The role of CD20 and CD19 with their flow cytometric parameters in differentiation between Chronic Lymphocytic Leukaemia / Small Lymphocytic Lymphoma and other B-cell Non Hodgkin Lymphoma

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Abstract: CD20 and CD19 are known to common B cell markers used widely in the identification of B cells. They are also available in most flow cytometry laboratories which is study of B cell disorders, so our choice of these CDs is to shed light on their expression in normal pattern and find out whether the way the pattern of expression in the cells may be useful in the diagnosis of some B cell disorders, especially to differentiate between chronic lymphocytic leukemic (CLL) and Non-Hodgkin lymphoma (NHL) , To study these changes we depend on some flow cytometric parameters like the percentage of positive cells, mean fluorescence intensity and the positive peak width. Hundred and thirteen samples were studied, 8 samples of them were normal control. The samples nature were venous blood, bone marrow aspiration samples and lymph node samples. We were found that the percentage of CD20 or/and CD19 which reflect the total B cells in the sample is the best parameter for indication of presence of B cell disorders. Mean Fluorescence intensity and positive peak width have a highly significant variation between CLL and NHL. Regarding the type of sample, we were found that the venous blood samples show the highest significance of the difference to distinguish between CLL and NHL then the bone marrow samples and samples from lymph node at last. We concluded that CD20 and CD19 are very useful markers in the identification and differentiation between B cell lymphoprliferative disorders especially in case of laboratories with limited resources. This finding similar to L. Ginaldi and his collage who concluded that the quantitative determination of CD19 and CD20 may provide useful diagnostic information for the study of B lymphoproliferative disorders (L. Ginaldi, *et al*, 1998). we can use them in this area to go a long way to distinguish between CLL and NHL while taking into consideration that the samples taken from a vein is the best in highlighting the difference between the two diseases. We strongly recommend inserting of CD20 and CD19 with their flow cytometric parameter in the scoring system for differentiation between CLL and NHL.

Key words: Immunophenotyping, Mean fluorescence intensity, Positive peak width.

INTRODUCTION

Over the last decade, flow cytometry has been established as a critical tool in the diagnosis of haematological malignancies. This review has summarised the contribution the technique currently makes to the diagnosis and management of mature lymphoid neoplasms. Recent improvements in technology are beginning to yield assays with much higher levels of disease specificity and sensitivity which can be applied in routine diagnostic laboratories. Many types of lymphoma can now be correctly classified by flow cytometric analysis independent of other diagnostic modalities which allows much more robust integrated diagnostic protocols to be developed. The development of highly sensitive techniques will play an increasing role in disease monitoring through and beyond treatment. Flow cytometry now has the potential to improve substantially the diagnosis and monitoring of patients with lymphoma (Ruth M. and de Tute. 2011).

Immunophenotypical analysis by Flow Cytometry (FC) appears to be very sensitive in the diagnosis and detection of minimal residual disease (MRD) in CLL. The role of FC for staging or re-staging of Non-Hodgkin Lymphoma (NHL) is not well defined, but it is thought to increase the sensitivity of bone marrow involvement by NHL over morphological evaluation alone in the bone marrow aspirates. Two of the most important markers that help in the immunophenotyping of B cells are CD20 and CD19 because they express in all types of normal mature B cell and B lymphoma (S P Sah, *et al*.. 2003). Lia Ginaldi and her partner's analysis show that in combination with other B cell antigenic markers, CD19 and CD20 might be useful to characterise B lymphoproliferative disorders with similar cytology (Lia G, *et al*.. 1998).

CD20 is a B-lymphocyte antigen, it is an activated-glycosylated phosphoprotein expressed on the surface of all B-cells beginning at the pro-B phase and progressively increasing in concentration until maturity (Tedder TF, *et al*.. 1988).

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In humans CD20 is encoded by the MS4A1 gene, this gene encodes a member of the membrane-spanning 4A gene family. Members of this nascent protein family are characterized by common structural features and similar intron/exon splice boundaries and display unique expression patterns among hematopoietic cells and nonlymphoid tissues. This gene encodes a B-lymphocyte surface molecule that plays a role in the development and differentiation of B-cells into plasma cells. This family member is localized to 11q12, among a cluster of family members. Alternative splicing of this gene results in two transcript variants that encode the same protein (Tedder TF, *et al.*. 1988).

Function of CD20, the protein has no known natural ligand and its function is to enable optimal B-cell immune response, specifically against T-independent antigens. It is suspected that it acts as a calcium channel in the cell membrane (Tedder TF, *et al.*. 1988).

CD20 is expressed on all stages of B cell development except the first and last; it is present from late pro-B cells through memory cells, but not on either early pro-B cells or plasma blasts and plasma cells. It is found on B-cell lymphomas, hairy cell leukemia, B-cell chronic lymphocytic leukemia, and melanoma cancer stem cells (Tedder TF, *et al.*. 1988).

Immunohistochemistry can be used to determine the presence of CD20 on cells in histological tissue sections. Because CD20 remains present on the cells of most B-cell neoplasms, and is absent on otherwise similar appearing T-cell neoplasms, it can be very useful in diagnosing conditions such as B-cell lymphomas and leukaemias. However, the presence or absence of CD20 in such tumours is not relevant to prognosis, with the progression of the disease being much the same in either case. CD20 positive cells are also sometimes found in cases of Hodgkins disease, myeloma, and thymoma (Tedder TF, *et al.*. 1988).

CD19 is a B-lymphocyte antigen CD19 also known as CD19 (Cluster of Differentiation 19), is a protein that in humans is encoded by the CD19 gene (Tedder TF, *et al.*. 1988).

Function of CD19: Lymphocytes proliferate and differentiate in response to various concentrations of different antigens. The ability of the B cell to respond in a specific, yet sensitive manner to the various antigens is achieved with the use of low-affinity antigen receptors. This gene encodes a cell surface molecule that assembles with the antigen receptor of B lymphocytes in order to decrease the threshold for antigen receptor-dependent stimulation (Tedder TF, *et al.*. 1988).

CD19 is expressed on follicular dendritic cells and B cells. In fact, it is present on B cells from earliest recognizable B-lineage cells during development to B-cell blasts but is lost on maturation to plasma cells. It primarily acts as a B cell co-receptor in conjunction with CD21 and CD81. Upon activation, the cytoplasmic tail of CD19 becomes phosphorylated, which leads to binding by Src-family kinases and recruitment of PI-3 kinase (Tedder TF, *et al*. 1988).

The main objective of this study is to examine the role of CD20 and CD19 in the discovery of B cell disorders and determine the characterization of normal B cell population and the peak which appear in the flow cytometer histograms and compare with the result of the patient. After that we can study the properties of abnormal B cells depending on the changes that occur in the antigenicity form of CD20 and CD19. This is done through the identification of the means of the percentages of positive cells in each sample and the mean intensity of antigen in the cell as well as to study the change in the intensity of antigen between cells of the same sample. All of these values are utilized to differentiate between B cell Chronic Lymphocytic Leukemia (CLL) and B cell Non Hodgkin Lymphoma (NHL).

MATERIALS AND METHODS

Hundred and thirteen samples from both genders (male and female) were collected. (Eight) healthy donors and (hundred and five) patients diagnosed as mature B cell neoplasm. Diagnosis of these patients was depending on their complete blood count, Bone marrow morphology and Immunophenotyping (CD5, CD23, CD22, CD79b, FMC7, kappa and lambda). All patients have no treatment and free of any immunological disorders. Samples were collected from different sites, 31 (27.4%) peripheral blood samples (PB), 59 (52.2%) Bone marrow aspiration samples (BM) and 15 (13.3%) Lymph node samples (FNA). PB and BM samples were collected in EDTA anticoagulant and FNA samples were collected in Phosphate Buffer Saline (PBS) (pH=7.2). PB and BM samples prepared through direct lysing procedure for whole blood and BM aspiration monoclonal antibody combination. 20uL of monoclonal CD20-FITC/CD19-PE antibody cocktail was added into a special flowcytometry tubes according to the sample size during the running time and 100uL of sample containing no more than 1 x 10 leukocytes / ml were added from each sample (Beckman Coulter, Miami, FL, USA, 2006). Mixing was done for all tubes and were incubated in room temperature (18-25 C) for 15 minutes in dark place. 1 ml of the "fix-and-lyse" mixture was added to the tubes and were incubated again in room temperature (18-25 C) for 15 minutes in dark place. Tubes were centrifugated at 150 x g for 5 minutes and the supernatant was aspirated and discharged. 3 ml of PBS was added to the tubes and centrifuged again 150 x g for 5 minutes and the supernatant was aspirated and discharged. The pellets of tubes were re-suspended by addition of 1.0 mL PBS (Beckman Coulter, Miami, FL, USA, 2006). FNA samples were filtered by Nylon mesh filter and the

suspensions were centrifugated at 150 x g for 5 minutes. Deposits were collected and re-suspended by addition of 1.0 mL PBS. All suspensions were stained by previous direct lysing procedure for whole blood monoclonal antibody combination. All tubes were analysed by the EPICS XL Beckman Coulter flowcytometer.

CD20-FITC/CD19-PE running protocol was created. The protocol contained the main three parameters of study: 1- Percentage (%) of cells were positive for the target antibody among the total population of lymphocytes, this parameter express about the total amount of antigen positive lymphocytes. 2- Mean fluorescence intensity (MFI) of the total lymphocytes population with express about the amount of antigen per individual cell. 3- Positive Peak width (Pw) of the antibody positive peak were taken from the base of peak, This parameter express about the amount of antigenic diversity between cells through the target population which were expressed to the same antibody. All samples were gated around the lymphocytes population in the Forward scatter/Side scatter histogram (FS/SS) and the region of the interested population was called A (System II software). The auto-stopping time was done when the total events arrived 10,000 cells passing the laser. The speed of the hydrodynamic focussing was adjusted as low speed to enable the maximum instrument sensitivity and accuracy.

Data of all samples were acquired on Beckman Coulter EPICS XL Flow cytometer using System II software (Beckman Coulter, Miami, FL, USA, 2006). Alignment was checked daily using Flow-Check (Miami, FL, USA). Flow-Set (Miami, FL, USA) was used weekly to monitor standardization. Compensation was checked continually with normal lymphocytes and adjusted if required.

CD20-FITC and CD19-PE percentage, MFI and Pw were measured on four decade logarithmic scale and all results were recorded. All acquired data were stored in the list mode. Statistical Package for Social Sciences (version 19) was used for analysis and to perform Pearson Chi-square test for statistical significance (*P value*) and one way Anova for numerical data. The 95% confidence level and confidence intervals was used.

Results:

(Table 1) show distribution of CD20 and CD19 with their parameter means among disease and control samples. In the study population there are 71(62.8%) were CLL, 34(30.1%) were NHL and 8(7.1%) were normal samples. In the field of percentage parameter (Table: 1), the mean of CD20% in CLL was (73.6), (88.9) in NHL and in the control sample was (7.7), while CD19 % in CLL was (83.8), (66.9) in NHL and (9.1) in control sample. The ratio between CD20 and CD19 always is less than 1.00 (CD20% < CD19%), (CLL=0.88, NHL=0.99, control=0.97).

In the field of mean fluorescence intensity (Table: 2), CD20 MFI was (1.74) in CLL, (8.99) in NHL and (5.02) in control samples. While CD19MFI in CLL was (6.81), (6.63) in NHL and (5.02) in control samples. CD20/CD19 MFI ratio was (0.32) in CLL, (2.05) in NHL and (1.01) in control samples.

In the field of positive peak width (Table: 3), the mean of CD20 Pw in CLL was (7.08), (5.69) in NHL and (3.03) in control samples. While the mean of CD19 Pw in CLL was (5.27), (4.95) in NHL and (5.02) in control samples. CD20/CD19 Pw ratio always was more than 1.00 (CD20Pw > CD19Pw), (CLL- 1.38, NHL=1.17, Control=1.18).

Table 1: show means distribution of CD20 and CD19 with their percentages among disease.

Disease	N	Means		
		CD20 %	CD19 %	CD20%/CD19% Ratio
CLL	71	73.6	83.8	0.88
NHL	34	66.9	66.9	0.99
Normal	8	07.7	09.1	0.97
Total	114	66.9	73.4	0.92

Table 2: show means distribution of CD20 and CD19 with their mean intensity among disease.

Disease	N	Means		
		CD20 MFI	CD19 MFI	CD20MFI/CD19MnI Ratio
CLL	71	1.74	6.81	0.32
NHL	34	8.99	6.63	2.05
Normal	8	5.02	4.73	1.01
Total	114	4.15	6.61	0.89

Table 3: show means distribution of CD20 and CD19 with their peak width among disease.

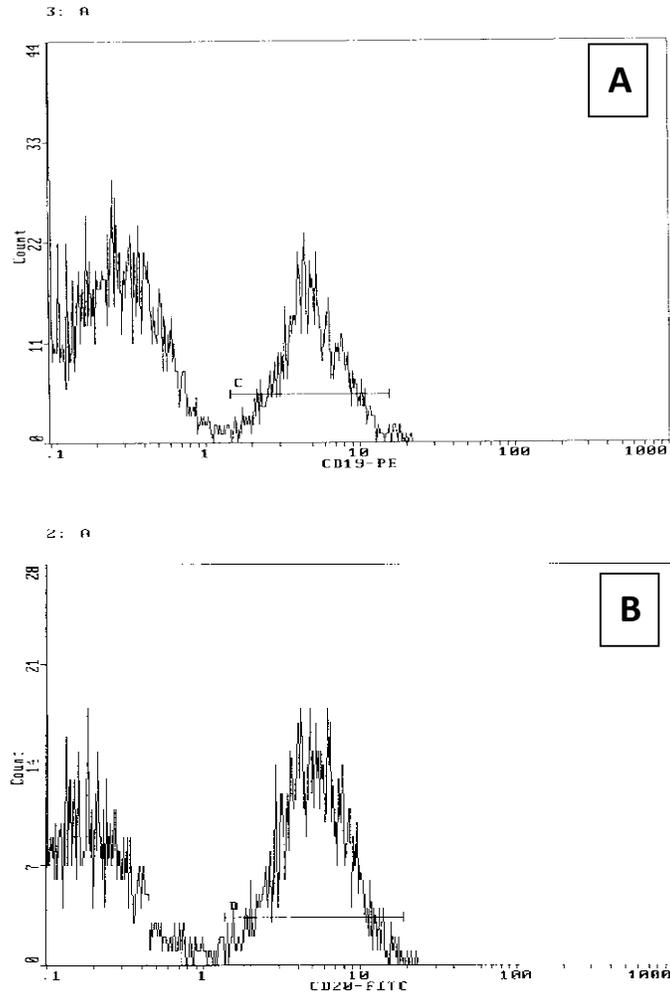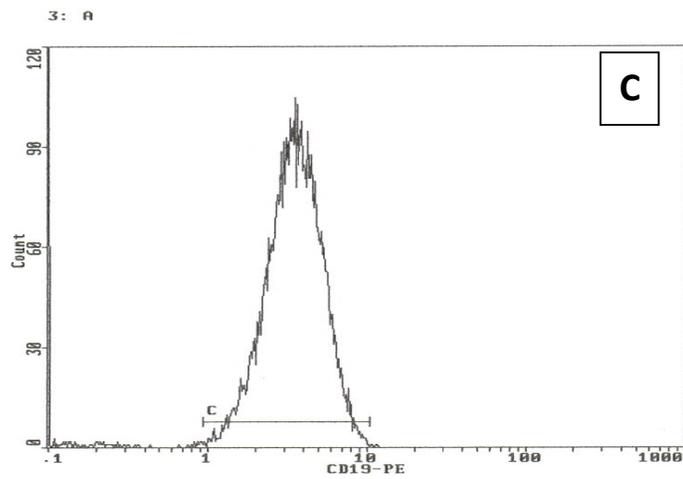| Disease	N	Means		
		CD20 Pw	CD19 Pw	CD20Pw/CD19Pw Ratio
CLL	71	7.08	5.27	1.38
NHL	34	5.69	4.95	1.17
Normal	8	3.03	3.18	1.18
Total	114	6.38	5.02	1.30

Fig. 1: Histograms (A)-(B) show the expression of CD19 and CD20 respectively in normal B cells from vinous blood sample, MFI and Pw of both are approximately equal.
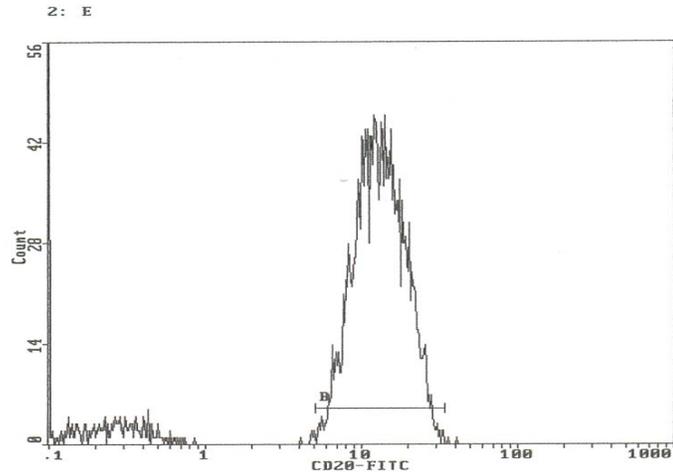
Fig. 2: Histograms (C)-(D) show the expression of CD19 and CD20 in patient with NHL, peaks display that CD20 was brighter than CD19 in MFI and slightly narrower than CD19 in width.
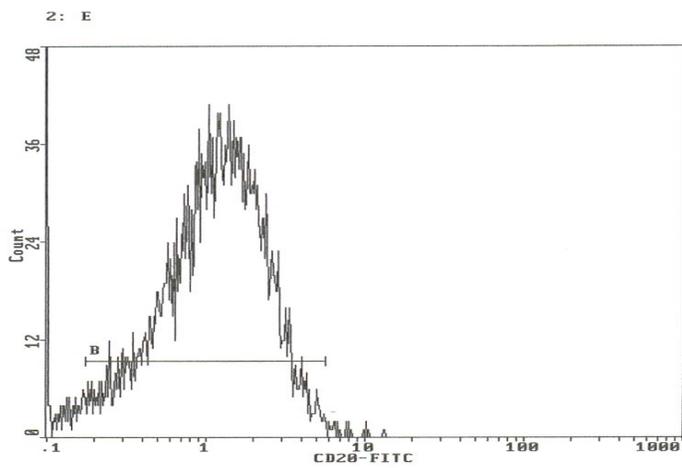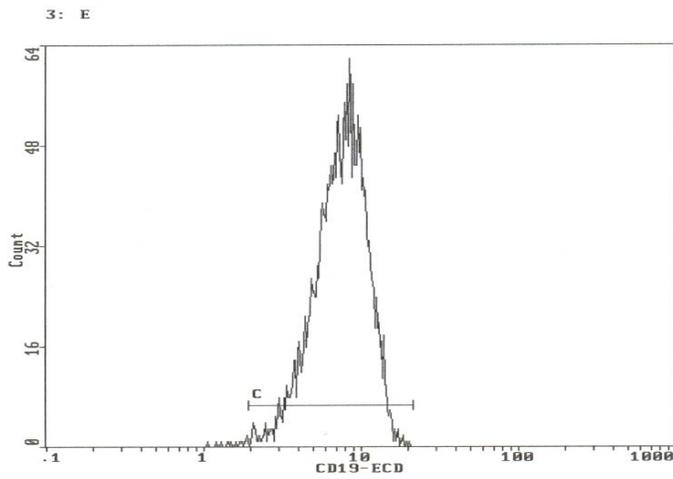

Fig. 3: Histograms (E)-(F) showing patient with CLL, peaks display CD20 was dimmer than CD19 in MFI and highly wider than CD19 in width.

Discussion:

Depending on the results of statistical data, the study of the characteristics of each of the CD20 and CD19 between normal cells and status of disease between CLL and NHL, we were found that there is significant difference between the implications of these results. This difference supports the decisions that confirm the presence of disease or denies. It also helps to distinguish between the two diseases (CLL & NHL). This variation depends on the different antigenic properties of cells positive for both CD20 and CD19. Since both CDs expressed as naturally in the normal cells with all types of mature B cells, we did not find that the percentage of positive cells to change much between the two diseases, where it is approximately equal to the CD20 (*p.value* = 0.119) but differ slightly for CD19 (*p.value* =0.000) Where they are higher in the CLL.

The changes that occur in the properties of the cells differ in showing of the antigenicity in the form of a single cell. This shows clearly the difference in the CD20 is more than CD19, Since the CD19 MFI is not significantly different between CLL & NHL (p.value =0.886), where CD20 MFI is the highest significantly different (*p.value* =0.000) compared to other indications. The reason for this severe difference is that CD20 MFI is reflected exactly between the two diseases, where more than the normal range of natural samples in the NHL and less than normal in the natural samples with CLL, this finding is similar to Lia Ginaldi`s study who found that only one case of CLL had a CD20 value within the normal range, whereas all other cases had CD20 Antigen Binding Capacity (ABC) below the normal range and the number of CD20 molecules was lower in typical CLL than in CLL with atypical morphology (Lia G, *et al..* 1998), also this finding accepted with J. Delgado who reported that CLL cells showed weaker CD20 expression compared with those in B-cell NHLs and other B-cell leukemias (J. Delgado, 2003) .That making it easier to distinguish between them, Therefore, CD20 MFI is considered as the most indicator that can be relied upon to distinguish between CLL and NHL (Figure 1,2).

The comparison of CD20 MFI:CD19MFI Ratio reflected a significant difference between the two conditions (*p.value* =0.000). When using the Ratio between CD20 and CD19 mean intensity we were found that this ratio is greater than 1.00 in NHL (CD20 MFI > CD19 MFI) and less than 1.00 in CLL (CD20 MFI < CD19 MFI). These results agree with the study of D. Nguyen who mentioned that the brightness of CD20 MFI and CD20 MFI > CD19 MFI represents two features of B cells in NHL (Doyen N., *et al*, 2007)

In our study, the Positive Peak width (Pw) which represents a measure reflects the change in the level of antigenicity between cells in the same sample, we were found that CD19 Pw was not significantly different between CLL & NHL (*p.value* =0.094) while comparing it with CD20 Pw we found that it is significantly different (*p.value* =0.000) where it was wide in the cases of CLL and narrow in cases of NHL. J. Hulkkonen`s results revealed that a large group of CLL surface membrane antigens with a heterogeneous expression pattern (J. Hulkkonen, *et al* 2002) .As well as when calculating the significant ratio between CD20 Pw and CD19 Pw we were found that there was a significant difference between the two diseases (*p.value* =0.001) so that its value was above the 1.00 in CLL and less than 1.00 in cases of NHL (Figure 1,2).

This study has shown that there is no any difference between CD20 MFI and CD19 MFI in normal B cells and that the focus of concentration is almost equal as well the Peak width which was also equal (Figure 1). Calculating the ratios between each of the parameters we were found that all values are significantly closer than the 1.00 and confirm that there is no increasing of one over the other.

With meditation to previous indications of the characteristics of each of CD20 and CD19, we were observed that there are significant differences appear on the positive cells can be used as successful indicators to distinguish between CLL & NHL, which can be arranged in order of importance as follows:

- CD20 mean intensity, then
- CD20 MFI : CD19 MFI Ratio, then
- CD20 Positive Peak Width, then
- CD19 percentage, then
- CD20 Pw : CD19 Pw Ratio

Regarding the type of sample, we were found that the venous blood samples show the highest significance of the difference to distinguish between diseases then the bone marrow samples and samples from lymph node at last, in addition the change in the type of sample does not change much in the order of priority in the use of these parameters.

Conclusions:

In conclusion of this study it is noticeable that the abnormal B cells by taking a different pattern for normal cells, which necessarily help to identify the disease, as these changes are the same that will help determine the nature of the disease among CLL or/and NHL cells depending on the characteristics of the these cells that express to the CD20 & CD19. So we can use both in this area to go a long way to distinguish between CLL and NHL while taking into consideration that the samples taken from the vein is the best in highlighting the difference between the two diseases. We strongly recommend inserting of CD20 and CD19 with their flow cytometric parameter in the scoring system for differentiation between CLL and NHL.

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