Haematologica 1998; 83:681-685 original paper

Rapid and simple immunophenotypic characterization of lymphocytes using a new test

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ABSTRACT

Background and Objective. **In this paper, we report our experience of lymphocyte phenotyping of a series of 108 consecutive samples using a simple flow cytometry test (Lymphogram®). The kit consists of a combination of 5 different markers conjugated with three fluorochromes (CD8-FITC, CD19-FITC, CD56-PE, CD3-PE, CD4-PECy5) in the same tube. This allows identification of different T-cells, NK subpopulations and B lymphocytes.**

Design and Methods. **The samples were divided into three groups: samples with absolute lymphocytosis (>5×10⁹/L) (n=50), samples with relative lymphocytosis (>50%) (n=24) and other categories for which a lymphocyte immunophenotype was required (T-cell lymphoma and estimation of blood involvement in chronic lymphoproliferative disorders (CLPD) (n=34). When CD19⁺ cells exceeded the normal range or there was a suspicion of CLPD without B-cell lymphopenia, clonality was investigated by means of light chain restriction analysis.**

Results. **In the first group, 29 samples were abnormal (10 CLPD, 3 polyclonal B-cell lymphocytosis, 13 inversions of the CD4/CD8 ratio and 3 cases with CD4 lymphocytosis) and 21 samples were regarded as normal. In the second group 7 samples showed abnormalities (2 CLPD, 3 inverted CD4/CD8 ratios and 2 with a relative increase in CD4 cells). In one sample from the third group B-cell clonality without lymphocytosis was detected whereas in 18 samples a polyclonal pattern was observed. The presence of B-cell lymphopenia precluded further clonality study in 13 samples.**

Interpretation and Conclusions. **Lymphogram® associated with clonality analysis is a rapid, easy and cheap method of assessing lymphocyte phenotypes in the majority of clinically relevant situations.**

Key words: immunophenotype, flow cytometry, lymphocytosis, lymphoproliferative disorders, clonality

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Flow cytometry (FC) is a useful and powerful technique for analyzing specific features of cell subsets present in a sample with mixed cellularity because it can analyze a large number of cells in a few seconds. As a result of the expression of different surface antigens, distinct lymphocyte subtypes can be identified, enumerated and characterized.[1] As a consequence, this method has been widely used in the study of lymphoid neoplastic diseases, immunodeficiency disorders and in transplantation medicine. Until now, the initial approach to the immunophenotypic characterization of peripheral blood lymphoid cells in patients suffering from lymphocytosis has been based on the use of a panel of monoclonal antibodies (MoAb) conjugated with different fluorochromes.[2,3] The disadvantage of this strategy, however, is that it requires preparation of different tubes even for the enumeration of the major peripheral blood (PB) lymphoid subsets. Recently, a combination of 5 different monoclonal antibodies conjugated with three fluorochromes has been commercialized for the analysis of the major PB lymphoid subsets in a single measurement (Lymphogram®, Cytognos, Salamanca, Spain).

In this study, we used this new kit, which makes full use of the combined analysis of more than one antigen stained with the same fluorochrome, to investigate its usefulness in all PB samples referred to our laboratory for lymphocyte immunophenotypic analysis. In the cases in which there was an increased number of B-cells we added a tube with reagents for the analysis of B-cell clonality to enhance the information provided by the test. With only two tubes, it was thus possible to screen rapidly a wide range of pathologic samples at a relatively low cost.

Materials and Methods

Samples

One hundred and eight consecutive whole blood samples from 108 subjects were included in the study. The samples were selected from our hematologic laboratory and were divided into three groups:

1. samples with absolute lymphocytosis (>5×10⁹/L) (n=50);
2. samples with relative lymphocytosis (>50% lym-

phocytes) (n=24);
3. other categories: samples from patients with T-cell lymphomas (n=2) and from patients with clinical or morphological suspicion of CLPD (n=32).

All the cases were analyzed by means of Lymphogram® and in the case of an increase in B-lymphocytes or a possible CLPD, the κ/λ/CD19 combination was also assessed. The reference ranges chosen were those established by Reichert *et al.*[4] because they were based on healthy Caucasian subjects and were determined by FC methods. In our series there were only 9 samples from patients under the age of 18 years. For these samples, the reference values published by Denny *et al*.[5] for children were used.

Lymphogram® is a mixture of murine MoAbs against CD8, CD19, CD56, CD3 and CD4. CD8 and CD19 were conjugated with fluorescein-isothiocyanate (FITC), CD3 and CD56 with phycoerythrin (PE) and CD4 with phycoerythrin-cyanine 5 (Pe/Cy5). The recommendations of the manufacturer were followed when carrying out the analysis. For sample preparation a stain, lyse and then wash procedure was used; erythrocytes were lysed by means of FACS lysing solution [Becton Dickinson (BD)]. Clonality study of B lymphocytes was undertaken using a triple reagent consisting of a combination of κ-FITC, λ-PE and CD19 PE/Cy5 in a single tube (K/L, Simultest® purchased from Becton Dickinson, San José, CA, USA and CD19-PE/Cy5 from Caltag, San Francisco, CA, USA).

Data acquisition and analysis

Measurements were performed on a FACScan flow cytometer (BD). For data acquisition the LYSIS-II (BD) software program was used. Fifteen thousand events/tube were measured. The PAINT-A-GATE PRO software program (BD) was used for further data analysis.The analysis was carried out on gated lymphoid cells. For this purpose, all the positive cells for the FL-1 (FITC) and FL-2 (PE) with low SSC were selected and the rest of the events were removed. A similar gating protocol based on light scatter and immunofluorescence (CD45/CD14) has been suggested for the optimal analysis of lymphocyte populations.[6] The same principle, the combination of physical characteristics and fluorescence pattern, is exploited by Lymphogram®.

Calibration of the instrument was performed prior to data acquisition using well established protocols, and $CD8^+/CD4^+/CD3^+$ positive controls as well as CALIBRITE beads standards (BD).

Results

Immunophenotypic findings in samples with absolute lymphocytosis (n=50)

Twenty-one samples were regarded as normal, since the differential percentage of lymphoid subpopulations fell within the standard range.[4,5] Alterations were detected in 29 samples. Thirteen showed an increased number of $CD19^+$ cells (normal range: 7-23%), 10 had restriction of the light chains and 3 additional cases (two men and one woman) showed a polyclonal pattern. In the monoclonal cases, complete immunophenotypic study of B-cell markers was performed. Four cases displayed a Matutes *et al.* score of 4-5; therefore these patients were diagnosed as having classic CLL.[7] The other 6 cases corresponded to one mantle cell lymphoma, two follicular lymphomas (FL) and three atypical CLL. There was a concomitant diagnosis of chronic myelomonocytic leukemia with CLPD (FL) in one case (see Figure 2).

An increase in $CD4^+$ cells (normal range:28-58%) was detected in 3 cases. One of these cases corresponded to a chronic T-cell lymphoproliferative disorder in which a clonal TCR-β was found by molecular techniques (see Figure 2). This case was diagnosed as having Sézary cell leukemia.[8] There was an inverted $CD4^+/CD8^+$ ratio (normal range: 0.6-2.8) in 13 cases. Four patients were diagnosed as having infectious mononucleosis, two patients suffered from large granular leukemia (LGL), five patients underwent an autologous bone marrow transplantation and one patient was treated for a non-Hodgkin lymphoma. There was no information about the diagnosis or evolution in one patient.

Immunophenotypic findings in patients with relative lymphocytosis (n=24)

Percentages of the different lymphoid populations were within the normal ranges (CD4:28-58%, CD8:19-48%, CD56: 6-29%, CD19:7-23%) in 16 samples. The values were outside the normal ranges in 8 cases. An increase in $CD19^+$ cells was observed in three of these, two of which were clonal. After complementary phenotypic analysis, one case was diagnosed as having classic B-CLL. The remaining clonal case corresponded to a FL. In two samples there was an increased percentage of $CD4^+$ cells and in 3 an abnormal $CD4^+/CD8^+$ ratio was detected (two patients were diagnosed as having LGL and one patient suffered from amyloidosis and received an autologous bone marrow transplantation).

Patients suspected of suffering from a CLPD without lymphocytosis (n=34)

This group included samples obtained from patients with a suspected diagnosis of CLPD (n=32) and PB samples from patients with T-cell lymphomas (n=2).

A diagnosis of CLPD was suspected in 32 samples in the absence of lymphocytosis. B-cell clonality analysis was performed in 19 samples. Only one sample showed a monoclonal pattern (persistence of the neoplastic cells in one case of FL). In the remaining 18 cases, a polyclonal pattern indicating the absence of a predominant B-cell clone was identified. All the

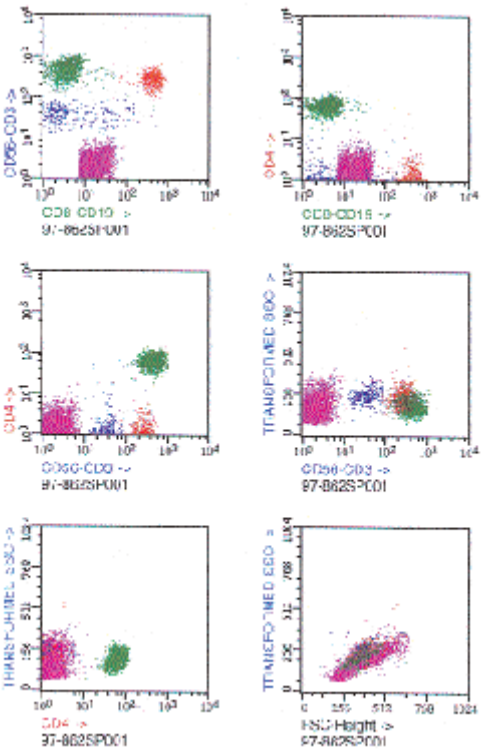

Figure 1. Representative flow cytograms obtained using Lymphogram®. In red CD8⁺CD3⁺ lymphocytes, in green CD4⁺CD3⁺ cells. Minor T-cells subpopulations are easily identified (double positives and double negatives) but are not shown in the figure. NK-cells are represented in blue. Violet cells correspond to B-lymphocytes.

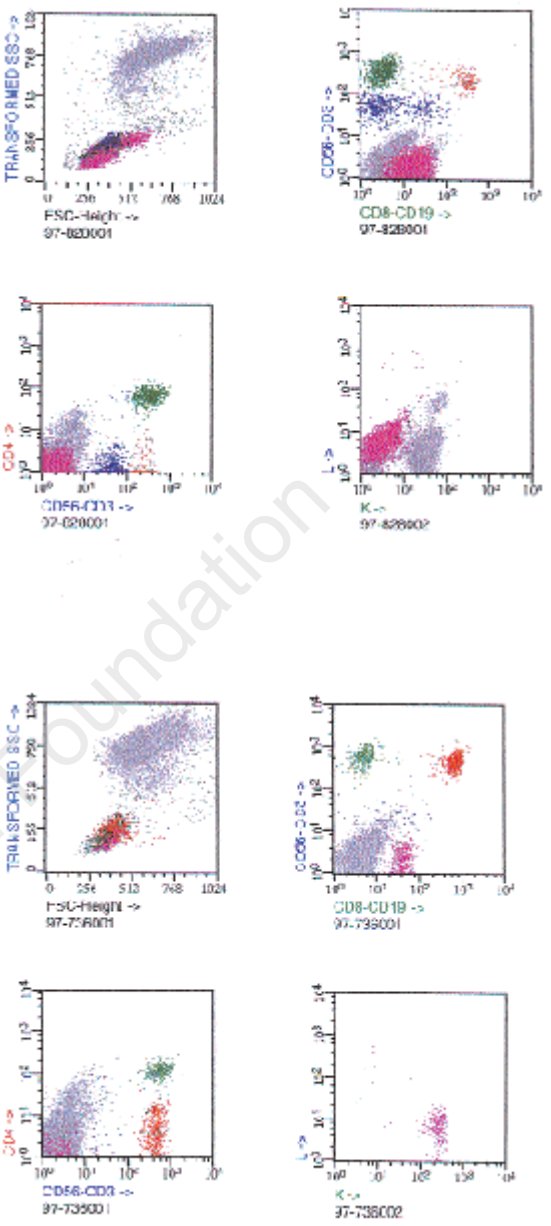

Figure 2. Identification of B-cell clonal populations. The 4 upper boxes correspond to a patient suffering from a classic B-CLL. The faint intensity of the surface immunoglobulins is characteristic of this disease. The remaining four boxes show the identification of a clonal population in a case of CMML. Note the different intensity of surface immunoglobulin expression with a clear light chain restriction (λ).

samples not tested for light chain restriction showed B-cell lymphopenia.

An increase in the proportion of CD4⁺ cells was observed in one sample obtained from a patient with T-cell lymphoma. In the other case the result was ambiguous. Conventional Lymphogram® analysis showed an increased percentage of CD4⁺ cells occupying a position identical to that of NK-cells with regards to PE staining; however, NK lymphopenia was detected when this apparent NK lymphocytosis was studied with additional antibodies (CD2,CD3, CD56, CD16, CD94, cytoplasmic CD3). These contradictory data could be explained by the fact that neoplastic cells express CD3 at a low intensity only detectable with PE-CD3 conjugates.

Discussion

The aim of this study was to establish the value of Lymphogram® in daily immunophenotypic characterization of peripheral blood lymphocytes. The major advantage of this technique lies in its simultaneous, rapid and cheap identification of B-lymphocytes, NK-cells and T-cell populations. The technique requires software designed to analyze three or more fluorescences.[9]

Our results suggest that Lymphogram®, when combined with K/L/19 analysis, is useful for detecting clonal B-cell populations even in cases without lym-

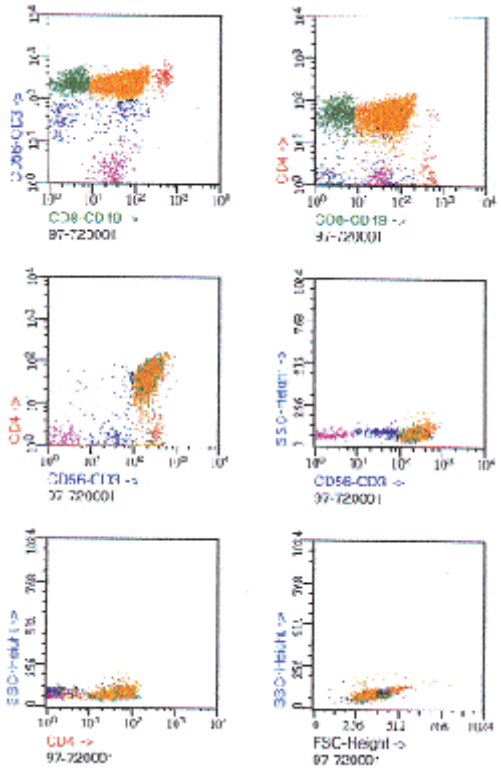

Figure 3. Lymphogram® images in a case of mature T-cell leukemia (Sézary cell leukemia) in which clonality was confirmed using molecular techniques. A clonal band rearrangement was detected when genomic DNA was hybridized to a Cβ probe (data not shown). Neoplastic population is indicated in yellow. The tumoral cells coexpressed CD4 and CD8.

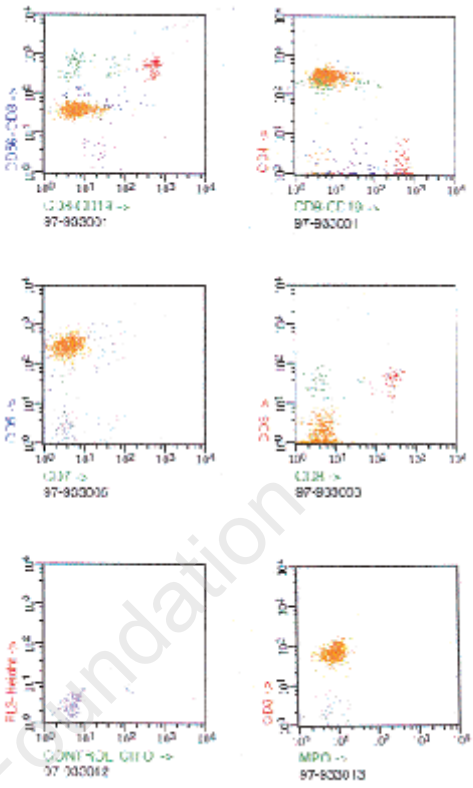

Figure 4. When Lymphogram® is used to study lymphocyte subsets in patients with cutaneous T-cell lymphoma, faint expression of CD3 can be potentially misleading. In this case a neoplastic CD4+CD3+faint is located in the region of NK-cells. Extensive immunophenotype disclosed its aberrant T-cell properties (CD3- with PerCp antibodies, CD7 loss, negativity for NK-markers).

phocytosis. Detection of monoclonal B-lymphocytes is one of the most important applications of FC in diagnostic hematology.[10-14] This technique, however, can could be time consuming and expensive when different combinations of antibodies are used. Recent developments in staining and software analysis have allowed more accurate and simple assessment of clonal B-cells. Furthermore, suitable antibody combinations such as the ones used in the present study obviate the need for cytophilic antibody-shedding by incubation at 37°C in fetal calf serum.[11] FC techniques compare favorably with APAAP methods in terms of speed and interpretation.[12] In this series, 13 samples were monoclonal and were included in the three groups. Furthermore, the polyclonal B-pattern might be of interest since persistent polyclonal lymphocytosis is a new entity that typically affects young women and follows a benign course.[15] This disease could be confused with other malignant conditions. Interestingly, two of our cases were males. When we studied the clinical records of these two patients we found that one was a child with whooping cough diagnosed as having an acute pertussis syndrome and the other was an adult under treatment with an experimental drug. Lymphogram® could be used to screen samples to search for B-cell clonality.

Lymphogram® can also be used to identify patients with inverted CD4/CD8 ratios. This group is important since it includes patients with acute viral infections (infectious mononucleosis), frequent immunodeficiency status (HIV) and lymphoproliferative disorders of large granular lymphocytes.[3,16] Analysis of sequential samples taken at periodic intervals, in conjunction with clinical and morphologic information, resolves most cases. If the alteration persists after three months without a clear diagnosis a clonality study of the T-cell receptor by means of Southern-blot should be carried out.[17] The mature T-lymphoproliferative disorders are a heterogeneous group of diseases which result from the clonal expansion of T-cells at various stages of differentiation.[16] Five main entities based on clinical, cytological and immunological features can be recognized: T-prolymphocytic

leukemia (T-PLL) and its variant Sézary cell leukemia (SCL),[8] adult-T cell leukemia lymphoma(ATLL), cutaneous T-cell lymphoma (CTCL), large granular lymphocyte leukemias of T-phenotype (LGL) and peripheral non-cutaneous T-cell non-Hodgkin's lymphoma (T-NHL). The immunophenotype of all these disorders shows negativity for TdT associated with the expression of T-cell markers (CD3, CD5, CD4, CD7, CD8, CD2). It is possible to find cases which lack one or more of these antigens in these diseases. In our series, there was one case with a faint expression of CD3. The interpretation of Lymphogram® in this context could be difficult. However, the use of 3 T-associated markers (CD4, CD8, CD3) in Lymphogram® minimizes possible misinterpretations of the test. In addition, it allows us to identify *empty spaces* which could be occupied by neoplastic populations.

One potential use of this test is to diagnose NK-malignancies. There are two major types of NK proliferations, chronic and acute form.[18] In this series, cases which fulfilled the criteria of NK-lymphocytosis were not detected. However, NK subpopulations are readily identifiable in all the samples. This test could also be applied to the detection of rare T-cell subsets (double positives $CD4^+$ $CD8^+$ $CD3^+$, and double negatives $CD4^-$ $CD8^-$ $CD3^+$) in the setting of bone marrow transplantation.

Lymphogram® associated with a simple clonality analysis provides a rapid and very informative immunophenotype at a low cost.

Contributions and Acknowledgements

JFN was responsible for the conception of the study, its design and for reviewing the manuscript. ER, JU, CE, OL and RM performed the test. MB was responsible for data handling and for drafting the article.

We are grateful to Professor Alberto Orfao for his comments and suggestions. We are also indebted to George von Knorring for improving the final version of the manuscript.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received January 30, 1998; accepted April 27, 1998.

References

1. Orfao A, Ruiz-Argüelles A, Lacombe F, Ault K, Basso G, Danova M. Flow cytometry: its applications to haematology. Haematologica 1995; 80:69-81.
2. Mandy FF, Bergeron M, Minkus T. Evolution of leukocyte immunophenotyping as influenced by the HIV/AIDS pandemic: a short history of the development of gating strategies for CD4+ T-cell enumeration. Cytometry 1997; 30:157-65.
3. Centers for disease control and prevention. 1994 revised guidelines for the performance of CD4+ T-cell determinations in persons with human immunodeficiency virus (HIV) infection. MMWR 1994; 43:1-21.
4. Reichert T, DeBruyère M, Deneys V, et al. Lymphocyte subset reference ranges in adult caucasians. Clin Immunol Immunopathol 1991; 60:190-208.
5. Denny T, Yogev R, Gelman R, et al. Lymphocyte subsets in healthy children during the first 5 years of live. JAMA 1992; 267:1484-8.
6. Loken MR, Brosnan JM, Bach BA, Ault KA. Establishing optimal lymphocyte gates for immunophenotpyping by flow cytometry. Cytometry 1990; 11:453-9.
7. Matutes E, Owusu-Ankomah K, Morilla R, et al. The immunological profile of B-cell disorders and proposal of scoring system for the diagnosis of CLL. Leukemia 1994; 8:1640-5.
8. Pawson R, Matutes E, Brito-Babapulle V, et al. Sézary cell leukaemia: a distinct T cell disorder or a variant form of T prolymphocytic leukaemia? Leukemia 1997, 11:1009-13.
9. Afar B, Merrill J, Clark EA. Detection of lymphocyte subsets using three color/single laser flow cytometry and the flourescent dye peridin chlorophyll a protein. J Clin Immunol 1991; 11:254-61.
10. General Haematology Task Force Of BCSH. Immunophenotyping in the diagnosis of chronic lymphoproliferative disorders. J Clin Pathol 1994; 47:871-5.
11. Fukushima P, Nguyen P, O'Grady P, Stetler-Stevenson M. Flow cytometric analysis of kappa and lambda light chain expression in evaluation of specimens for B-cell neoplasia. Cytometry 1996; 26:243-52.
12. Oertel J, Lipski H, Huhn D. Detection of light chain restriction in chronic B-lymphoid leukemia and B-non-Hodgkin´s lymphoma. Clin Lab Haematol 1991; 13: 33-40.
13. Reynolds GH, Williamsom AM, Smith GJ, Lane AC. A simple technique for the determination of κ and λ immunoglobulin light chain expression by-cells in whole blood. J Immunol Methods 1992; 151:123-9.
14. Ault KA. Detection of small numbers of monoclonal B lymphocytes in the peripheral blood of patients with lymphoma. N Engl J Med 1979; 300:1401.
15. Delage R, Roy J, Jacques L, Bernier V, Delage JM, Darveau A. Multiple bcl-2/Ig gene rearrangements in persistent polyclonal lymphocytosis. Br J Haematol 1997; 97:589-95.
16. Matutes E, Catovsky D. Mature T-cell leukemias and leukemia/lymphoma syndromes. Leuk Lymphoma 1991; 4:81-91.
17. Singh AK, Laffan M, Eridani S, Luzzatto L. Rearrangement of T-cell receptor (delta, gamma and beta) genes and its significance in T-cell chronic leukemias. Leuk Lymphoma 1990; 4:17-25.
18. Mateu R, Badell I, Alcala A, Estivill C, Rubiol E, Nomdedéu J, Soler J. Aggressive natural killer cell leukemia: report of a case in a caucasian boy. Haematologica 1998; 83:190-2.