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Malignant Lymphomas

Quantitative expression of CD23 and its ligand CD21 in chronic lymphocytic leukemia

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ABSTRACT

Background and Objectives. **Cells from the great majority of patients with chronic lymphocytic leukemia (CLL) express CD23. A recent histologic study has shown that CD23 is expressed more strongly in the proliferating centers of the lymph nodes, where the large prolymphocytoid cells are located. The aim of our study was to quantify the expression of CD23 and CD21 in small and prolymphocytoid cells from patients with CLL and B-cell lymphomas, and correlate this expression with clinical parameters.**

Design and Methods**. Using quantitative flow cytometry we analyzed the antigen density of CD23 and CD21 in: 1) 101 cases of chronic lymphocytic leukemia, 84 typical, 14 with increased prolymphocytes (CLL/PL) and 3 atypical, 2) 15 cases of CD23 positive B-cell lymphoma with circulating lymphoma cells and 3) 8 normal subjects. The results were correlated with morphology and clinical staging.**

Results. **Cells from CLL and CLL/PL have a significantly higher number of CD23 molecules than normal and lymphoma B-cells ($p<0.001$ and $p<0.001$, respectively). Differences were not significant for CD21. CLL and CLL/PL cases had similar values of CD23 and CD21 molecules, but analysis at a single level showed that prolymphocytes in typical CLL and CLL/PL expressed significantly higher CD23 ($p=0.001$, $p=0.006$) and CD21 ($p=0.001$, $p=0.001$) than small lymphocytes. There was no correlation between CD23 or CD21 antigen density and clinical stages although there was a trend for a brighter CD23 in stage C patients.**

Interpretation and Conclusions. **Since interaction between CD23 and CD21 is important for B-cell activation, proliferation and tumor formation, findings that both molecules are upregulated in prolymphocytes suggest that this is the proliferating cell component in CLL and underline the association between progression and increased prolymphocytes in typical CLL and CLL/PL.**

Key words: CD23, CD21, CLL, CLL/PL, quantitative flow cytometry

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The CD23 antigen is a 45 KDa transmembrane protein identified as the low affinity receptor of IgE and is an adhesion molecule expressed in activated mature B-cells. The molecule may also be induced in immature pre-B-cells after interleukin-4 (IL-4) stimulation.[1,2] Proteolysis of CD23 results in a rise of soluble CD23 (sCD23) molecules of variable molecular weight, which can be detected in the serum by an enzyme-linked immunosorbent assay (ELISA). sCD23 products have a variety of functional activities such as survival extension of B-cells and the induction of cell growth and differentiation not only of B-cells but also of myeloid and T-cells.[3,4] It has been shown that CD21, the receptor for the Epstein-Barr virus (EBV) is the ligand for CD23 and the interaction between CD21/CD23 is involved in cell adhesion of human B-lymphocytes.[5]

CD23 is a marker characteristically expressed by lymphocytes in chronic lymphocytic leukemia (CLL).[6-10] The great majority of CLL cases are CD23 positive, and most patients have increased serum levels of sCD23. The latter has been correlated with disease activity and thus, regarded as a prognostic factor.[11,12]

A recent histologic study[13] has shown that CD23 is expressed more strongly in the proliferating centers of lymph nodes, which contain large prolymphocytoid cells, than in the small cellular component, which is either CD23 weakly positive or negative. It has been suggested that this observation may explain the high sCD23 levels and the associated adverse prognosis in these patients. Although cells from the majority of CLL cases are CD23 positive, there are no data so far on the amount of CD23 antigen and its ligand CD21 in the membrane of CLL cells. We have investigated here the antigen density of CD23 and CD21 in cells from patients with CLL, B-cell lymphomas and normal B-cells using a flow cytometry quantitative method that estimates the number of antigenic determinants per cell. This study aimed to discover whether: 1) there are differences in the number of CD23 and CD21 molecules/cell

between normal B-cells and various types of malignant B-cells, 2) there is any correlation between CD23/CD21 expression and cell morphology, e.g. typical CLL versus CLL with increased prolymphocytes (CLL/PL) and clinical features, and 3) there are differences within a single CLL case between the expression of these molecules in the prolymphocytes compared to in the small lymphocytes.

Design and Methods

Patient and control samples

One hundred and sixteen newly diagnosed or previously treated patients referred to the Royal Marsden Hospital NHS Trust were studied. These patients included 101 with CLL and 15 with B-cell lymphomas in leukemic phase. The latter were selected because they were known to be CD23 positive. Out of the 101 cases of CLL, 84 had typical morphology, 14 had CLL with >10% prolymphocytes (CLL/PL) and the remaining 3 were atypical CLL with ≥15% cleaved and/or lymphoplasmacytic cells. The diagnosis was based on clinical features, cell morphology and immunophenotyping according to the FAB group criteria.[10,14,15] CLL patients were stratified according to Binet's staging system[16] as having stage A (58), stage B (13) and stage C (20) disease; data were not available for the remaining 10 cases. Peripheral blood mononuclear cells were analyzed in all cases and bone marrow in 9 of the CLL cases. Blood lymphocytes from 8 healthy donors were used as controls to establish reference ranges. Because it is well established that CD23 is negative in normal T-cells, the normal values refer to B-lymphocytes.

Flow cytometry

Direct immunofluorescence staining was performed with monoclonal antibodies (MoAb) conjugated to phycoerythrin (PE): PE-CD23 (MHM6) and PE-CD21 (MHCD2104-4) (DAKO, Glostrup, Denmark). Mononuclear cells were isolated by density gradient centrifugation with Histopaque (Sigma, Dorset, England). Cells were washed three times with Hank's solution balanced salt solution prior to immunostaining. After washing three times with Hank's, 1-2×10^6 cells were used per test. Cells were incubated for 10 minutes at room temperature with saturated amounts of MoAb. The cells were washed twice with PBS containing 0.02% sodium azide, and resuspended in 0.5 mL Isoton (Becton-Dickinson, San Jose, USA). Negative controls included the substitution of the MoAb by a FITC-conjugated mouse IgG1 and a PE-conjugated mouse IgG1R (Caltag, Buckingham, UK). In order to assess the immunostaining in large and small lymphocyte populations, a gate for each population, according to size and granularity, at the FSC/SSC dot plot was established (Figure 1).

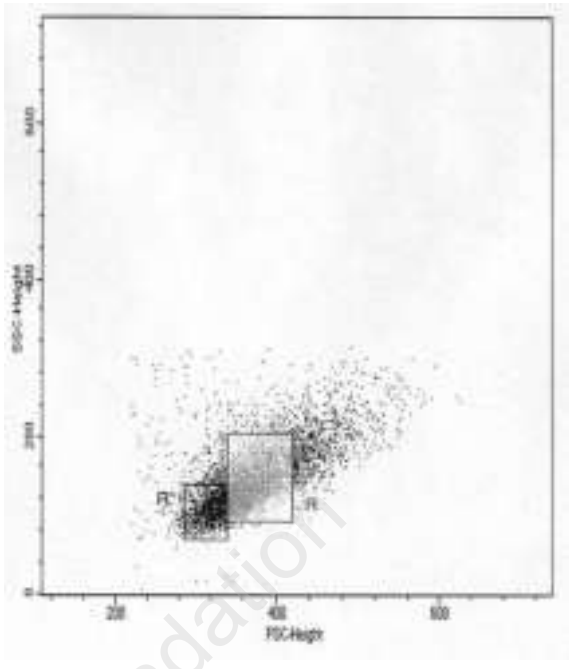

Figure 1. Illustrates the gating of small (R1) and large (R2) populations at the FSC/SSC dot plot.

All samples were acquired on a FACScan flow cytometer (Becton Dickinson) using CELL QUEST software. The fluorescence intensity was measured with detectors and amplifiers set on a logarithmic scale. For each sample, 5,000 mononuclear cells were acquired for list mode analysis by setting a live gate around the lymphocyte area in the forward scatter versus side scatter dot plot. The mean fluorescence intensity (MFI) values of the positive cells were converted into number of molecules of antigen per cell or antibody binding capacity (ABC) by using the Quantum Simply Cellular (QSC) microbeads kit (Sigma, St.Louis, Missouri, USA). This is a mixture of one blank and four microbead populations which differ by their incremental capacities to bind directly labeled mouse immunoglobulins. The accompanying software, the QUICK CAL software (FCSC, Sigma), regresses the binding capacities of the microbeads against their corresponding peak channels. The regression curve permits quantitative estimates of the MoAb molecules bound to the target cells, that is, the number of molecules of antigen expressed per cell. Ten microliters of each MoAb were added to 100 µL QSC beads. After one hour of incubation, the mixtures were washed and ana-

Table 1. ABC number of CD23 molecules in normal and leukemic B-cells.

	CD23 ABC All cells ($\times 10^3$)	*CD23 ABC Large cells ($\times 10^3$)*	*CD23 ABC Small cells ($\times 10^3$)*
CLL (PB)	24.7 (0.6-167.3)	27.1 (0.6-138.8)	9.8 (0.8-109.9)
CLL/PL (PB)	23.2 (1.1-85.2)	22.1 (1.9-148.6)	7.8 (0.9-25.9)
B-cell lymphoma (PB)	2.5 (0.6-42.7)	(Not applicable)	
Controls (PB)	1.1 (0.8-3.6)	(Not applicable)	
CLL (BM)*	3.3 (1.5-2.1)	3.4 (0.9-45)	1.4 (0.9-23.7)

*Results are shown as median (range). PB: peripheral blood; BM: bone marrow. *In all these cases, blood and bone marrow cells were analyzed. Differences in CD23 expression were statistically significant between CLL plus CLL/PL and normals ($p<0.001$), CLL and B-cell lymphomas ($p<0.001$) and between large and small lymphocytes in CLL ($p=0.001$) and CLL/PL ($p=0.006$).*

lyzed according to the method used for lymphocytes. All samples were acquired using the same instrument settings.

Statistical analysis

The ABC values of CD23 and CD21 antigens between normal and leukemic cells were compared using Wilcoxon's signed rank test. The Kruskal-Wallis test was used to correlate clinical stages and CD23/CD21 expression.

Results

CD23 expression (Table 1)

Cells from all CLL cases were CD23 positive with a median of CD23 molecules/cell of 24.7×10^3. Considering the whole blood lymphocyte population (small and large cells) the ABC number of CD23 molecules was higher in cells from CLL and CLL/PL patients compared to in normal blood lymphocytes and cells from B-cell lymphomas (Figure 2). These differences were statistically significant between CLL and controls ($p<0.001$), CLL/PL and controls ($p=0.001$), CLL and B-cell lymphomas ($p<0.001$) and CLL/PL and B-cell lymphomas ($p=0.002$). There were no differences, however, in CD23 expression between CLL and CLL/PL or between B-cell lymphomas and normal B-lymphocytes (Table 1). Considering as the upper normal limit the median value plus two standard deviations of normals, 81 of 87 (93%) CLL cases, 12/14 (86%) CLL/PL and 5/15 (33%) B-cell lymphomas had a number of CD23 molecules/cells above that of the normal cells.

The values of CD23 in the bone marrow specimens from CLL were similar to those of normal lymphocytes and B-cell lymphomas and significantly lower than those in the blood CLL lymphocytes ($p=0.006$), even within a single case in whom blood and bone marrow were available for analysis. The analysis of CD23 molecules (ABC) in the large and small lymphocytes within a single case of CLL showed that CD23 was significantly more strongly expressed by large lymphocytes (prolymphocytes) than by small lymphocytes (Figures 3 and 4). These differences were significant in both CLL ($p=0.001$) and CLL/PL ($p=0.006$) (Table 1).

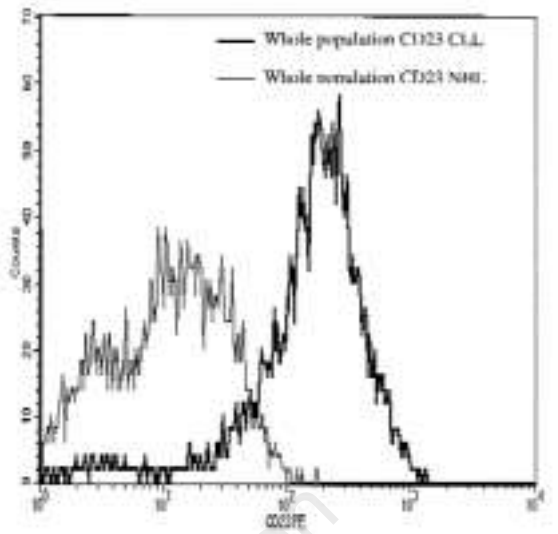

Figure 2. Flow cytometry plot illustrating the expression of CD23 in a case of CLL compared to a B-cell non-Hodgkin's lymphoma.

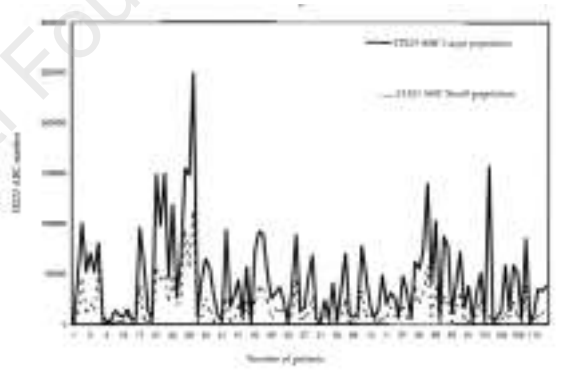

Figure 3. The figure compares the number of CD23 molecules in large cells (dark line) and small cells (dot line) for each individual case.

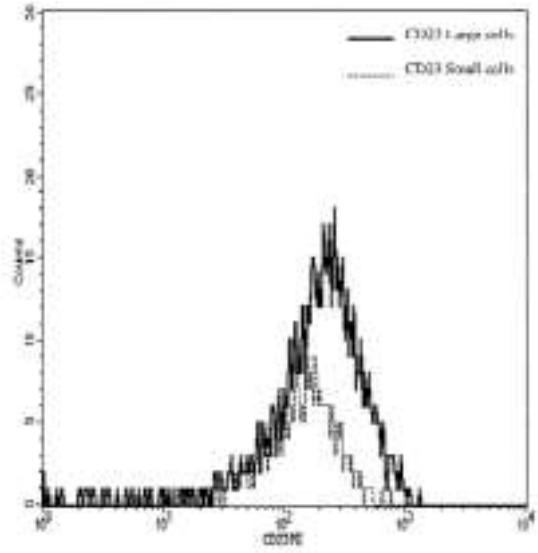

Figure 4. Flow cytometry plot illustrating the higher number of CD23 molecules in large prolymphocytoid cells (dark line) compared to in the small cells (dot line).

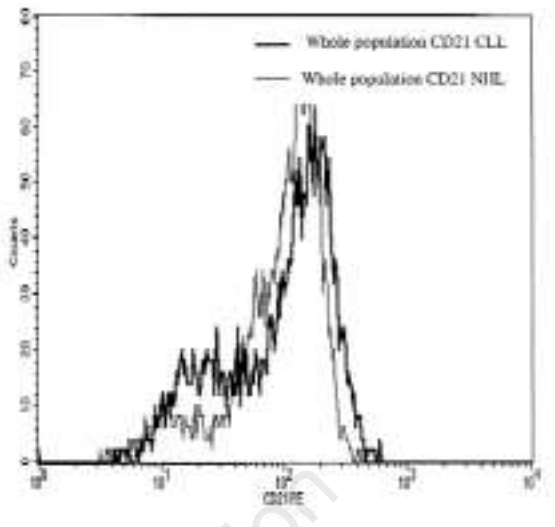

Figure 5. Flow cytometry plot illustrating CD21 expression in CLL and B-cell non-Hodgkin's lymphoma.

Table 2. ABC number of CD21 molecules in normal and leukemic B-cells.

	CD21 ABC All cells (×10³)	*CD21 ABC Large cells (×10³)*	*CD23 ABC Small cells (×10³)*
CLL (PB)	40.1 (15.7-156.5)	43.2 (3.5-120.7)	31.2 (2.9-108.2)
CLL/PL (PB)	36.4 (16-86.7)	48.3 (17.2-74.2)	23 (13.3-45.19)
B-cell lymphoma (PB)	37.7 (16.9-79)	(Not applicable)	
Controls (PB)	49.1 (33.1-82.9)	(Not applicable)	
CLL (BM)*	34 (12.4-168)	35.5 (15.1-70.8)	29.5 (3.2-80.7)

*Results are shown as median (range). PB: peripheral blood; BM: bone marrow. *In all these cases, blood and bone marrow cells were analyzed. Differences in CD21 expression were statistically significant between large and small lymphocytes in cases of CLL and CLL/PL (p=0.001).*

CD21 expression

The median values and range of CD21 molecules in the patient and control samples are shown in Table 2. Considering the whole lymphocyte population, there were not significant differences in the expression of CD21 between CLL, CLL/PL, B-cell lymphomas and normal lymphocytes (Figure 5). The highest values of CD21 were found in normal B-cells and few cases (5 CLL and 1 CLL/PL) had CD21 expression above the upper normal limit (median plus 2 standard deviations). However, when the expression of CD21 was considered in large and small lymphocytes within a single CLL case, CD21 expression was significantly stronger in the large cells compared to in small cells (Figure 6). This was observed in cases of CLL (p=0.001) and CLL/PL (p=0.001). There were no significant differences in the ABC values of CD21 molecules between peripheral blood and bone marrow in the 6 CLL cases in which both these specimens were examined.

Correlation between CD23/CD21 expression and clinical stages

There were no statistically significant correlations between clinical stages and CD23 and CD21 expression considering either the whole lymphocyte population or the large and small cells individually (Table 3). There was, however, a trend towards a higher expression of CD23 in the large lymphoid population in stage C CLL.

Discussion

A recent immunohistochemistry study in CLL showed that CD23 is more strongly expressed in the proliferating cells from the splenic white pulp and lymph nodes than in the small CLL cells outside the proliferating centers.[13] The authors suggested that this might be linked to the high soluble CD23 levels in CLL and the poor outcome of these patients. This study[13] did not investigate the intensity of CD23 expression in the circulating CLL cells. Our findings have shown that the number of CD23 molecules/cell in CLL, whether typical or CLL/PL, is significantly

Table 3. CD23/CD21 expression and clinical stages in CLL.

Stage	CD23 (×10³)	CD23 large (×10³)	CD23 small (×10³)	CD21 (×10³)	CD21 large (×10³)	CD21 small (×10³)
A	23 (0.6-165.5)	25.3 (0.6-102.5)	10.5 (0.9-56.7)	39.5 (4.9-168.0)	43.2 (3.5-120.8)	32.7 (2.9-79.8)
B	23 (0.9-167.3)	27.1 (0.9-138.8)	10.1 (1.1-109.9)	40.1 (15.7-75.3)	40.7 (13.3-67.7)	27.9 (13.3-80.7)
C	23.7 (1.1-102.6)	33.7 (1.3-148.6)	7.5 (0.8-44.1)	40.1 (16.1-67)	45.1 (15.4-65.8)	27.6 (8.8-45.7)

Results are shown as median (range).

higher than that in normal lymphocytes and cells from CD23⁺ B-cell lymphomas presenting with leukemia. The differences between CLL and B-cell lymphomas may help the differential diagnosis between these two conditions in cases which are CD23⁺ and difficult to classify by morphology and other markers. The high number of CD23 molecules in CLL can also explain the high levels of soluble CD23 found in the patients.

Although our overall results did not show differences in CD23 intensity between CLL and CLL/PL, when analyzing the number of CD23 molecules at a single cell level, there was a significantly stronger expression of CD23 in the large cells (prolymphocytes) than in the small lymphocytes. This parallels findings in tissue samples where proliferating prolymphocytoid cells and paraimmunoblasts strongly expressed CD23, compared to the small lymphocytes which were weakly positive. This might explain to some extent the prognostic impact of serum sCD23 levels in CLL as an increased number of prolymphocytes, both in typical CLL and in CLL/PL, has been shown to be an adverse prognostic factor for disease progression particularly in cases with stage A disease.[11,17,18]

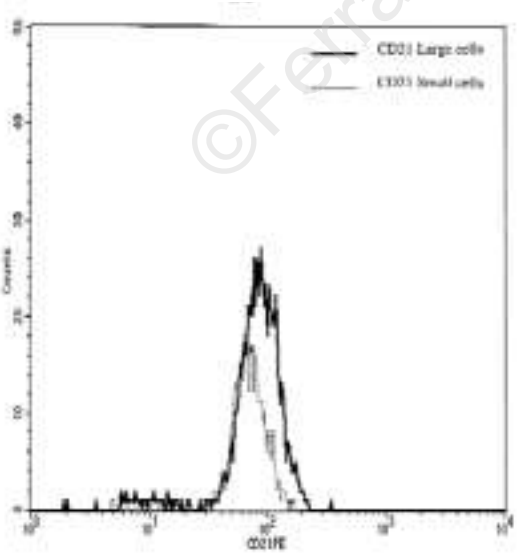

Figure 6. Flow cytometry plot illustrating a higher number of CD21 molecules in the prolymphocytoid cells (dark line) compared to in the small cells (dot line) in a case of CLL.

In the few CLL cases in which bone marrow was investigated, CD23 expression was relatively weak even in the large cells (data not shown) and within the same range as that found in normal B-cells. It is possible that CD23 is upregulated in circulating blood cells and/or that the number of prolymphocytes is higher in blood than in bone marrow. Nevertheless, because of differences in the values between the two different tissues, blood and bone marrow, it seems important to estimate this marker in peripheral blood samples. In contrast to CD23, our results have shown that, compared to normal B-lymphocytes, cells from most CLL cases have low expression of CD21, although this expression was significantly higher in large cells than in small cells. Previous studies using standard flow cytometry showed that CLL lymphocytes are weakly positive or negative with CD21[9,19] and that cases with bright CD21 expression might have a more aggressive course.[9] Our study has further confirmed, by a sensitive flow cytometry quantitative method, that the number of CD21 molecules in CLL is lower than in normal B-cells. However, CD21 intensity did not correlate in this series with clinical stage and/or progressive course. It was of interest however that within a single case and like CD23, prolymphocytoid cells had much brighter expression of CD21 compared to the small CLL lymphocytes. It has been shown that the increase of CD23 antigen facilitates the entry of normal B-cells into cell cycle[8] and that the interaction between CD23 and its ligand CD21 is important for B-cell activation, increases expression of proto-oncogenes such as c-fos[20] leading to cell proliferation and facilitates tumor mass formation. Our findings that both molecules, CD23 and CD21, are expressed at significantly higher levels in the large prolymphocytoid cells, substantiate the belief that these cells are the main cellular component responsible for proliferation and disease progression and

explain, to some extent, the association between increased numbers of prolymphocytes and disease progression.

Contributions and Acknowledgments

ML, RM and MR performed the flow cytometry analyses. EM and DC collected clinical data and reviewed morphology.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential implications for clinical practice

- The differential quantitative expression of CD23 between CLL and B-cell lymphomas helps to distinguish the two disorders when cells from both are CD23+
- At present quantitative estimation of CD23 in CLL has no clinical implications as a prognostic factor.[21,22]

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