ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN — POLONIA
VOL. LV, 14 SECTIO D 2000

[1] Department of Clinical Immunology, University School of Medicine, Lublin
Zakład Immunologii Klinicznej Akademii Medycznej w Lublinie
[2] Department of Pulmonology, University School of Medicine, Lublin
Klinika Chorób Płuc i Gruźlicy Akademii Medycznej w Lublinie

PAWEŁ KRAWCZYK[1], ROBERT KIESZKO[2],
MAREK MICHNAR[2], JACEK ROLIŃSKI[1], MICHAŁ DRAGAN[1],
JAROSŁAW BUCZKOWSKI[1], JANUSZ MILANOWSKI[2]

Expression of bcl–2 protein and Fas antigen in lymphocytes from bronchoalveolar lavage fluid (BALF) and peripheral blood in different clinical presentations of sarcoidosis

Ekspresja proteiny bcl–2 i antygenu Fas na limfocytach z płynu płukania oskrzelowo–pęcherzykowego i z krwi obwodowej od chorych w różnym stadium zaawansowania sarkoidozy

INTRODUCTION

Sarcoidosis, a systemic, granulomatous disorder with predominant involvement of the hilar lymph nodes and the pulmonary parenchyma, is characterised by accumulation of T cells in the lung, more specifically, the helper subset [7]. These activated T lymphocytes CD4+ may modulate the histologic changes in this disease. Most studies showed that intensity of lymphocytic alveolitis have an influence on the outcome of sarcoidosis but presented controversial data [17]. The role of apoptosis of lymphocytes in the regulation of the immune response in sarcoidosis is still unknown.

The control of cell survival plays an important role in the immune system. Apoptosis is an active mode of cell death [1]. The protein encoded by the bcl–2 (B cell lymphoma/leukemia–2) protooncogen has been implicated in the regulation of lymphocyte cell survival. Upregulation of bcl–2 appears to be normal mechanism for positive selection of developing lymphocytes in thymus and bone marrow. These findings indicate that high expression of bcl–2 protein is capable of inhibiting apoptotic cell death during normal lymphopoiesis. Only those lymphocytes bearing antigen receptors with appropriate specificity are selected for survival. Apoptosis is also involved in the termination of an immune response. Most activated T cells downregulate of bcl–2 and die by apoptosis at the end of immune response and the peripheral lymphoid organs revert to their normal cellular content [15].

In contrast to the bcl–2 protein, the CD95 (Fas/APO–1) antigen is able to induce apoptosis. The CD95 is a member of the tumour necrosis factor receptor superfamily and induces apoptotic cell death upon binding of the natural ligand or agonistic antibodies [4]. The CD95 is expressed on the surface of many transformed cell lines and chronically stimulated T cells [10].

These data suggest that bcl–2 expression prevents the apoptosis of lymphoid cells induced by the Fas antigen – dependent mechanism and that apoptosis of lymphocytes is exquisitely controlled by regulation of the bcl–2 and Fas genes.

The aim of the present study was undertaken to determine the expression of bcl–2 mitochondrial oncoprotein and Fas antigen in CD3, CD4, CD8, CD19 positive lymphocytes obtained from BALF and peripheral blood from patients with sarcoidosis. We have analysed our results in respect to the presence and type of the clinical symptoms.

MATERIALS AND METHODS

Patients. The study population consisted of 31 newly diagnosed, untreated patients with sarcoidosis (median age 38, range 23 to 65, 23 women). The diagnosis of sarcoidosis was based on clinical presentation, chest x–ray abnormalities and histopathological analysis.

We analysed the follows pairs of groups: patients with Löfgren Syndrome (n = 14) vs patients without Löfgren Syndrome (n = 17), patients with constitutional and respiratory symptoms (n = 23) vs symptomless patients (n = 6) and patients with hilar lymphadenopaty (I RTG stage, n = 19) vs patients with parenchymal infiltration (II and III RTG stage, n = 12).

Bronchoalveolar lavage (BALF). The bronchoscope (Olympus BF20) was wedged in a middle lobe bronchus, and 140 ml sterile saline sodium at 37° was instilled in seven aliquots of 20 ml. After each instillation, the fluid was gently aspired with a syringe. The first aliquot recovered were used for bacteriologic analysis and the remaining were pooled The fluid was mixed and strained through double layer of nets. Cells were pelleted at 500 x g for 5 min. at 4°C and than resuspended in phosphate buffered saline (PBS) at 10^6 cells for future staining. Cells were counted in a Bürker chamber.

Preparation of cells from peripheral blood. Mononuclear cells were isolated by density gradient centrifuging on Lymphoprep (Nycomed, Norway). Interphase cells were removed, washed twice in PBS and than resuspend at 10^6 cells for future staining.

Antibodies. Double colour immunofluorescence studies were performed using combinations of phycoerythrin (PE) and fluorescein isothyocyanate (FITC) conjugated monoclonal antibodies. Monoclonal antibodies were obtained from Ortho Diagnostic Systems (Germany), Becton Dickinson (Germany), Serotec (U.K.) or Dako (Denmark). The following antibody combinations were used: IgG1 FITC/IgG2a PE (negative control), anti–CD3 PE, anti–CD19 PE, anti–CD4 PE, anti–CD8 PE, anti–

CD95 FITC. 1 x 10^6 cells were incubated with antibodies for 30 min. at 4°C and washed twice with PBS afterwards.

Intracellular staining. For the immunodetection of the intracellular bcl-2 protein, cells after binding of monoclonal antibodies with surface antigens were fixed with 0.25% paraformaldehyde (15 min. at RT) and permeabilised with cold 70% methanol for 60 min. at 4°C before incubation (30 min. at 4°C) with FITC conjugated anti-bcl-2 mouse monoclonal antibody (Dako, Denmark) [2].

Flow cytometric analysis. All samples were measured on a Cytoron Absolute flow cytometer (Ortho Diagnostic Systems). To determine the fluorescence intensity of a stained cells, the logarithmic fluorescence channel intensity was converted to arbitrary units based on the ImmunoCount 2.0 software. The mean fluorescence intensity (MFI) of bcl-2 and CD95 were measured from then upper limit of the negative control.

Statistical analysis. Data are expressed as means ±SD. Wilcoxon's matched pairs test and Mann-Whitney U test were applied for the statistical comparison of the mean fluorescence intensity of bcl-2 and CD95 on different subtypes of lymphocytes for each group of patients. $P < 0.05$ was considered significant.

RESULTS

The expression of the bcl-2 protein and Fas antigen measured as a mean fluorescence intensity was studied in pre-treatment BALF and blood samples from 31 patients with sarcoidosis. Nearly 100% lymphocytes from BALF and peripheral blood of all patients expressed bcl-2 protein. T BALF cells expressed a higher level of the bcl-2 protein in comparison with T lymphocytes from peripheral blood in all groups of patients. In contrast to patients with Löfgren Syndrome and with constitutional and respiratory symptoms of sarcoidosis, the symptomless patients have shown higher level of bcl-2 protein in all subtypes lymphocytes (CD3, CD19, CD4, CD8) from BALF and peripheral blood (Figure 2). The results show that the bcl-2 protein was significant weakly expressed in CD4+ T cells from patients with Löfgren Syndrome (106,69 ±11,89 MFI) and with respiratory symptoms of sarcoidosis (107.6 ±11.46 MFI) than in CD4+ lymphocytes from patients without Löfgren Syndrome (112.3 ±13.08 MFI) and symptomless patients (118.31 ±12.45 MFI). Expression of the bcl-2 protein in CD4+ cells was significantly higher in comparison with CD8+ lymphocytes from blood in symptomless patients and significantly lower in BALF cells in all groups of patients (Figures 1, 3).

The mean percentage of Fas+ lymphocytes from peripheral blood was significantly higher in group of symptomless patients (48.91 ±9.59%) than group of patients with Löfgren Syndrome (34.63 ±12.65%) and patients in II/III RTG stage of sarcoidosis (48.47 ±8.38%) than patients in I stage of disease (38.87 ±13.19%). Blood lymphocytes from patients without acute symptoms expressed Fas antigen more intensely (71.98 ±4.54 MFI) in comparison with patients with Löfgren Syndrome (69.01 ±6.02 MFI). Mean fluorescence intensity of CD95 on blood lymphocytes in other

groups of patients was similar (Figure 4). The percentage of Fas+ cells and expression of Fas antigen on BALF lymphocytes was higher in groups of patients without acute symptoms (47.01 ±14.56%, 73.48 ±7.34 MFI), symptomless patients (50.42 ±12.77%, 72.37 ±7.41) and patients in II/III RTG stage of sarcoidosis (45.77 ±15.49%, 73.22 ±7.37 MFI) than groups of patients with Löfgren Syndrome (39.64 ±16.62%, 69.83 ±10.08 MFI), with constitutional and respiratory symptoms (41.95 ±16.33%, 71.15 ±9.05 MFI) and in I RTG stage of disease (42.43 ±16.32%, 71.01 ±9.22 MFI).

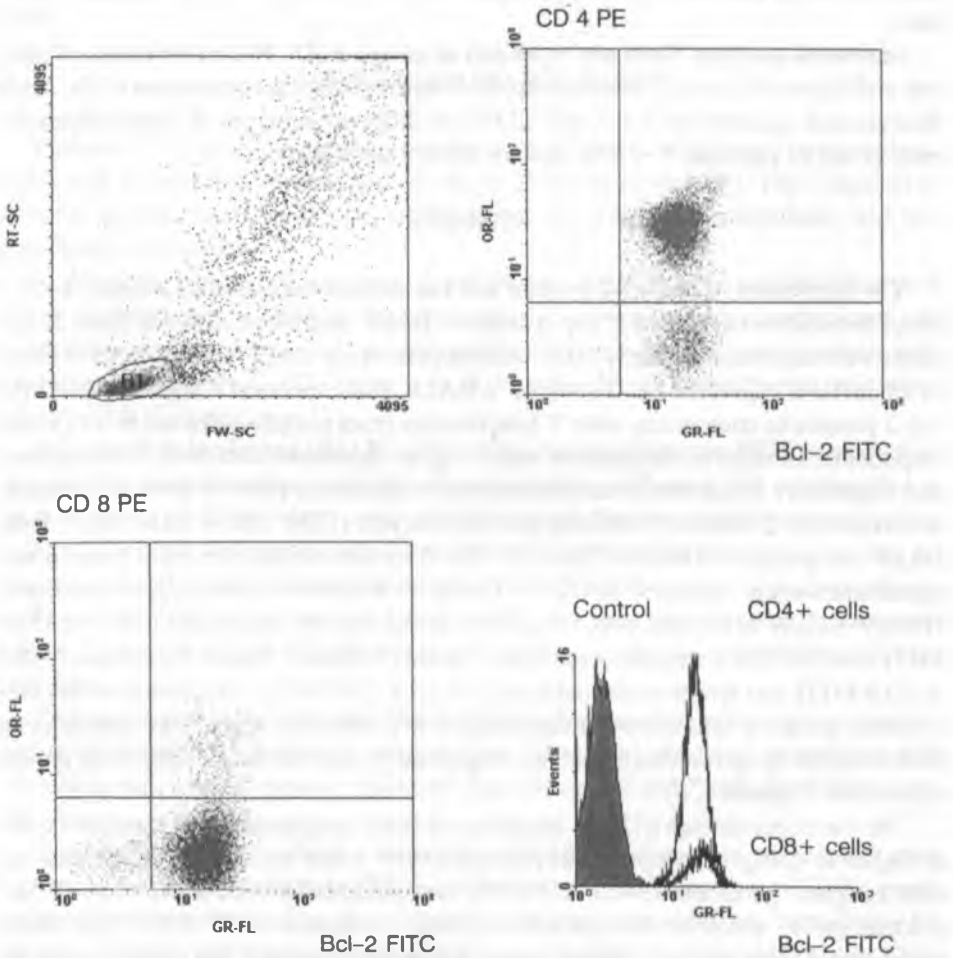

Fig. 1. Expression of bcl–2 protein in CD4+ and CD8+ cells from BALF from patients with pulmonary sarcoidosis

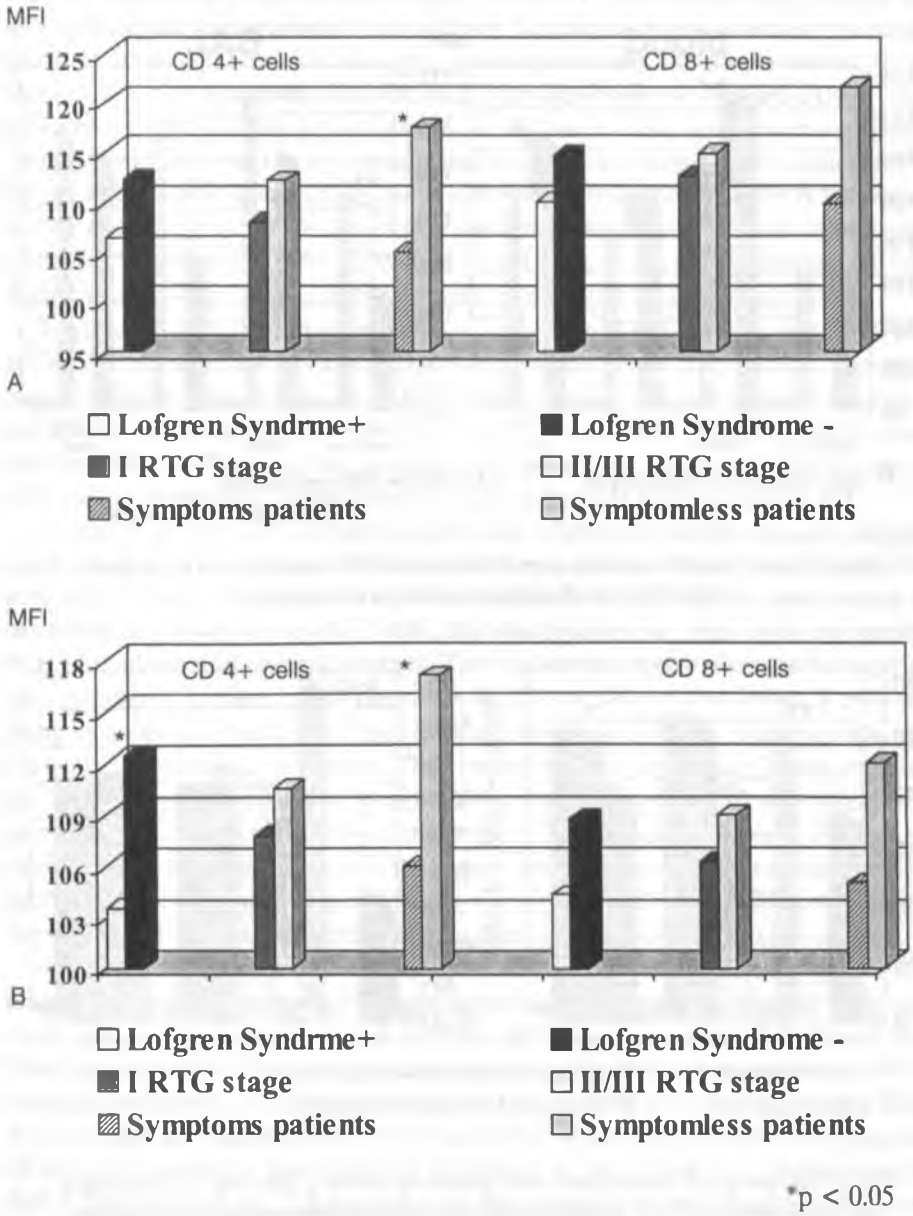

Fig. 2. Expression of bcl-2 protein in CD4+ and CD8+ lymphocytes from BALF (A) and peripheral blood (B) from patients with different clinical presentations of sarcoidosis

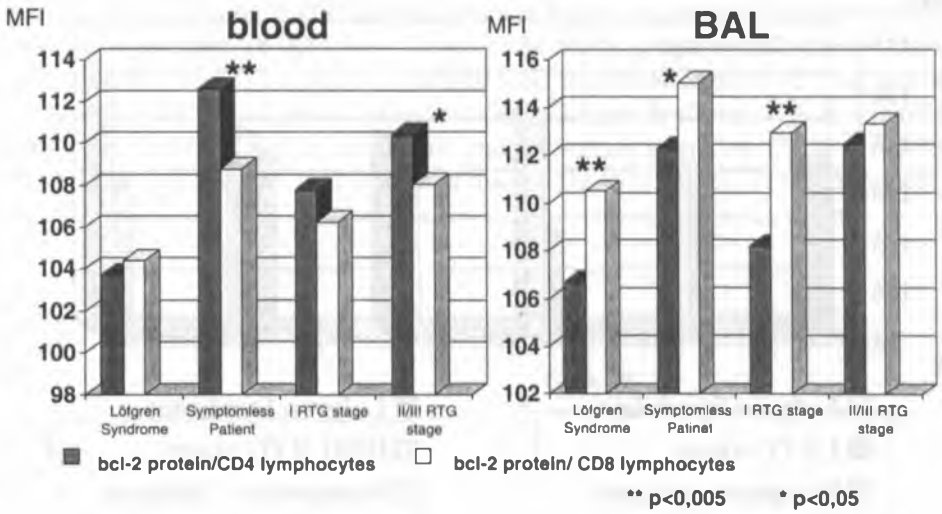

Fig. 3. Bcl–2 protein expression on CD4+ and CD8+ lymphocytes in patients with different clinical presentations of sarcoidosis

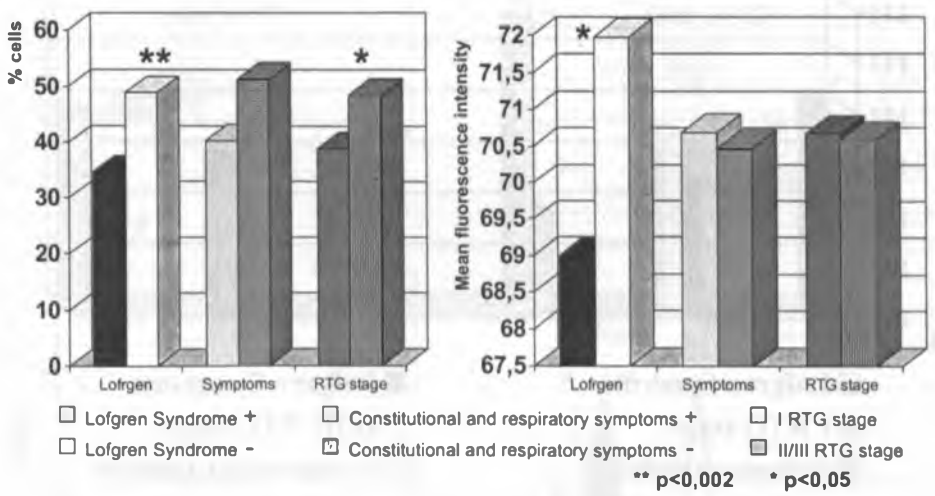

Fig. 4. Percentage of lymphocytes from peripheral blood presenting of Fas antigen and expression of CD95 antigen in different clinical presentation of sarcoidosis

DISCUSSION

Development in flow cytometric analysis has led to the spread of procedures which enable us to quantify precisely both membrane and intracellular antigens [2]. In this study we have applied this technique to determine the expression of the bcl–2 protein

and Fas antigen by lymphocytes obtained from BALF and blood from patients with different clinical presentations of sarcoidosis. Sarcoidosis is characterised by accumulation of activated T cells in the lung, more specifically, the helper subset [7, 13]. Results of our earliest study demonstrated increased levels of activated CD4+ T lymphocytes from BALF. We noticed that HLA DR and CD25α activation markers were strongly expressed by BALF T cells populations from patients with sarcoidosis [11]. Immunophenotypic characterisation of subtypes of lymphocytes from BALF can be achieved by using monoclonal antibody and flow cytometry technique. Combination of surface (CD19, CD3, CD4, CD8, CD95) and intracellular (bcl-2) antigen allows to distinguish between cells with the different expression of the bcl-2 protein.

High levels of bcl-2 found in CD4+ T cells from BALF suggest that bcl-2 is involved in their accumulations and these lymphocytes escape apoptosis [3]. The apoptotic process in lymphocytes from BALF mediated by Fas can be influenced by an antiapoptotic mechanism, such as bcl-2. The combination of high level bcl-2 protein and resistance to Fas mediated apoptosis may contribute to the extended *in vivo* survival and accumulation lymphocytes in lungs of patients with sarcoidosis [9].

Herry et al. [6] have analysed *in vitro* susceptibility to death through apoptotic mechanism recently activated lymphocytes recovered from BALF and isolated from peripheral blood. The progressive loss of viable cells following *in vitro* culture was considerable greater for lavage T cells, and was observed for cells from both patients with sarcoidosis and control subjects. The evaluation of morphologic features and flow cytometric profiles of fragmentation of DNA indicated that lavage T cells were dying by apoptotic mechanism. Authors suggested that CD4+ T cells appeared to be particularly sensitive to apoptosis. They observed that most lavage T cells expressed Fas antigen but the viability of lavage T cells was not improved by incubation in the presence of a monoclonal antibody that inhibits Fas-induced apoptosis. Their studies emphasise that T cells on the alveolar surface are in a different state of activation and differentiation compared with circulating T cells, and offer a possible explanation for the impaired functional capacities observed for lavage T cells in *in vitro* studies.

Activation of lymphocytes seemed to be associated with a simultaneous downregulation of the bcl-2 protein and upregulation of Fas antigen. Variation in the level of expression of bcl-2 protein in all subtypes of lymphocytes from BALF and blood from patients with different clinical presentation of sarcoidosis suggested key role of expression of bcl-2 gene in the accumulation lymphocytes in the lasting process. Hence it follows that lymphocytes from BALF and blood from patients with acute symptoms of sarcoidosis manifest lower level of expression of bcl-2 protein. Lymphocytes from BALF and blood from all groups of patients demonstrate the expression of Fas antigen, which might be one of mechanisms of elimination of activated T cells in the periphery [15].

Other factors (e.g. bax and other proteins, cytokines) might be responsible for modulation of intensity of apoptosis. Huang et al. [8] have found protective effect of IL-2 against spontaneous programmed cell death cultured B and T cells from patients with chronic lymphocytic leukaemia. There is mounting evidence that activated

IL-2-releasing lymphocytes play a central role in the immunopathogenesis of sarcoidosis by directing inflammatory reactions and granuloma formation [5, 14]. This data suggested that control of cells survival in the lung of patients with sarcoidosis depended on many different factors and further studies are needed to confirm and generalise our observations.

REFERENCES

1. *Abastrado J.P.*: Apoptosis: function and regulation of cell death. Res. Immunol., 1996; 147: 443–456.
2. *Aiello A., Delia D., Borello M.G., Biassoni D., Giardni R., Fontanella E., Pezzela F., et al.*: Flow cytometric detection of the mitochondrial bcl-2 protein in normal and neoplastic human lymphoid cells. Cytometry, 1992; 13: 502–509.
3. *Akbar A., Salmon M.*: Cellular environments and apoptosis: tissue microenvoriments control activated T-cell death. Immunol. Today, 1997; 18: 72–71.
4. *Cheng J., Zhou T., Liu C., Shapiro J.P., Brauer M.J., Kiefer M.C., Barr P.J., Mountz J.D.*: Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. Science, 1994; 263: 1759–1762.
5. *Girgis R.E., Basha M.A., Maliarik M., Popowich J.J., Iannuzzi M.C.*: Cytokines in the bronchoalveolar lavage fluid of patients with active pulmonary sarcoidosis. Am. J. Respir. Crit. Care Med., 1995; 152: 71–75.
6. *Herry I., Bonay M., Bouchonnet F., Schuller M.P., Lecossier D., Tazi A., Lynch D.H., Hance A.J.*: Extensive apoptosis of lungs T-lymphocytes maintained in vitro. Am. J. Respir. Cell. Mol. Biol., 1996; 15: 339–347.
7. *Hole B.E., Hintzen R.Q., Van Lier R.A., Alberts A., Out T.A., Jansen H.M.*: Soluble and cellular markers of T cell activation in patients with pulmonary sarcoidosis. Am. Rev. Respir. Dis., 1993; 148: 643–649.
8. *Huang R.-W., Tsuda H., Takatsuki K.*: Interleukin-2 prevents programmed cell death in chronic lymphocytic leukaemia cells. International Journal of Haematology, 1993; 58: 83–92.
9. *Iwai K.., Miyawaki T., Takizawa T., Konno A., Ohta K., Yachie A., Seki H., Tanguchi N.*: Differential expression of bcl-2 oncoprotein and susceptibility to anti-Fas-mediated cell death in peripheral blood lymphocytes, monocytes and neutrophils. Blood, 1994; 84: 1201–1208.
10. *Ju S.T., Panka D.J., Cui H. et al.*: Fas (CD95)/FasL interactions required for programmed cell death after T-cell activation. Nature, 1995; 373: 444–448.
11. *Kieszko R., Michanr M., Milanowski J., Fąfrowicz B., Koktysz M., Roliński J., Dmoszyńska A.*: Lymphocyte phenotypes in bronchoalveolar lavage (BAL) and peripheral blood in different clinical presentations of sarcoidosis. Pneumol. Alerol. Pol., 1996; 64: 52–58.
12. *Ormerod M.G.*: The study of apoptosis cells by flow cytometry. Leukemia, 1998; 12: 1013–1025.
13. *Mukae H. et al.*: Two-colour analysis of lymphocyte subsets of bronchoalveolar lavage and peripheral blood in Japanese patients with sarcoidosis. Chest, 1994; 105: 1474.
14. *Müller Quernheim J., Pfeifer S., Kienast K., Zissel G.*: Spontaneous interleukin 2 release of bronchoalveolar lavage cells in sarcoidosis in a codetermination of prognosis. Lung, 1996; 174: 243–253.
15. *Nagata S., Golstein P.*: The Fas death factor. Science, 1995; 267: 1449–1456.
16. *Nunez G., Merlino R., Grillot T. et al.*: Bcl-2 and bcl-x regulatory switches for lymphoid death and survival. Immunol. Today, 1994; 15: 582–588.

17. *Suzuki K,. Tamura N., Iwase A., Dambara T., Kira S.*: Prognostic value of Ia+ T lymphocytes in bronchoalveolar lavage fluid in pulmonary sarcoidosis. Am. J. Resipr. Crit. Care. Med., 1996; 154: 707–712.

STRESZCZENIE

Różnice w przebiegu sarkoidozy wydają się być uwarunkowane wieloczynnikowo. W niniejszej pracy starano się udowodnić istnienie związku pomiędzy manifestacją kliniczną sarkoidozy, a występowaniem czynników regulujących apoptozę limfocytów. W różnych subpopulacjach limfocytów otrzymanych z BALF i krwi obwodowej od chorych na sarkoidozę, określono ekspresję mitochondrialnej onkoproteiny bcl-2, hamującej apoptozę i antygenu Fas (CD95, APO-1), stymulującego programowaną śmierć komórki. W tym celu posłużono się przeciwciałami monoklonalnymi i techniką cytometrii przepływowej. We wszystkich badanych grupach pacjentów stwierdzono, że limfocyty T z BALF wykazują wyższą ekspresję bcl-2 w stosunku do komórek T z krwi obwodowej. U pacjentów bezobjawowych wszystkie subpopulacje limfocytów z BALF posiadały wyższą ekspresję bcl-2 w porównaniu do komórek pobranych podczas płukania oskrzelowo-pęcherzykowego od chorych z zespołem Löfgrena i innymi objawami sarkoidozy. Dodatkowo ekspresja proteiny bcl-2 była istotnie wyższa w komórkach CD4+ niż w komórkach CD8+. Odsetek limfocytów z BALF i krwi obwodowej z antygenem Fas na powierzchni był wyższy u pacjentów bezobjawowych w stosunku do chorych z ostrymi objawami sarkoidozy. Wysoka ekspresja bcl-2 przy jednoczesnej oporności komórek na apoptozę stymulowaną antygenem Fas, wydaje się być jedną z przyczyn wydłużenia życia i akumulacji limfocytów w trakcie zapalenia pęcherzyków płucnych w przebiegu sarkoidozy.