression of certain markers such as CD20, CD22, surface membrane immunoglobulins (SmIg), and FMC7 was suggested as a valuable discriminator of typical CLL from atypical CLL and other BCLPDs.[3,7] For example, the expression of C22 is dim or even negative in CLL and rare cases have FMC7.[7] Although the measurement of these markers may be helpful, there are some exceptions, and in addition, the importance of CD20 in CLL has been questioned.[8] However, accurate distinction of typical CLL from atypical and from non-CLL BCLPD is still an important issue

Shah et al[9] have shown that mature T- and B lymphoid cells typically have a bright expression of CD45 both in neoplasia and in reactive states. A combination of CD45 and right-angle light scatter (RALS) gating strategy to analyze normal bone marrow specimens and leukemic blasts showed that CD45 is expressed at different densities according to cell maturation stage.[10,11] This gating strategy as a procedure, often termed CD45 gating, is an analysis tool capable of identifying changes in CD45 density during development of the cell lineages.[10] The malignant cells could be separated from the normal lymphocyte on the CD45 gating.[11] Thus, assessment of CD45 is of particular interest in flow cytometry analysis of neoplastic cells in heterogeneous samples, such as CLL cases, which are defined as mature cell type leukemia.[1,8] Our objective was to study whether CD45 increased the diagnostic accuracy of our flow cytometry panel which includes antibodies to CD2, CD3, CD5, CD7, CD10, CD16, CD19, CD20, CD22, CD23, CD25, CD38, CD45, CD56, CD79b, CD103, CD138, FMC7, TdT, and SmIg in the distinction between typical CLL and atypical CLL or non-CLL cases. The issue regarding typical and atypical morphology has not been investigated previously.

MATERIALS AND METHODS

Patients: Twenty-three patients with typical B-CLL, seven patients with atypical B-CLL (including one case with CLL/PLL), three patients with HCL, one patient with B-PLL, and three with B-cell non-Hodgkin's lymphoma (B-NHL) in leukemic phase, who were evaluated at the department of hematology and medical oncology referral center and were all previously untreated adults with absolute lymphocytes during 2002 to 2004, were included in this study and patients who had no complete Para clinical evaluation were excluded. Binet clinical staging was performed on CLL cases. A diagnosis of B-NHL was

made by bone marrow and / or lymph node/ spleen histology. Wright-Giemsa stained peripheral / bone marrow films were reviewed and all cases with typical B-CLL had a varying proportion of cells with features characteristic of CLL: clumped chromatin, no visible nucleoli, scanty high nuclear cytoplasmic ratio, and regular nuclear outline.[1,3,7] Cases with atypical B-CLL had a cleaved nucleus/irregular nuclear outline or lymphoplasmacytoid features.[3,7] A diagnosis of CLL/PLL was made when the proportion of prolymphocytes was greater than 10% out of all the lymphoid cells.[1,2] A diagnosis of PLL was made by the observation of a well spread area of blood film and 55% or greater prolymphocytes was the level used to differentiate B-PLL from CLL/PLL.[1,2] A diagnosis of HCL was made by the careful analysis of peripheral blood films and bone marrow trephine biopsy and, subsequently, confirmed by demonstration of tartrate-resistant acid phosphatase that is specific for HCL.[1] All the diagnoses were confirmed by membrane markers as shown in Table 1.

Patient and control Samples

Ethylendiaminetetraaceticacid (EDTA, code no. 8421, MERCK, Germany) anticoagulated blood samples were obtained from each patient and eight healthy age-matched controls that were selected from the hospital staff after a detailed evaluation of clinical features and laboratory investigations including a CBC, ESR, blood biochemistry tests and complete urine analysis to exclude the presence of illnesses. Controls were not having any form of infection and abnormal clinical and para-clinical findings. Informed consent was obtained from all subjects and ethical clearance from the university was taken to conduct this study. The samples were analyzed within 6 hours of collection.

Mononuclear cells

Mononuclear cells were separated from the samples by density gradient centrifugation with Ficoll (code no. 8206, Bahar Afshan,) and washed three times in Hanks balanced salt solution prior to study.[2]

Table 1: Immunological markers* in 37 patients with B-cell leukemia

Cases (no)	*Markers*†
Typical B-CLL (23)	CD5+, CD19+, CD20+, CD22+‡, CD23+, CD25-, CD38±, CD45+, CD79b-, CD103-, FMC7-, SmIg-/+
Atypical B-CLL (6)	CD5+, CD19+, CD20+/CD22+, CD23+, CD25-, CD38±, CD45+, CD79b-, CD103-, FMC7-, SmIg+
CLL/PLL (1)	CD5+, CD19+, CD23+, CD25-, CD38-, CD45+, CD103-,FMC7+, SmIg +
HCL (3)	CD5-, CD19+, CD20+, CD22+, CD23-, CD25+, CD38-, CD45+, CD79b+, CD103+, FMC7+, SmIg +^{s}
B-NHL (3)	CD5+, CD19+, CD20+, CD22+, CD23-, CD25+, CD38-, CD45+, CD79b+, CD103-, FMC7-, SmIg+^{s}
	CD5-, CD19+, CD20+, CD22+, CD23-, CD25-, CD38-, CD45+, CD79b+, CD103-, FMC7-, SmIg+^{s}
	CD5+, CD19+, CD20+, CD22+, CD23-, CD25-, CD38+, CD45+, CD79b+, CD103-, FMC7+, SmIg+^{s}
B-PLL (1)	CD5+, CD19+, CD20+, CD22+, CD23+, CD25-, CD38+, CD45+, CD79b+, CD103-, FMC7+, SmIg+^{s}

*(-): Negative expression, (-/+): dim expression, (+): positive expression, S: bright expression.
†All cases were CD2-, CD3-, CD7-, CD10-, CD16-, CD56-, CD138-, and TdT-. All cases were HLA-DR+.
‡B cell markers particularly CD22 was dim in typical CLL. ± 9 cases of typical CLL, and five cases of atypical CLL were CD38+.

Detection of CD45 as a cell surface marker was performed by direct immunofluorescence on FACS Calibur (Becton Dickinson, USA) flow cytometer. The diagnostic reagent used in this study was fluorescence isothiocyanide (FITC)-conjugated mouse McAb to human CD45 (code no. F 0861 from DAKO, DK-2600 Glostrup, Denmark).

1 x 10^6 mononuclear cells were used for each test tube. The cells were incubated with 10 ul FITC conjugated anti-CD45 for 15 min in a dark at room temperature. The cells were washed twice with 0.01 mol/L PBS, pH 7.4 and resuspended in 0.5 ml 1% Para formaldehyde (code no. 4005, MERCK, Germany) in 0.01 mol/L PBS, pH 7.4 and run on a FACS Calibur flow cytometer.[2] 10,000 cells were acquired by the instrument for each test and control tubes.

Blocking of FC receptors was performed by adding to the buffer 2% human AB serum in incubation step. FITC-conjugated negative control (cod no. X 0927, DAKO) was used for control tube and all the subsequent steps were the same as for the test.[2]

To ascertain the intensity of CD45 expression on the cell surface, in the dot plot histogram, the RALS (SSC) parameter was set as the X-axis and CD45, as the Y-axis.[10,12,13] In this setting, neoplastic cells and lymphocytes from healthy individuals were usually located in an isolated dot cluster in which a manual gate was set (Gate 1 or R1 in Figure 1).

The cells in CD45/RALS dot plot were analyzed on a FACS Calibur flow cytometer with a CELL Quest software program (Becton Dickinson, USA) and the mean, median, and peak channel fluorescence intensity that was automatically calculated by the instrument software were recorded for the CD45, based on a lymphocyte or tumor gated cell population.

Instrument setting for the cells was saved and used consistently for subsequent samples. A set of calibrated fluorescence standard beads (code no. 349502, Becton Dickinson) was used for the determination of instrument linearity and stability.

Statistical analysis

The data obtained was analyzed using the SPSS Statistical software (SPSS Version 12 for Windows, USA). The mean, median, and peak channel CD45 scores for each patient and controls were recorded and then the mean scores ± SD were calculated for the four study groups and compared among themselves using one-way analysis of variance (ANOVA) test to find out whether there were any significant differences. Also, the mean scores ± SD obtained for mean, median, and peak channel for each of the four study groups were compared with normal and other groups scores using Independent Samples t-test to ascertain whether there were any significant differences. A P value < 0.05 was considered statistically significant.

RESULTS

The main demographic, laboratory features and the immunophenotype of the 37 patients are shown in Tables 1 and 2. Twenty-three patients had typical B-CLL, seven patients had atypical B-CLL (including one case with CLL/PLL), three cases had HCL, three patients had

Table 2: Demographic and laboratory features from 37 patients with B-cell chronic lymphoproliferative disorders

Variable	*Value in Typical CLL*	*Value in Atypical CLL*	*Value in HCL*	*Value in B-NHL*	*Value in B-PLL*
Age (yr) [median (range)]	67(23-76)	75(52-83)	63(50-65)	52(50-57)	71
Male: female	17 : 6	6 : 1	2 : 1	1 : 2	1 : -
WBC x 1000/µl [median (range)]	65(14-288)	46(18-193)	13(9.5-15)	21(10-70)	44
Lymphocyte x 1000/µl [median (range)]	56(9-217)	41(13-157)	10(8.3-11)	16(7-58)	NA
Hemoglobin (g/dl) [median (range)]	13(7-18.5)	14(9-7)	9(5.4-9)	10(10-15.6)	15.3
Platelet x1000/µl [median (range)]	166(38-455)	177(125-312)	65(47-98)	180(100-248)	181

Binet stage for typical CLL were (number of patients): 0(10), I (2), II (4), III (4), IV (3)
Binet stage for atypical CLL were (number of patients): 0(2), I (2), II (0), III (2), IV (1)

B-NHL in leukemic phase, and the remaining one B-PLL. All were adults with median age of 67 years (range 23-83) and there were 27 males and 10 females. All cases had a mature B cell phenotype and were positive with CD19, CD20+ or CD22+. Cells from all patients were negative with CD2, CD3, CD7, CD10, CD16, CD56, CD138, and TdT. The median value of CD22 as determined by the intensity of fluorescence was 21(range 12-31), 46(37-73), and 818(272-1147) in typical CLL, atypical CLL, and non-CLL BCLPD cases respectively. The median value of SmIg expression was 12 (5-39) in typical, 18(7-53) in atypical and 117(55-701) in non-CLL BCLPD.

The level of expression of CD45 as determined by the intensity of fluorescence is shown in Table 3. In the normal samples, the mean of means, medians, and peak channels fluorescence intensity were 237± 20.42, 230 ±17.30, and 237±25.40 respectively. These figures were 81±27.85, 75±26.47 and 74±27.47 in typical CLL; 191± 43.27, 185±43 and 192±41.39 in atypical CLL; and 247±109.64, 251±106.52 and 249± 105.71 in non-CLL. Expression of these is shown in Figure 1. Typical CLL had the lowest CD45 intensity within BCLPDs and the mean, medians, and peak channels differences between typical CLL and atypical were statistically significant (P <0.001, df=28, 95% CI -82.13, -138.35), (P<0.001, df=28, 95% CI -81.92, 136.38), and (P<0.001, fd=28, 95% CI -91, -145.9) respectively. The mean, medians, and peak channels differences between typical CLL and non-CLL were significant (P<0.001, df=28, 95% CI -116, -215), (P<0.001, df=28, 95% CI -127.8, -224.4), and (P<0.001, df=28, 95% CI -77, -127.8) respectively. The mean, medians, and peak channels differences between typical CLL and normal control were significant (P<0.001, df=29, 95% CI -134.7, -178.8), (P,0.001, df=29, 95% CI -134.5, -175.7), and (P<0.001, df=29, 95% CI -140, -186) respectively. No differences were found between normal lymphocytes and atypical and non-CLL BCLPD cases. The mean, median, and peak channel expression of CD45 in non-CLL BCLPD was significantly higher than atypical CLL (P=0.014, df=12, 95% CI -152.8, +41.38), (P=0.044, DF=12, 95% CI -161.6, +27) and (P=0.035, df=12, 95% CI -150, +36) respectively.

Table 3: Level of expression of CD45 in 37 patients with chronic B-cell Leukemia and eight healthy donors

*Variable**	*Typical CLL*	*Atypical CLL*	*Non CLL BCLPD*	*Blood donors*
Mean of means	81±28†	191±43	262±110	237±20
Mean of medians	75±26	185±43	251±107	230±17
Mean of peak Channels	74±27	192±41	249±106	237±25

*Number (or antibody binding sites) of CD45 on the cells surface that initially were recorded as mean, median, and peak channel values for each patient and blood donors in comparison to negative control tubes and then these values statically compared. (See text); † ± Standard deviation

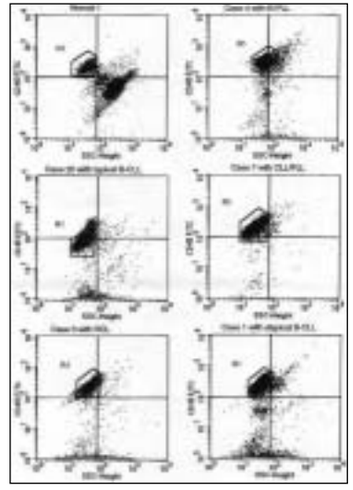

Figure 1: Examples of dot plot histogram analysis of the level of CD45 in Normal lymphocytes and cells from patients with B cell leukemia. R1: the Gated population in the CD45/RALS dot plots

DISCUSSION

Flow cytometry analysis of the intensity of certain antigens has an important role in the diagnosis of BCLPDs. From previous studies it is believed that, immunophenotypic panels using a few key antibodies correlate with particular subclasses of BCLPDs.[2,14,15] For example, CLL (typical and atypical) and MCL usually express CD5, and often, can be discriminated by expression of CD23.[5] Other BCLPDs such as HCL, FL, PLL, and splenic marginal zone lymphoma that are usually CD5- can be distinguished by the morphologic features and expression of other antigens such as CD10 for FL, and CD25 and CD103 for HCL.[2] As shown in Table 1, all these findings were confirmed. Quantitation of CD20, CD22, CD79b, and SmIg, also has been shown to be an important diagnostic tool in BCLPDs. For instance, in contrast to atypical CLL, CLL/PLL and B-PLL, dim expression of CD20, CD22, CD79b, and SmIg are associated with typical CLL.[2,3,15] As in the reported cases by Matutes et al[7] and D Arena et al,[3] our typical CLL group had a lower intensity of CD22 and SmIg than atypical CLL. Although the expression of CD45 in our atypical CLL relatively closed to normal lymphocytes, but, in contrast to the Shah et al[9] and Lavaber-Bertrand et al[16] expression of CD45 in the typical CLL were not same as mature lymphoid cells as seen in normal blood or bone marrow specimens. However, according to descriptive morphology, all 23 of our typical CLL showed lower CD45 intensity, distinguishable from the atypical CLL, non-CLL, and normal lymphocytes. Our data indicate that CD5+, CD19+ and CD23+ cells in typical CLL are not mature as normal lymphocytes, and like D Arena et al,[3] atypical CLL (also with CD5+, CD19+ and CD23+ immunophenotype), represent a more mature disease than typical CLL. Since CD45/RALS gating improve the purity of tumor cell isolation, we have provided new findings in the field that indicate that the intensity of CD45 as a discriminator marker can differentiate typical B-CLL from other BCLPDs, the issues that previously have not been investigated. Thus, we highly recommend using the same combination for routine immunophenotyping of

BCLPDs, in particular, for the discrimination of typical CLL from atypical CLL and atypical CLL from B-NHL.

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