OExperimental
NCOLOGY

PERIPHERAL BLOOD LYMPHOCYTE PHENOTYPE OF ZAP-70+ AND ZAP-70− PATIENTS WITH B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA

*A. Rivkina[1−4, *], I. Holodnuka Kholodnyuk[4], M. Murovska[4], M. Soloveichika[3], S. Lejniece[1−3]*
[1]Riga Stradins University, Internal Diseases Department, Riga LV1007, Latvia
[2]Riga Eastern Clinical University Hospital, Chemotherapeutic and Hematological Clinic, Riga LV1038, Latvia
[3]Riga Hematology Centre, Riga LV1006, Latvia
[4]Riga Stradins University, A. Kirchenstein Institute of Microbiology and Virology, Riga LV1067, Latvia

Background: Up to now, the immune status of chronic lymphocytic leukemia (CLL) patients in association with the expression of zeta-chain-associated protein kinase 70 (ZAP-70) in leukemic cells has not been evaluated. *Aim*: The aim of this work was the study of the peripheral blood (PB) T-lymphocyte phenotypes in ZAP-70-positive (ZAP-70+) and ZAP-70-negative (ZAP-70−) untreated patients with CLL. *Materials and Methods*: ZAP-70-, CD25-, CD3-, CD4-, and CD8-positive lymphocytes were enumerated by flow cytometry in PB of 120 untreated CLL patients. CD8+, CD3+CD4+ and CD3+CD25+ cells were counted for the non-leukemic lymphocytes. *Results*: The patients were distributed into two groups: the ZAP-70+ group of high CLL progression (n = 61), and the ZAP-70− group of low CLL progression (n = 59). In the ZAP-70+ group, the ratio CD4/CD8 (0.33 ± 0.62; $p = 0.001$) and the numbers of the CD3+ ($34.8 \pm 8.1\%$; $p = 0.01$), CD3+CD4+ ($24.4\% \pm 4.8$; $p = 0.001$), and CD3+CD25+ ($6.2 \pm 0.91\%$; $p = 0.001$) lymphocytes were reduced and the percentage of the CD8+ cells ($73.1 \pm 4.6\%$; $p = 0.0001$) was above the norm. In the ZAP-70− group, the number of the CD3+CD4+ cells ($36.9 \pm 6.1\%$; $p = 0.001$) was within the norm, but the numbers of the CD8+ ($11.3 \pm 1.1\%$; $p = 0.0001$) and CD3+ ($41.2 \pm 5.3\%$; $p = 0.05$) lymphocytes were reduced; the ratio CD4/CD8 (3.26 ± 0.88; $p = 0.001$) and the percentage of the CD3+CD25+ cells ($27.1 \pm 3.4\%$; $p = 0.0001$) were above the norm. *Conclusions*: Our data show that the increased CD4/CD8 ratio, caused by the reduced number of the CD8+ lymphocytes, and the increased number of CD3+CD25+ cells are characteristic for the ZAP-70− group (slow progressing) of untreated CLL patients. In ZAP-70+ patients, the CD4/CD8 ratio was significantly below the norm indicating an active disease process. Results of our study contribute to identification of CLL patients with different prognosis in routine diagnostic/prognostic procedures.
Key Words: chronic lymphocytic leukemia, ZAP-70, immune status, CD4/CD8 ratio, regulatory T cell.

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia among all neoplastic diseases of the hematopoietic and lymphoid tissues. CLL is heterogeneous in its course and is characterized not only by rates of progression but also by survival of CLL patients. Currently, morphological structure and clinical manifestations of CLL are described fully and detailed, whereas the pathophysiological features of this form of leukemia are not completely investigated and are the subject of many research studies [1–4].

A significant role in CLL is assigned to the interaction of leukemic cells with the microenvironment, which ensures their survival, proliferation, and resistance to the action of pro-apoptotic stimuli [5, 6]. Clarification of the relationship between the immune system and the development of the tumor in the body is of current importance in modern oncoimmunology, as it plays a significant role in the delay of growth and regression of tumors [7]. CLL immune deficiency caused by the both the tumor lesions of lymphoid tissue and the influence of chemotherapy on hema-

topoiesis, results in the inhibition of the cellular and humoral responses [8, 9].

T cells in CLL have been poorly studied despite researchers' growing interest in the peculiarities of the functional activity of T-cell subpopulations and their participation in the anti-tumor immune responses. It has been shown that in CLL, there is a decrease in CD3+ T-lymphocytes [9]. The ratio of lymphocytes with the CD4 and CD8 receptors or the helper/suppressor (CD4/CD8) ratio (norm 1.5–2.5) in the PB is an assessment of the immune status [10]. The increase in lymphocytes with the CD8 receptor (the reduced CD4/CD8 ratio, < 1.5) is characteristic for tumor development [11, 12]. In CLL, an absolute CD8+ lymphocytosis correlates with clinical stage of the disease [13]. A number of recent studies have shown that CLL patients usually have abnormalities in the phenotype of CD4 and CD8 T cells, including inversion of the normal CD4/CD8 ratio [14–16].

CD25 is the alpha chain of the IL-2 receptor (critical for maintenance and expansion of the T-cell response to antigen presentation) present on activated T and B cells. When the immune system is hyperactive, the count of the CD25+ lymphocytes in PB is increased. Normally, the percentage of cells with the CD25 receptor in the blood of a health adult is 13–24% [17]. In CLL patients, the frequency of CD25+ regulatory T cells is higher, when compared with healthy donors, and

is further increased in patients with advanced stage disease [16, 18, 19].

One of the most differentially expressed lymphocytes genes is the gene encoding the Zetachain-associated protein kinase of 70 kDa (ZAP-70), which is normally expressed in T cells and NK cells. Crespo *et al.* [20] were the first who confirmed the value of ZAP-70 as a surrogate marker for the immunoglobulin variable region heavy chain (IgVH) mutation status in CLL. In a number of studies of recent years, it has been shown that, in CLL, high expression of ZAP-70 is significantly associated with an unmutated IgVH gene [21, 22] and high expression of CD38 (> 30% of leukemic cells) and is correlated with shorter treatment-free survival indicating an adverse clinical prognosis [23, 24]. Expression of the ZAP-70 protein above a certain threshold of cells (> 20%) has proven to be an independent marker of clinical outcome [25].

Up to now, the association between the levels of the T-lymphocyte subpopulations in the subgroups of CLL patients, positive for the ZAP-70 (ZAP-70+, rapidly progressive) and negative for the ZAP-70 (ZAP-70–, slowly progressive), has not been evaluated. The aim of our study was to analyze the immune status (the CD4/CD8 ratio) and the proportion of the CD8+, CD3+CD4+, and CD3+CD25+ lymphocytes in PB of ZAP-70+ and ZAP-70– groups of untreated CLL patients.

MATERIALS AND METHODS

Selection of patients and clinical assessments. The study group consisted of 120 patients who were diagnosed with CLL for the first time. The patients enrolled in this study (60 males, 60 females) had an age range of 34–86 years (median age was 67 years). The median age of males and females was 65.6 and 69.3 years, respectively. The Rai classification for the estimation of clinical stages was used. The selection of patients was carried out at the Chemotherapeutic and Hematological Clinic (Riga, Latvia) in the period from July 2007 to July 2008. The study design, patients' information, and consent forms were approved by the Central Ethics Commission at the Latvian Medical Association, Riga, Latvia (22.03.2007. N. A-9).

Flow cytometry analyses. The indices of CD19, CD5, CD23, CD3, CD4, CD8, CD25, and ZAP-70 in PB were determined by flow cytometry (FC) for all new CLL patients. After establishing the presence of the CD19+CD5+ population and diagnosis of CLL, analysis of ZAP-70 expressing cells was performed using the PN 772587 kit (Beckman Coulter Inc., USA). The pairs of fluorochrome-labeled reagents from Beckman Coulter, CD3-FITC and CD4-PE, CD4-FITC and CD8-PE, and CD3-FITC and CD25-PE were used and the FC analyses were carried out according to the standard protocol from Beckman Coulter using the flow cytometer EPICS XL (Beckman Coulter). The number of the CD3+ cells and the CD4/CD8 ratio were counted for the total number of gated lymphocytes. The percentages of the CD8+, CD3+CD4+,

and CD3+CD25+ cells were calculated for the non-leukemic lymphocytes. Statistical analysis of the data was performed using the D'Agostino-Pearson test and the Mann — Whitney U test [26].

Statistical analysis. All data were statistically processed, using the statistic programs GraphPad-Prism version 5 (San Diego, California, USA). The data were presented as M ± SE, $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

All 120 CLL patients in the study were classified by Rai stages (Table 1). The largest numbers of patients were in Rai stage I (n = 38) and Rai stage II (n = 40).

Table 1. Number of patients in the study with low and high levels of ZAP-70 in CLL stages of Rai classification

Rai classification		ZAP-70+ subgroup (rapid rate of CLL progression)	ZAP-70– subgroup (slow rate of CLL progression)
Stage	Number of patients, n (%)	Number of patients with the ZAP-70 level > 20%	Number of patients with the ZAP-70 level 0–20%
0	7 (6.0)	1	6
I	38 (32.0)	14	24
II	40 (33.0)	26	14
III	19 (16.0)	12	7
IV	16 (13.0)	8	8

In practice, flow cytometry turned out to be the preferred technique for assaying ZAP-70 expression in CLL cells. Crespo *et al.* [20] were the first, who had described such a flow cytometric method and who confirmed the value of ZAP-70 as a marker for the IgVH mutation status. In our study, we had defined two levels of ZAP-70 expression: low (within the interval of 0–20% of positive cells), corresponding to a slowly progressing CLL, and high (with more than 20% of positive cells), corresponding to the rapidly progressing disease [3, 4, 21, 22]. The CLL patients were distributed into two groups: the ZAP-70+ subgroup, with high level of ZAP-70 (61 patients), and the ZAP-70– or slowly progressing CLL subgroup (59 patients), with the low ZAP-70 level (see Table 1).

Table 2 presents the data of the levels of CD3+, CD8+, CD3+CD4+, and CD3+CD25+ lymphocytes in the ZAP-70+ and ZAP-70– subgroups of patients. In the ZAP-70+ subgroup, the CD4/CD8 ratio below the norm was due to the decrease in the number of CD4+ lymphocytes and the increase in the number of CD8+ cells (cytotoxic T-lymphocytes and NK cells). In the ZAP-70– subgroup, the CD4/CD8 ratio was above the norm. In this case, the amount of CD8+ cells was below the norm and the amount of CD4+ lymphocytes corresponded to the norm. In the ZAP-70+ subgroup, the amount of CD3+CD4+, and CD3+CD25+ cells were below the norm with the statistical significance $p = 0.001$, but the level of CD8+ lymphocytes was increased. On contrary, in the ZAP-70– subgroup, the count of CD8+ lymphocytes was below the norm, the count of the CD3+CD25+ cells was above the norm, whereas the count of CD3+CD4+ cells corresponded to the norm, indicating the activity of the immune cells. There was a significant correlation between the parameters of CD25, CD8, CD4 and the ratio of CD4/

CD8 in both subgroups. The correlation for the parameter CD3 was not identified in the above-mentioned subgroups.

Table 2. Parameters of PB T-lymphocytes in the ZAP-70+ and ZAP-70− subgroups of CLL patients

Parameters (the normal reference range, norm)	ZAP-70+ subgroup			ZAP-70− subgroup		
	> 20% of positive cells (n = 61)	p	r	0–20% of positive cells (n = 59)	p	r
CD3+ cells*, % (norm: 58–76%)	34.8 ± 8.1	0.01	0.33	41.2 ± 5.3	0.05	0.15
CD4/CD8 ratio* (norm: 1.5–2.5)	0.33 ± 0.62	0.001	0.33	3.26 ± 0.88	0.001	0.37
CD8+ cells**, % (norm: 17–37%)	73.1 ± 4.6	0.0001	0.41	11.3 ± 1.1	0.0001	0.48
CD3+CD4+ cells**, % (norm: 35 – 65%)	24.4 ± 4.8	0.001	0.44	36.9 ± 6.1	0.001	0.34
CD3+CD25+ cells**, % (norm: 13–24%)	6.2 ± 0.91	0.001	0.32	27.1 ± 3.4	0.0001	0.49

CD marker(s) positive cells were detected within the lymphocyte analysis gate defined on the basis of light scatter (FSC *vs* SSC). *The number of the CD3+ cells and the CD4/CD8 ratio were counted for the total number of lymphocytes; **the percentages of the CD8+, CD3+CD4+, and CD3+CD25+ cells were calculated for the non-leukemic lymphocytes; norm — the normal reference ranges for percentages of CD marker(s) positive cells in lymphocytes of health adults [17, 31].

To our knowledge, no research has been conducted on the immune status of the ZAP-70+ *vs* ZAP-70− CLL patients. In the study of Zhang *et al.* [27] of 67 patients with B-cell CLL, the number of CD3+, CD4+, CD8+, and NK cells was reduced significantly and the CD4/CD8 ratio was not severely changed. However, the group of CLL patients was not divided into rapidly progressive (ZAP-70+) and slowly progressive (ZAP-70−) subgroups and the CD25 immunophenotype was not analyzed.

In a number of studies of recent years, it has been shown that T cells with the phenotype CD3+CD4+CD25+ suppress the activity of the effector T-lymphocytes and thus may contribute to tumorogenesis [11]. Indeed, Hus *et al.* [28] showed that stimulation of the tumor immunity reduce lymphocytosis in CLL patients and a decrease in the number of CD4+CD25+FOXP3+ (forkhead box P3) regulatory T cells was detected. Another study has demonstrated that, in patients at later CLL stages, the levels of CD4+FOXP3+ T cells were significantly increased and the immune response was reduced [29]. However, in the study by Gowda *et al.* [30], it was shown that the high levels of cells with CD4 and CD25 receptors contributed to better survival of patients with CLL. Recently, it was reported that inverted CD4/CD8 ratios (< 1) are associated with shorter lymphocyte doubling time, shorter time to first treatment, and reduced progression-free survival in CLL disease, suggesting that T-cell dysfunction contributes toward disease progression [16].

Our study also revealed that in the slowly progressing ZAP-70− subgroup of CLL patients, the number of CD3+CD25+ cells and the CD4/CD8 ratio were significantly higher than in the rapidly progressing ZAP-70+ subgroup. In ZAP-70+ patients, the CD4/CD8 ratio was substantially below the norm, indicating a cell cytotoxic activity and an active disease process. The increased number of the CD3+CD25+ cells,

the level of the CD3+CD4+ cells within the normal reference range and the CD4/CD8 ratio above the norm in the ZAP-70− subgroup of CLL patients, evidence the better functional immune activity and the low rate of the disease progression.

ACKNOWLEDGMENTS

REFERENCES

1. **Montillo M, Hamblin T, Hallek M, *et al*.** Chronic lymphocytic leukemia: novel prognostic factors and their relevance for risk-adapted therapeutic strategies. Haematol 2005; **90**: 391–9.

2. **Van Bockstaele F, Verhasselt B, Philippé J.** Prognostic markers in chronic lymphocytic leukemia: a comprehensive review. Blood Rev 2009; **23**: 25–47.

3. **Chiorazzi N.** Implications of new prognostic markers in chronic lymphocytic leukemia. Hematology Am Soc Hematol Educ Program 2012; **2012**: 76–87.

4. **Siddon AJ, Rinder HM.** Education Committee of the Academy of Clinical Laboratory Physicians and Scientists. Pathology consultation on evaluating prognosis in incidental monoclonal lymphocytosis and chronic lymphocytic leukemia. Am J Clin Pathol 2013; **139**: 708–12.

5. **Seiler T, Dohner H, Stilgenbauer S.** Risk stratification in chronic lymphocytic leukemia. Sem Oncol 2006; **33**: 186–94.

6. **Burger JA.** Nurture versus nature: the microenvironment in chronic lymphocytic leukemia. Hematol Am Soc Hematol Educ Program 2011; **2011**: 96–103.

7. **Mueller MM, Fusenig NF.** Friends or foes — bipolar effects of the tumor stroma in cancer. Nature Rev Cancer 2004; **4**: 839–49.

8. **Zamai L, Ponti C, Mirandola P, *et al*.** NK cells and cancer. J Immunol 2007; **178**: 4011–6.

9. **Hamblin AD, Hamblin TJ.** The immunodeficiency of chronic lymphocytic leukaemia. Br Med Bull 2008; **87**: 49–62.

10. **Inoshita T, Whiteside TL.** Imbalance of T-cell subpopulations does not result in defective helper function in chronic lymphocytic leukemia. Cancer 1981; **48**: 1754–60.

11. **Chatila TA.** Role of regulatory T cells in human diseases. J Allergy Clin Immunol 2005; **116**: 949–59.

12. **Godfrey DI, Berzins SP.** Control points in NKT-cell development. Nat Rev Immunol 2007; **7**: 505–18.

13. **Catovsky D, Lauria F, Matutes E, *et al*.** Increase in T gamma lymphocytes in B-cell chronic lymphocytic leukaemia. II. Correlation with clinical stage and findings in B-prolymphocytic leukaemia. Br J Haematol 1981; **47**: 539–44.

14. **Gonzalez-Rodriguez AP, Contesti J, Huergo-Zapico L, *et al*.** Significance of CD8 and CD4 T cells in chronic lymphocytic leukemia. Leuk Lymphoma 2010; **51**: 1829–36.

15. **D'Arena G, Laurenti L, Minervini MM, *et al*.** Regulatory T-cell number is increased in chronic lymphocytic leukemia patients and correlates with progressive disease. Leuk Res 2011; **35**: 363–8.

16. **Nunes C, Wong R, Mason M, *et al*.** Expansion of a CD8(+)PD-1(+) replicative senescence phenotype in early stage CLL patients is associated with inverted CD4:CD8 ratios and disease progression. Clin Cancer Res 2012; **18**: 678–87.

17. **Belkaid Y, Piccirillo CA, Mendez S, *et al*.** CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature 2002; **420**: 502–7.

18. **Beyer M, Kochanek M, Darabi K, *et al*.** Reduced frequencies and suppressive function of CD4+CD25hi regulatory

T cells in patients with chronic lymphocytic leukemia after therapy with fludarabine. Blood 2005; **106**: 2018—25.

19. Giannopoulos K, Schmitt M, Kowal M, *et al*. Characterization of regulatory T cells in patients with B-cell chronic lymphocytic leukemia. Oncol Rep 2008; **20**: 677—82.

20. Crespo M, Bosch F, Villamor N, *et al*. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. N Engl J Med 2003; **348**: 1764—75.

21. Wiestner A, Rosenwald A, Barry TS, *et al*. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. Blood 2003; **101**: 4944—51.

22. Orchard JA, Ibbotson RE, Davis Z, *et al*. ZAP-70 expression and prognosis in chronic lymphocytic leukaemia. Lancet 2004; **363**: 105—11.

23. Schroers R, Griesinger F, Trumper L, *et al*. Combined analysis of ZAP-70 and CD38 expression as a predictor of disease progression in B-cell chronic lymphocytic leukemia. Leukemia 2005; **19**: 750—8.

24. Deaglio S, Vaisitti T, Aydin S, *et al*. CD38 and ZAP-70 are functionally linked and mark CLL cells with high migratory potential. Blood 2007; **110**: 4012—21.

25. Rassenti LZ, Jain S, Keating MJ, *et al*. Relative value of ZAP-70, CD38, and immunoglobulin mutation status in predicting aggressive disease in chronic lymphocytic leukemia. Blood 2008; **112**: 1923—30.

26. D'Agostino RB, Belanger AJ, D'Agostino RB, Jr. A suggestion for using powerful and informative tests of normality. J American Statistician 1990; **44**: 316—21.

27. Zhang L, Zhu L, Wang HW, *et al*. Analysis of immunophenotype, lymphocytic subsets and NK cells in patients with B cell chronic lymphoid leukemia. Intern Med J 2009; **17**: 36—9.

28. Hus I, Schmitt M, Tabarkiewicz J, *et al*. Vaccination of B-CLL patients with autologous dendritic cells can change the frequency of leukemia antigen-specific CD8+ T cells as well as CD4+CD25+FoxP3+ regulatory T cells toward an antileukemia response. Leukemia 2008; **22**: 1007—17.

29. Piper KP, Karanth M, McLarnon A, *et al*. Chronic lymphocytic leukaemia cells drive the global CD4+ T cell repertoire towards a regulatory phenotype and leads to the accumulation of CD4+ forkhead box P3+ T cells. Clin Exp Immunol 2011; **166**: 154—63.

30. Gowda A, Ramanunni A, Cheney C, *et al*. Differential effects of IL-2 and IL-21 on expansion of the CD4+ CD25+ Foxp3+ T regulatory cells with redundant roles in natural killer cell mediated antibody dependent cellular cytotoxicity in chronic lymphocytic leukemia. MAbs 2010; **2**: 35—41.

31. Janeway ChA, Travers JrP, Walport M, *et al*. Immunobiology: the immune system in health and disease. 5th edn. New York: Garland Science, 2001.