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Markers of activation can help distinguish Extrinsic Allergic Alveolitis from stage III sarcoidosis

Znaczenie markerów aktywacji w różnicowaniu Alergicznego Zespolenia Pęcherzyków Płucnych i sarkoidozy w III stopniu radiologicznym

INTRODUCTION

Sarcoidosis is a systemic disorder of unknown cause that is characterized by its pathological hallmark, the noncaseating granuloma. Its presenting features are protean, ranging from asymptomatic but abnormal findings on chest radiography in many patients to progressive multiorgan failure in an unfortunate minority. The illness can be self-limited or chronic, with episodic recrudescence and remissions. Because the lungs and thoracic lymph nodes are almost always involved, most patients report acute or insidious respiratory problems, variably accompanied by symptoms affecting the skin, eyes, or other organs. Especially intense immunological response is characteristic to acute sarcoidosis (Löfgren syndrom) with good perspectives in recovery while chronic sarcoidosis with constant antigen stimulation requires intensive corticosteroid therapy. The diverse manifestations of this disorder help fuel the prevailing hypothesis that sarcoidosis has more than one cause, each of which may promote a different pattern of illness [5].

From the pathogenesis point of view sarcoidosis is a disease characterized by the accumulation of activated T cells and macrophages at sites of disease activity. T lymphocytes are stimulated by unknown antigen or antigens which are presented by Antigen Presenting Cells, mainly macrophages and dendritic cells. Expresion of co-stimulating molecules e.g. CD28 and VLA-1 on lymphocytes surface is necessary to their cooperation. Apart from these co-stimulating molecules on the cell surface appear activation markers. Activation of alveolar T lymphocytes can be deduced from an

increased expression of activation markers on their surface [2]. These immunological events lead to the typical granuloma formation, characteristic of type IV hypersensitivity.

The intensity of the immunological processes in sarcoidosis differs in patients which results in different clinical manifestations. The quantification of T cell activation in the lungs of patients is of interest for obtaining insight in the pathogenesis, development, and prognosis of the disease.

Extrinsic Allergic Alveolitis (*EAA*) presents as a clinical syndrome of cough and dyspnoea in some individuals who are exposed to organic dusts, chemicals or drugs and is an immunological, inflammatory response involving alveoli, bronchioles and interstitium. The inciting antigens reach alveoli by inhalation, e.g. organic dusts, but may also do so by bloodstream, e.g. drugs [1]. EAA is an important disease since it may result in acute or chronic illness. In patients with chronic EAA, unless the diagnosis is established and exposure to the antigen terminated, progressive irreversible lung disease may occur with fibrosis and pulmonary insufficiency [3]. Cells involved in inflammation of the alveolar and interstitial structures include neutrophils, mast cells, lymphocytes and monocyte-macrophages. Lung involvement in EAA is commonly characterized by lymphocytic alveolitis, characterized by excess numbers of CD8+ lymphocytes. The CD8 T-cells count recovers to normal levels in patients who are not further exposed to specific antigens that cause EAA [8].

Some cases of III X-ray stage lung sarcoidosis are clinically difficult to distinguish from Extrinsic Allergic Alveolitis — in both of the diseases there are possible diffuse bilateral reticular infiltrates. III X-ray stage lung sarcoidosis may be the first one to be accidentally diagnosed and evaluation of possible exposures to antigens known to cause EAA may be very difficult especially if there is no employment or hobbies with exposures to antigens. Therefore the aim of the study was to assess value of activation markers in order to help distinguish some doubtful cases of sarcoidosis from EAA.

MATERIALS AND METHODS

Patients. The study population consisted of 22 patients with sarcoidosis III X-ray stage (mean age 40±10 years), 16 patients with Extrinsic Allergic Alveolitis (mean age 47±9 years) and 13 normal subjects (mean age 43±13 years). Members of the control group had no previous history of pulmonary diseases. The diagnosis of pulmonary sarcoidosis was based on clinical presentation, X-ray abnormalities and histopathological analysis. The diagnosis of Extrinsic Allergic Alveolitis was based upon exposure history, clinical assessment, radiographic and physiologic findings, removal of the patient from the suspected etiologic exposure and inhalation challenge to the suspected antigen in a hospital setting.

We analysed the following pairs of groups: patients with sarcoidosis III X-ray stage vs patients with Extrinsic Allergic Alveolitis, normal subjects vs patients with Extrinsic Allergic Alveolitis and normal subjects vs patients with sarcoidosis III X-ray stage.

Bronchoalveolar lavage fluid (BALF). The patients were pre-medicated intramuscularly with atropine (0.5 mg). After local anaesthesia with 4% lignocaine a flexible fibreoptic bronchoscope (Olympus BF-20) was wedged into a middle lobe bronchus and 140 ml sterile physiological saline solution at body temperature was instilled in seven aliquots of 20 ml. The first aliquot recovered was 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 degrees C and resuspended in phosphate buffered saline (PBS) for future staining. Cells were counted in Bürker chamber.

Preparation of cells from peripheral blood. Mononuclear cells were isolated by density gradient centrifugation on Lymphoprep (Nycomed, Norway). Interphase cells were removed, washed twice in PBS and than resuspended for future immunostaining.

Monoclonal antibodies. Double colour immunofluorescence studies were performed using combinations of phycoerythrin (PE) and fluoroscein isothyocyanate (FITC) conjugated monoclonal antibodies. Fluorescein isothiocyanate (FITC) conjugated CD4, CD8, CD 25, CD 45RO, CD 69, anti-human lymphocyte antigen-DR (HLA-DR) antibodies, and phycoerythrin (PE) conjugated anti-CD3, CD4 and CD8 antibodies. Monoclonal antibodies were obtained from Ortho Diagnostic Systems (Germany), Becton Dickinson (Germany), Serotec (UK) or Dako (Denmark). 1 x 10^6 cells were incubated with antibodies for 30 min. at 4 degrees C and washed twice with PBS afterwards.

Flow cytometric analysis. All samples were measured on a Cytoron Absolute flow cytometer (Ortho Diagnostic Systems). To determine the fluorescence intensity of stained cells, the logarithmic fluorescence channel was converted to arbitrary units based on the ImmunoCount 2.0 Software.

Statistical analysis. Mann–Whitney U test was applied for the statistical comparison, the $p<0.05$ was considered significant. Kruskal-Wallis test was applied to figures.

RESULTS

Sarcoidosis vs Extrinsic Allergic Alveolitis

In BAL fluid the percentages of CD3+, CD4+, CD4+/CD8+ ratio (Fig. 1), HLA-DR+3+ (Fig. 3) and CD4+45RO+ cells were significantly higher in patients with sarcoidosis than in patients with EAA. In patients with EAA the percentages of CD8+ cells and macrophages in BAL fluid were significantly higher than in sarcoidosis. (Tab. I and II).

Control group vs Extrinsic Allergic Alveolitis

In BAL fluid the percentages of CD19+ was significantly higher and CD4+45RO+ cells nearly significant ($p=0.0596$) in patients from the control group than in patients with EAA (Tab. I and II).

Control group vs Sarcoidosis

In BAL fluid the percentages of CD8+, CD19+ cells and macrophages were significantly higher and CD25+ cells nearly significant ($p=0.0618$) in patients with sarcoidosis than in patients from the control group.

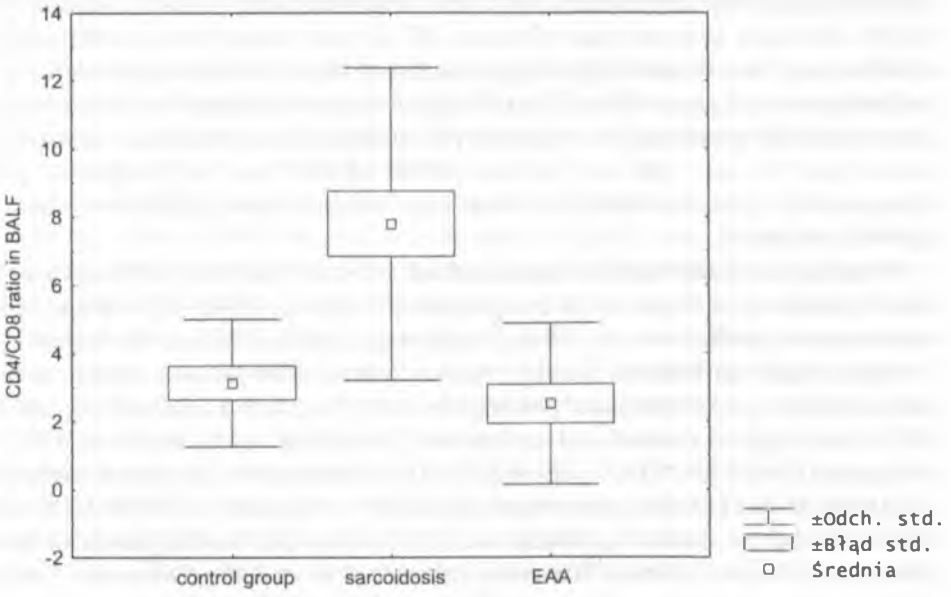

Fig.1. Comparison of CD4/CD8 ratio in BALF from the control group, patients with sarcoidosis and EAA

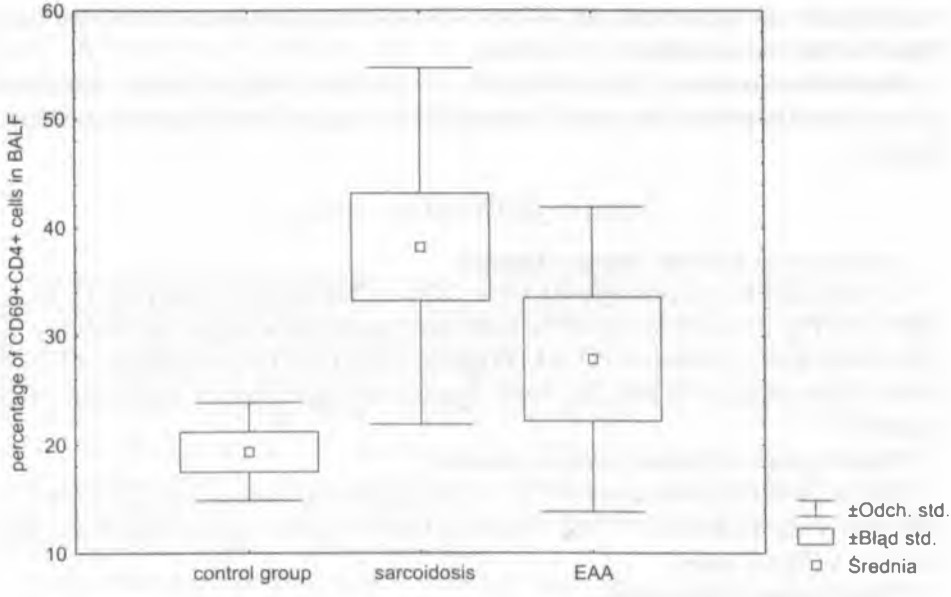

Fig.2. Comparison of percentages of CD69+CD4+ cells in BALF from the control group, patients with sarcoidosis and EAA

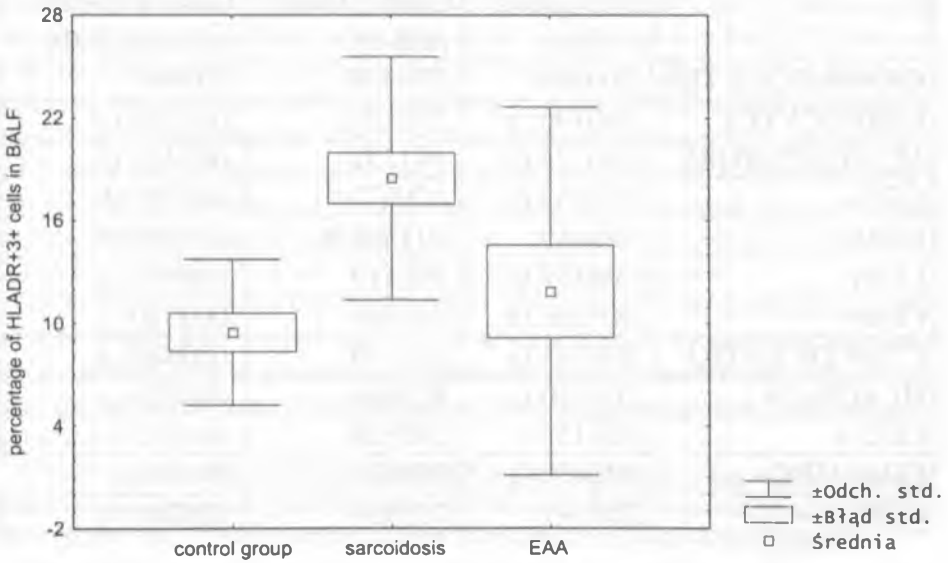

Fig.3. Comparison of percentages of HLA-DR+CD3+ cells in BALF from the control group, patients with sarcoidosis and EAA

Table I. Results describing cells in BALF

	Control group	Sarcoidosis	EAA
GRANULOCYTES	3,7±5,1	2,6±2,1	6,1±6,4
LIMFOCYTES	31,2±19,9	55,7±18,7	28,2±21,1
MACROPHAGES	59,7±22,8	35,0±17,6	56,7±25,8
CD3+	83,1±5,6	87,7±6,1	81,7±8,7
CD19+	3,2±2,7	1,6±1,3	1,5±0,8
CD8+	26,9±15,5	13,8±8,5	33,3±20,7
CD4+	58,0±15,1	74,1±9,9	48,4±20,7
CD4/CD8 RATIO	3,1±1,9	7,8±4,6	2,5±2,4
HLADR+3+	9,5±4,2	18,5±7,1	11,9±10,8
CD25+	7,8±3,6	5,9±2,7	6,2±3,0
CD4+45RO+	33,0±18,8	41,8±20,0	19,6±19,4
CD69+4+	19,3±4,5	38,3±16,4	27,9±14,1

Table II. Values of p in Mann–Whitney U test in BALF

	Sarcoidosis vs EAA	Control Group vs EAA	Control Group vs Sarcoidosis
GRANULOCYTES	,200906	,279439	,745494
LIMFOCYTES	,000196 (a	,570107	,001246 (a
MACROPHAGES	,006324 (b	,891131	,001413 (a
CD3+	,007558 (b	,829677	,006254 (b
CD19+	,645489	,011568 (b	,005058 (b
CD8+	,000132 (a	,262117	,000964 (a
CD4+	,000010 (a	,149269	,000370 (a
CD4/CD8 RATIO	,000043 (a	,186379	,000846 (a
HLADR+3+	,001430 (a	,853826	,000204 (a
CD25+	,626153	,245529	,061833
CD4+45RO+	,022150 (b	,059676	,247091
CD69+4+	,256141	,179654	,020200 (b

In case of patients with sarcoidosis the percentages of CD3+, CD4+, CD4+/CD8+ ratio (Fig. 1), HLA-DR+3+ (Fig. 3), CD69+4+ (Fig. 2), cells and lymphocytes in BAL fluid were significantly higher than in the control group (Tab. I and II).

In peripheral blood from patients with sarcoidosis and EAA we have not found any significant changes vs. the control group. This part of the studies was made in order to assess the immunological compartmentalization of alveolitis.

DISCUSSION

The pathogenesis of sarcoidosis is closely associated with a predominance of activated T helper lymphocytes, especially in the early stage of the disease, and this is reflected by an increased CD4/CD8 ratio. However, patients with longer duration of disease may also have an increase in the numbers of CD8 cells in BALF [5].

Lung involvement in EAA is commonly characterized by lymphocytic alveolitis with an increase in both percentage and absolute number of CD8+ T cells in BALF. The CD8 T-cell count recovers to normal levels in individuals who are not further exposed to specific antigens that cause EAA [8].

Our studies have confirmed the literature data concerning the major immunological markers in sarcoidosis and EAA.

Previous studies demonstrated presence of persistently activated lymphocytes in the lung compartment by an unknown antigen or antigens. The activation markers can be classified as very early (CD69), early (CD25), late (HLA-DR) and very late, depending on their expression in time after activation [2]. Significantly higher percentage of HLA-DR+CD3+ and CD69+CD4+cells in sarcoidosis vs the reference

group in BALF in our researches seems to confirm this theory. Therefore these markers of activation, from clinical point of view, can be very useful in the diagnostic process. Wahlstrom and coworkers pointed at high percentage of CD69 cells [9], whereas Iida and coworkers obtained high percentage of HLA-DR+CD3+ in BALF [3]. Hol and co-workers had similar results according to HLA-DR and CD69 markers. They also pointed at low levels of sCD25 in the unconcentrated BALF below detection level [2]. In our studies percentage of CD25+ cells in sarcoidosis was nearly significantly higher ($p=0.0618$) than in the reference group. Furthermore, Iida and co-workers showed that CD4+ CD29+ memory T cells were significantly increased in patients with sarcoidosis than in normal subjects [3]. Prasse and coworkers obtained an increased expressions of both CD25 and HLA-DR on BALF lymphocytes [7].

α-chain CD 25 is a part of IL-2 receptor. Sarcoidosis shows predominantly Th1 cytokine profile with the key role of IL-2 as an inflammatory mediator. Increased percentage of CD 69 as an early stage activation marker (present on the cells 4 hours after the exposition) and HLA-DR (a later one), suggests constant immunological stimulation.

Percentage of markers of activation in EAA vs the reference group in BALF showed no statistical significance, yet there are significances in EAA vs sarcoidosis: both HLA-DR+CD3+ and CD4+CD45RO+ cells have higher expression in sarcoidosis.

Sarcoidosis is an chronic disease, whereas EAA is rather an acute one where immunological markers recover to normal value without further exposition to the antigens. This results in constant presence of activation markers on the surface of lymphocytes in stage III sarcoidosis and their absence in EAA. In conclusion, our data reveal an interesting diagnostic suggestion which may help distinguish the diseases.

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STRESZCZENIE

W niniejszej pracy postanowiono ocenić przydatność markerów aktywacji w diagnostyce różnicowej Allergicznego Zapalenia Pęcherzyków Płucnych (AZPP) oraz sarkoidozy w III stopniu radiologicznym. III radiologiczny stopień sarkoidozy może być pierwszym wykrytym stadium choroby, zwłaszcza jeśli przebiega skąpo- lub bezobjawowo. Diagnostyka części przypadków AZPP również napotyka na szereg trudności, zwłaszcza gdy nie można ustalić ewentualnego narażenia na powszechnie uznane czynniki wywołujące AZPP. W obydwu przypadkach możemy mieć podobny obraz radiologiczny. U pacjentów z rozpoznanym EAA, III stopniem sarkoidozy oraz w grupie kontrolnej oznaczano markery aktywacji: CD69, CD25 i HLA-DR zarówno na limfocytach uzyskanych z popłuczyn oskrzelikowo-pęcherzykowych (BALF) jak i w krwi obwodowej. W BALF w sarkoidozie stwierdzono istotnie statystycznie wyższe odsetki CD69+ i HLA-DR w porównaniu z grupą kontrolną oraz istotnie statystycznie wyższe odsetki CD45RO+ i HLA-DR w porównaniu z AZPP. Powyższe wyniki mogą mieć duże znaczenie w diagnostyce różnicowej trudnych klinicznie przypadków AZPP i sarkoidozy.