Detection of CD3,19 and CD4,8 markers on lymphocyte of lymphocytic leukemia Iraqi patients

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Abstract

This work aimed to study expression of CD marker on surface of lymphocyte of (acute lymphocyte leukemia ALL; chronic lymphocyte leukemia CLL, and control), To achieve this goal, blood samples were collected from 39 leukemic cases (21 ALL and 18 CLL), and isolation of lymphocyte than proliferation of leukemia lymphocyte culture for detection of CD Marker on surface of lymphocyte by immunofluorescence technique, the results, appeared elevated levels of CD8+ cells in both ALL and CLL patients were detected, in contrast, lower levels of CD4+ cells and CD4/CD8 ratio were noticed in these patients. However, high level of CD3+ cells, CD8/CD19 ratio and CD19+ cells were noticed in ALL patients and low level in CLL patients.

Introduction

Leukemia is a cancer originating in any of hematopoietic cell that tends to proliferate as single cells within bone marrow [1]. Four types of leukemia are classified; chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL), and acute myelogenous leukemia (AML). Tumor cells may evade immune responses by losing expression of antigens and major histocompatibility complex (MHC) molecules by producing immunosuppressive cytokines. The development of a malignant cell clone is due to the dysregulation of the balance between cell proliferation and the programmed cell death–apoptosis. Acute lymphoblastic leukemia (ALL) is a malignant proliferation of lymphoid cells blocked at an early stage of differentiation and accounts for ¾ of all cases of childhood leukemia [2]. Although ALL is a disease primarily of the bone marrow and peripheral blood, any organ or tissue may be infiltrated by the abnormal cells [3]; [4]. B cell chronic leukemia is characterized by the relentless accumulation in the blood, bone marrow and lymphoid tissues of mature monoclonal B lymphocytes that express the CD5 surface molecule [5]. The B-CLL accumulation appears to be a result of defective apoptosis and uncontrolled proliferation [6] [7], the cells of origin in most of the patients are clonally B cells arrested in the B-cell differentiation pathway, intermediate between pre-B cells and mature B cells [8]. The result is the uncontrolled growth of lymphocytic cells in the marrow, leading to an increase in the number of lymphocytes in the blood [5]. B-CLL lymphocytes usually show B-cell surface antigens, as demonstrated by CD19, CD20, CD21, and CD23 monoclonal antibodies, additionally, they express CD5, which is more typically found on T cells [9]. B-CLL has a distinctive immunophenotype characterized by the expression of mature B cell markers CD19, CD20 and CD23, weak expression of surface immunoglobulin and co-expression of the T cell marker, CD5 [10].

Materials and Methods

Sample collection

Five ml of blood was collected by vein puncture from 39 cases (ALL and CLL) who were admitted to the National Center of Haematology/ Al Mustanisyria University from May 2010 till March 2011. The disease was clinically diagnosed by the consultant medical staff at the centre. In addition, 15 apparently healthy controls (blood donors) were also included.

Isolation of Lymphocytes

Preparation of solutions and media were done according to the methods described by [11];[12] unless mentioned. The lymphocytes were isolated from the heparinized whole blood using the method described by [13] as follows: three ml of blood was centrifuged at 1000rpm for 15min. The plasma was collected for perforin estimation, buffy coat was collected in a 10 ml centrifuge tubes and diluted with 5ml RPMI 1640 (cell suspension), five ml of the diluted cell suspension was layered on 3ml of ficoll-isopaque separation fluid, the tubes were centrifuged at 2000rpm for 30min in a cooled centrifuge at 4ºC. After centrifugation, the mononuclear cells were visible as cloudy band between the RPMI1640 and lymphoprep layers. The band was collected in a 10ml test tube and the cells were suspended in 5 ml RPMI 1640. The tube was centrifuged at 2000rpm for 5min (first wash), the supernatant was discarded and the cells were resuspended in 5 ml RPMI 1640 (repeated twice). The suspension was centrifuged at 1000rpm for 10min, the supernatant was discarded. The precipitated cells were resuspended in 1ml RPMI solution and used in the planned experiments. Counting the cells were performed before experiment according to [5], the numbers of lymphocytes were counted by light microscope and the cells concentration was adjusted to $1X10^6$ cell/ml.

Analysis of T-lymphocyte Subset based on Immunostaining Technique (Dual-Immunofluorescence Technique)

Preparation of solutions and media were done according to the methods described by [14]. Lymphocytes subpopulations were identified by two sets of dual staining monoclonal antibodies labeled

with fluorescein isothiocyanate (FITC) in the order of anti-CD3, anti-CD19 and anti-CD4, anti-CD8.

Lymphocyte suspension 10µl was placed in each well of an immunofluorescent slide and allowed to air-dry at room temperature. Acetone or ethanol absolute 10ml was added and left about 10min for fixation, allowed to air- dry at room temperature 20-25ºC, wrapped in foil and stored at -70ºC until used. The immunofluorescent slide pre coated with lymphocytes were removed from the freezer and allowed warm to room temperature about 20-25ºC. For blocking the non-specific binding site, 100µl of a protein-blocking reagent was placed onto lymphocyte and incubated for 10mins in a humid chamber at room temperature 20-25ºC, then the slides were drained and blotted gently. Diluted antibody 50µl was placed onto lymphocyte and incubated for 1 hour at 37ºC in humid chamber then the slides were drained. Antibodies were removed by washing with 100ml washing buffer for 5-10min in humid chamber. The slides were drained and mounting medium was added to slide and quickly covered with a cover slip. Slides were examined under 40X magnification of a fluorescent microscope.

The statistical analysis is a very important final step in the research to analyse and evaluate the obtained results. Medical statistics of this study was conducted via computer based statistical program which was :

1- SPSS for Windows computer package (Programmer 11.5).
2- Microsoft Excel 2003.

The statistical analysis tests which used in this were as follows:

Duncan test is non-parametric test which used to determine whether there is a significant difference between the expected frequencies with respect to two variable. It is a well used test for the medical statistics. P value <0.05 is considered a significant correlation.

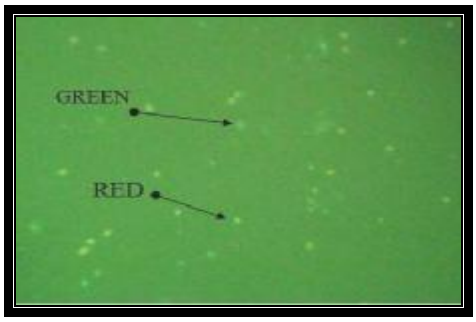
Result and discussion

Samples Collection

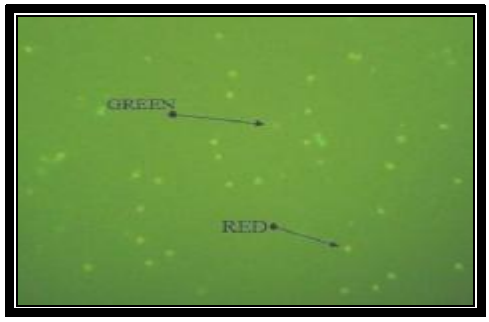
Five ml of blood was collected by vein puncture from 39 leukemia cases (18 Chronic lymphocyte leukemia; CLL and 21 Acute lymphocyte leukemia; ALL) who were admitted to the National Center of Haematology/ Al Mustanisyria University from May 2010 till March 2011 and 15 cases of healthy control. The patients were first diagnosed by the consultant medical staff at the centre. The age mean of CLL patients was 61.5± 2.9 years (range 50-80 years), for ALL patients was 29.1±3.1years (range 20-40 years), while for controls, the age mean was 38.4±1.9 years (range 30-45 years). The mean of age in the two groups of patients, as well as, in controls showed a significant difference (P≤0.001). With respect to the isolation of lymphocytes, the viability was greater than 98% for fresh sample, and it was greater than 95% for overnight samples.

Immunofluorescent Expression of CD on Surface of Cells

Lymphocytes isolated from patients and controls were inspected for the expression of CD3 (Pan T cells), CD4 (T- helper cells), CD8 (cytotoxic T-lymphocytes) and CD19 (B-Cells) markers by means of dual immunofluorescence staining as shown in figure (1).



A



B

Figure (1): Immunofluorescence staining of lymphocytes. (A): lymphocytes stained with Anti-CD3+ and Anti-CD19+ (dual staining) antibodies; cells stained red is CD19+ and cells stained green is CD3+. (B): lymphocytes stained with Anti-CD4+ and Anti-CD8+ (dual staining) antibodies; cells stained red is CD4+ and cells stained green is CD8+.

T- Helper cells (CD4+ Cells)

Both groups of patients (CLL and ALL) shared an approximated percentage mean of CD4+ cells (20.19±1.33% and 21.4±1.6%, respectively); but both means were significantly (P≤ 0.001) lower than the observed percentage mean in controls (42.42 ±1.01%), as shown in table (1).

Table (1): Percentage frequency of CD4+ cells in leukemia patients (CLL and ALL) and controls.

Groups	Number	Percentage frequency of CD4+ Cells (Mean ± S.E.)*
CLL	18	20.19 ± 1.33^{B}
ALL	21	21.40 ± 1.60^{B}
Controls	15	42.42 ± 1.01^{A}

*Different letters:Significant difference (P≤0.001) between means (Duncan test).

T-cytotoxic cells (CD8+ cells)

Both groups of patients (CLL and ALL) shared an approximated percentage mean of CD8+cells (81.34±1.48 and 80.04±1.54%, respectively); but

both means were significantly (P≤0.001) higher than the observed percentage mean in controls (57.61± 1.01%) as shown in table (2).

Table (2): Percentage frequency of CD8+ cells in leukemia patients (CLL and ALL) and controls.

Groups	Number	Percentage frequency of CD8+ Cells (Mean ± S.E.)
CLL	18	81.34 ± 1.48^{A}
ALL	21	80.04 ± 1.54^{A}
Controls	15	57.61 ± 1.01^{B}

*Different letters: Significant difference (P ≤ 0.001) between means (Duncan test).

CD4+/CD8+ Ratio

The CD4+/CD8+ ratio was significantly decreased in CLL (0.34± 0.08) and ALL (0.27 ± 0.03) patients as compared with controls (1.08±0.08). The ratio in CLL patient was higher than the corresponding ratio in ALL, but the difference was not significant as shown in table (3).

Table (3): Percentage frequency of CD4+/ CD8+cells in leukemia patients (CLL and ALL) and controls.

Groups	Number	Percentage Frequency of CD4+/CD8+ Cells (Mean ± S.E.*)
CLL	18	0.34 ± 0.08^{B}
ALL	21	0.27 ± 0.03^{B}
Controls	15	1.08 ± 0.08^{A}

*Different letters: Significant difference (P ≤ 0.001) between means (Duncan test).

In comparison with other result, B-cell chronic lymphocyte leukemia (B-CLL) is the most common adult leukemia in the western world and is characterized by the accumulation of circulating sIgM+, CD19+, in addition, higher absolute number of circulating T-cells and increase in CD8 T-cells results and a low CD4/CD8 ratio, but CD4+ cells were eightfold lower than control healthy [15]. The B-cell patients appeared lower expression of CD4, CD8 and CD4/CD8 ratio (6.2± 0.5%; 15.8±1.67 and 0.79± 0.51) compared with healthy control (45.9± 9.8; 27± 7.6 and 1.79± 0.68) respectively [16], however, the experiment of [17] showed higher expression of CD4+ and CD8+ (4.2±0.63 and 2.8±0.29) compared with healthy control (2.1±0.5 and 2±0.7) respectively of B-CLL patient.

T-cells (CD3+cells)

The highest percentage mean of CD3+ cells was observed in ALL patient (82.11 ±1.06%), while in CLL patients (16.10±1.22%), as well as, controls (47.67±2.18%), a significantly lower mean was observed. The percentage frequency of CD3+ cells in CLL also significantly decreased as compared with controls as shown in table (4) and figure (1B).

Table (4): Percentage frequency of CD3+ cells in leukemia patients (CLL and ALL) and controls.

Groups	Number	Percentage frequency of CD3+ Cells (Mean ± S.E.*)
CLL	18	16.10 ± 1.22^{C}
ALL	21	82.11 ± 1.06^{A}
Controls	15	47.67 ± 2.18^{B}

*Different letters: Significant difference (P≤0.0001) between means (Duncan test).

[8] referred to B-CLL reported increasing in number of B-lymphocytes that express CD19, CD8, weak florescence of surface immunoglobulin, and decrease concentration of CD3, but when receiving high dose of chlorambucil treatment, an increase in CD3 Positive cells, aberrant expression of CD8 antigen and a decrease in CD8/CD19 positive clone occur. This result was the same result obtained in this study which is lower expression of CD3+ of CLL patients.

B-cells (CD19+ cells)

The ALL patients recorded the lowest percentage mean of CD19+ cells (18.37 ± 1.23 %), which was significantly different from the observed means in controls (51.43±1.66%) and CLL patients (84.33±1.46%). The later groups of patients recorded the highest mean of the cells, and the difference was significant as compared with ALL patients or controls shown in table (5). In comparison with other results appeared similar to this result, [18]; [19] referred to high expression of CD19+, with weak expression of sIg (surface immunoglobulin) in B-CLL patients.

Table (5): Percentage frequency of CD19+ cells in leukemia patients (CLL and ALL) and controls.

Groups	Number	Percentage Frequency of CD19+ Cells (Mean ± S.E.*)
CLL	18	84.33 ± 1.46^{A}
ALL	21	18.37 ± 1.23^{C}
Controls	15	51.43 ± 1.66^{B}

*Different letters: Significant difference (P ≤ 0.001) between means (Duncan test).

CD8/CD19 Ratio

The CD8/CD19 ratio was significantly decreased in CLL (0.95 ± 0.02) and increased in ALL (4.79±0.33) patients as compared with controls (1.11±0.03). There was significant difference between both ALL and CLL groups as compared with controls as shown in table (6).

Table (6): Percentage frequency of CD8+/ CD19+cells in leukemia patients (CLL and ALL) and controls.

Groups	Number	Percentage Frequency of CD8+/CD19+ Cells (Mean ± S.E.*)
CLL	18	0.95 ± 0.02^{C}
ALL	21	4.79 ± 0.33^{A}
Controls	15	1.11 ± 0.03^{B}

*Different letters: Significant difference (P ≤ 0.001) between means (Duncan test).

The results of this study showed that fluctuated levels of CD types were recognized among CLL and ALL lymphocytes. CD8+showed elevated level in both CLL and ALL in opposite to CD4+ which detected in low levels as well to CD4+/CD8+ ratio in both patient groups. On the other hand, CD3+, CD8+/CD19+ and CD19+ found to have a high level in ALL and lower level in CLL group. [20] through studying ALL broadly classified B-lineage and T-lineage ALL according to the degree of B-lymphocyte differentiation of the blast cells. However, T-lineage ALL observed in patients could also characterized as four variants (T-I; pro-T-ALL, T-II; pre-T-ALL, T-III; with cortical thymocytes phenotype, T-VI; mature T-ALL and all variants have CD3+ expression. B-lineage ALL was defined according to expression of the three early B-cell markers as CD19 +, cytoplasmic CD79a and CD22, four subtypes of B-Lineage ALL from B-I to B-IV (B-I; pro B-ALL, B-II; Common ALL, B-III; pre-B-ALL, B-IV; B cell ALL). [21] refer to higher expression of CD19+ and CD20+ of 55 cases of B-cell ALL patient but higher expression of CD10 and non expressed CD19 and CD20 of 18 cases of T-cell ALL patient when samples taken from patients of acute leukemia from different part of Pakistan.

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تحديد المعلمات المناعية 3و19 والمعلم 4 و8 على الخلايا اللمفاوية السرطانية للمرضى العراق

نجوى شهاب احمد ، ايمان عضبان خليل و حيدر فيصل غازي ، اديب عباس الشامي ،صبحي جواد حمزه ، عبد الحسين مويت الفيصل

الملخص

تضمنت الدراسة قياس التعبير المعلمات المناعية على سطح الخلايا اللمفاوية للمصابين بابيضاض الدم الحاد والمزمن والسيطرة ، جمعت 39 عينه من الدم وتوزعت 21 عينه من ابيضاض الدم الحاد و18 عينه من ابيضاض الدم المزمن بدون علاج وعزل وتنمية الخلايا للمفاويه وتحديد المعلمات المناعية على سطحها باستخدام تقنية التعبير للمعلمات المناعية حيث تم الحصول على نتائج تشير إلى ارتفاع التعبير المناعي CD8+ للمرضى ابيضاض الدم الحاد والمزمن بالمقارنة مع السيطرة وانخفاض CD4/CD8 , CD4 + للمجموعتين بالمقارنة مع السيطرة ، بينما أظهرت النتائج ارتفاع التعبير المناعي CD8/CD19, CD9, CD3 في ابيضاض الدم الحاد وانخفاضها في ابيضاض الدم المزمن بالمقارنة مع السيطرة .