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Local CD4+, CD8+ and CD56+ T-lymphocite Reaction on Primary Lung Cancer

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Original paper
SUMMARY
Objective: The primary goal of this study was to determine the difference of abundance of CD4+, CD8+ and CD56+ bronchoalveolar fluid's lymphocytes and their subpopulations between cancerous lung and healthy lung from the same patient. **Methods:** Mini-bronchoalveolar lavage was taken from 55 patients from lung with cancer and healthy lung. After laboratory processing and addition of CD4, CD8, CD27, CD28 and CD56 antibody, the material was analyzed by flow cytometer. Results from lung with cancer were compared to the ones from the healthy lung. The examined patients were the test and the control group at the same time. **Results:** CD27+28+ forms of CD4+ and CD8+ lymphocytes are more activated in the cancerous lung compared to healthy lung, while the CD27-28- forms are less activated in diseased lung. CD4+ forms of CD56+ lymphocytes are more activated in cancerous lung compared to the health lung, while the CD8+ forms are less activated in diseased lung. **Conclusion** Immature helper and cytotoxic T lymphocyte response, as well as regulatory NK and NKT cell response are more activated in cancerous lung compared to the health lung of the same patient.
Key words: Bronchoalveolar lavage, CD4+ lymphocytes, CD8+ lymphocytes, CD56+ lymphocytes, Lung cancer.

1. INTRODUCTION

The primary goal of this study was to determine the difference of abundance of CD4+, CD8+ and CD56+ bronchoalveolar fluid's lymphocytes and their subpopulations between cancerous lung and healthy lung from the same patient. The hypothesis of this work was that T cells immunological reaction on lung cancer was very different form the one in healthy lung in the same patient.

At the start of the 21 century the lung cancer has become one of the leading causes of death among preventable diseases (1, 2). There are several different types of antigens on the tumorous cells such as (NY-ESO-1, WT1 antigen, MRP3, MAGE and BAGE family, gp 100, SART-1, tirozinaze, MUC-1, etc) (3, 4). These antigens are believed to be the major factors in the activation of numerous immune reactions to the tumor, including T-lymphocyte reaction that believed to have a central role. T-lymphocytes are a numerous, but heterogeneous population of the entire number of effector cells. (5, 6). CD4+ T lymphocytes posses a helper function and are activated by antigen presenting cells. These lymphocytes show a significant immunoregulatory activity (7). CD8+ T lymphocytes are effector cells of the immune system and possess cytolytic and cytotoxic function. (6, 8). Currently there are only limited number of studies that address the phenotypic classification of human CD4+ and CD8+ T lymphocytes. On the other hand the path of their differentiation is quite well established. We know that CD27+28+ subpopulations are immature memory cells, and CD27-CD28- subtype are mature forms of CD4+ and CD8+ T lymphocytes (6, 8, 9). NK cells are lymphocytes with particularly cytolytic activity, capable of spontaneously destroying tumor cells and even metastatic tumor cells in the systemic circulation (10). NKT cells represent 0,2% of all T-cells in the peripheral blood, and are characterized by swift and abundant secretion of cytokines (IL-2, IFNγ, TNFα and IL-4) (11, 12). NK and NKT cells are of CD56+ type. All CD56+ cells are further classified to CD4+ and CD8+, each one having a different biological base (10, 11, 12).

2. MATERIALS AND METHODS

The study was cross sectional. All laboratory analise were completely done in The Department of Clinical Immunology and Molecular Genetics at University Clinic of Respiratory and Allergic Diseases Golnik, Slovenia. It included 55 patients in study on CD4+ and CD8+ lympho-

cyte, and 46 patients in study on CD56+ lymphocyte. Inclusion criteria was the pathohistological confirmed diagnose of primary lung cancer. Exclusion criteria were: previous chemotherapy, radiotherapy or other anti-cancer therapy; absence of lymphocytes in the cytological analyses of material; non-representative number of events in flow-cytometric analyses of material (lower than 80 for lymphocytes in cancerous lung and lower than 70 in healthy lung).

All patients had a mini bronhoalveolar lavage (mini-BAL)[1] taken during a routine diagnostic flexible bronchoscope. During the procedure 20 ml of 0,9% NaCl solution was instilled. Mini-BAL was taken from the cancerous lung and from the healthy lung of the same patient. The material obtained with the mini BAL was centrifuged at 1600 rpm, the supernatant was poured out, and 0,5ml of azid Hemacel-a solution was added followed by 1 ml of solution for deep freezing. The material was frozen at -70 °C preserved until further processing.

Flow cytometer was used to count the lymphocytes in the 100 µl samples. The number of lymphocytes and the percentage of T-lymphocyte subpopulations in the mini-BAL was determined, including the immature (CD27+28+) mature (CD27-28-) forms of CD4+ and CD8+ T lymphocytes, also analysis included the regulatory (CD4+) and activated (CD8+) NK and NKT cells. Proportions of lymphocyte subpopulations between the cancerous and the healthy lung of the same patient were compared.

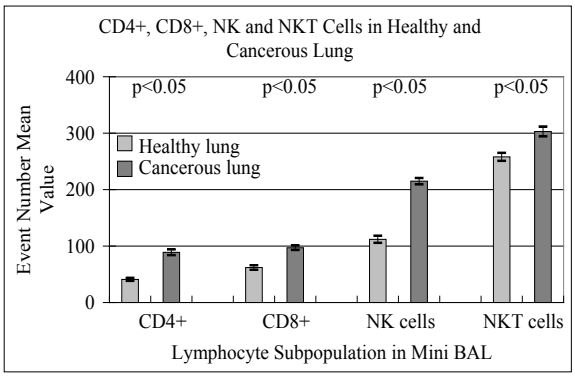
Values of T-lymphocyte miniBAL parameters in the healthy lung were designated as reference values, so that the non cancerous influences in the BAL results were excluded. Mini-BAL values of different cellular subpopulations in the healthy lung were divided in three classes, for every cellular subpopulation:

First class, low percentage class (more and equal 0% and less and equal 33%);

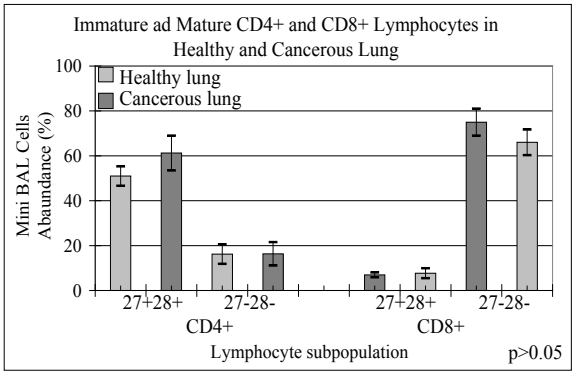
Second class moderate percente class (more than 33% and less and equal 66%); Third class, high percentage class (more than 66% and less and equal 100%).

Finally average percentage of different classes of lymphocyte subpopulations in mini-BAL of the healthy lung, and average percentage of the same classes of T-lymphocyte subpopulations of the cancerous lung in the same patient were compared.

Material obtained using mini-BAL was stained with monoclonal antibodies and prepared for flow cytometry using the procedural technique described by the manufacturer. Following antibodies were used: CD4-FITC: BD-2010; CD8-APC: BD-2010; CD27-PE: BD Pharmigen-2014, CD28-Cy-Chrome: BD Pharmigen-2010 and CD56-PE: IQ Products 2010. The prepared material was then processed by the flow cytometer FACS Calibur basic G4, manufactured in 2001. by Becton Dickinson. Following parameters were used: FSC-H/SSC-H for lymphocytes, FL4-H/SSC-H for CD4+ lymphocytes and FL1-H/SSC for CD8+ lymphocytes, FL2-H/FL3-H for CD27/CD28, FL3-H/FL1-H for CD3/CD8, FL3/FL4-H for CD3/CD4, FL1-H/FL2-H for CD8/CD56 and FL4-H/FL2-H for analyzing CD4/CD56. Nonparametric and parametric methods were used for calculating statistical significance. Mann-Whitney test, Student's t-test, χ^2 test and Fisher test were used to calculate differences between groups. Statistical hypotheses were tested with level of α=0.05, in other words difference between groups in the sample was considered significant if p<0.05. All the data was analyzed using SPSS statistical program (Version 10 SPSS Inc, Chicago, IL).



Graph 1. The event mean value of CD4+, CD8+, NK and NKT lymphocytes in the lung with primary cancer and in the healthy lung of the same patients



Graph 2. The average percentage of immature and mature forms both CD4+ and CD8+ T lymphocyte subpopulations in BAL from the cancerous and healthy lung of the same patient

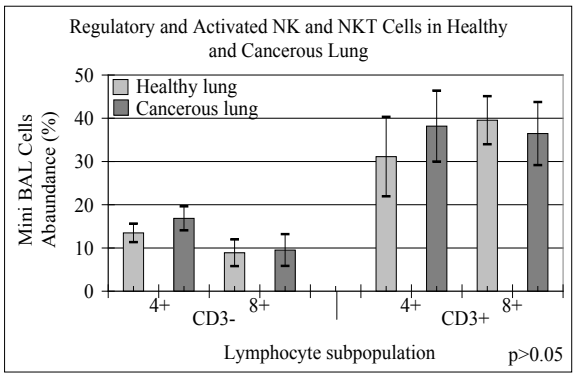
The study was undetaken with the agreement of Republic Ethical Commitee in Republic of Slovenia.

3. RESULTS

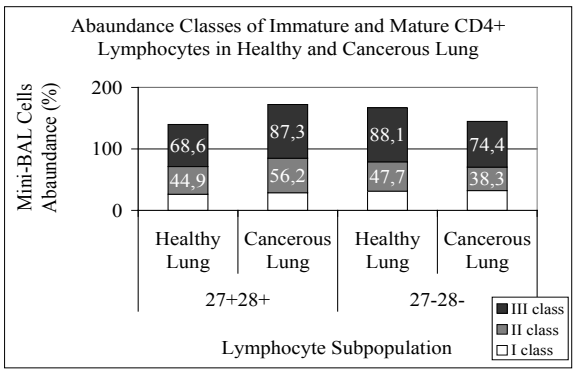
We included 40 men aged 66 ± 8.58 years and 15 women aged 74.47 ± 5.12 years Men were significantly more numerous compared to women (p<0.05). Also, male patients were significantly younger than female (p<0.05).

The highest incidence was for squamous cell carcinoma (49.09%) and adenocarcinoma of the lung (25.45%). Non small lung cancer incidence was 85.45%. Significantly (p<0.05) most common type of lung cancer in males was squamous cell carcinoma.

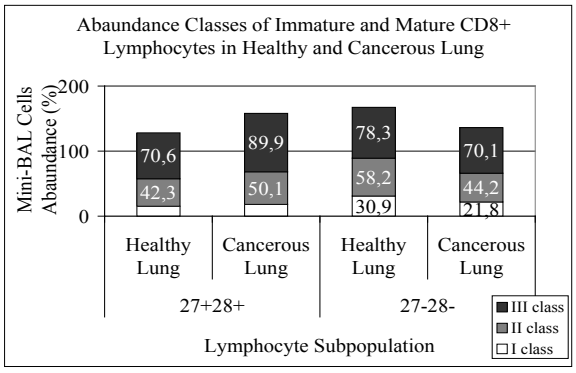
For women the most common types of lung cancer was adenocarcinoma and squamous cell carcinoma, but there were no statistically significant differences (p>0.05) in the frequency of these two types of lung cancer in women. The frequency of lung adenocarcinoma was significantly



Graph 3. The average percentage of regulatory and activated NK and NKT lymphocyte subpopulations in BAL from the cancerous and healthy lung of the same patients.



Graph 4. The average proportional representation of immature forms and CD4+ T-lymphocyte subpopulations in regard to low, moderate and classes with high lymphocyte abundance.



Graph 5. The average proportional representation of immature forms of CD8+ T-lymphocyte subpopulations in regard to low, moderate and classes with high lymphocyte abundance.

higher in women than in men suffering from lung cancer ($p<0.05$).

Most patient were in IV, and then in III clinical stage (III and IV stage – 74.54% of patients) ($p<0.05$).

The event mean value of CD4+, CD8+, NK and NKT lymphocytes in the lung with primary cancer were significantly higher ($p<0.05$) than the event mean values of corresponding T-lymphocytes in the healthy lung of the same patients (graph 1). The average percentage of immature and mature forms both CD4+ and CD8+ T lymphocyte subpopulations in BAL from the cancerous and healthy lung of the same patient did not differ significantly ($p> 0.05$) (Graph 2)., and also the average percentage of regulatory and activated NK and NKT lymphocyte subpopulations in BAL from the cancerous and healthy lung of the same patients did not differ significantly ($p> 0.05$) (Graph 3).

The average proportional representation of immature forms and CD4+ T-lymphocyte subpopulations were significantly higher ($p<0.05$) in diseased lungs only for grades of moderate and high prevalence, while for the same classes, the representation of mature forms and CD4+ T-lymphocyte subpopulations were significantly higher ($p<0.05$) in healthy lungs (graph 4).

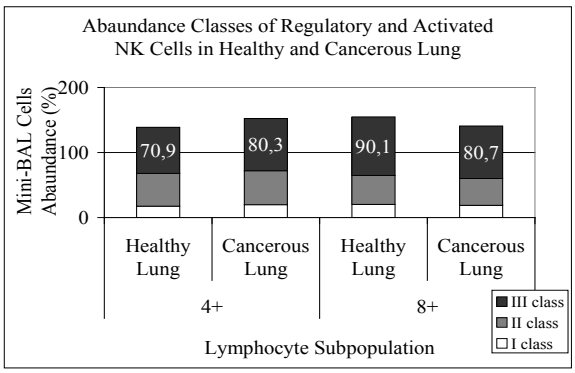
The average proportional representation of immature forms of CD8+ T-lymphocyte subpopulations were significantly higher ($p<0.05$) in diseased lungs only for grades of moderate and high prevalence. The representation of mature forms of CD8+ T-lymphocyte subpopulations were significantly higher ($p<0.05$) in healthy lungs for all classes of representation (Graph 5).

The average proportional representation of regulatory forms of NK cells was significantly higher ($p<0.05$) in diseased lungs only for high-class representation, while the prevalence of activated forms of NK cells was significantly higher ($p<0.05$) in healthy lungs, also only for high-class representation (Graph 6). The average proportional representation of CD4+ forms of NKT cells was significantly higher ($p<0.05$) in diseased lungs only for moderate grade and high-grade classes of representation, and the presence of CD8+ forms of NKT cells was significantly higher ($p<0.05$) in healthy lungs, also for classes of moderate and high representation (Graph 7).

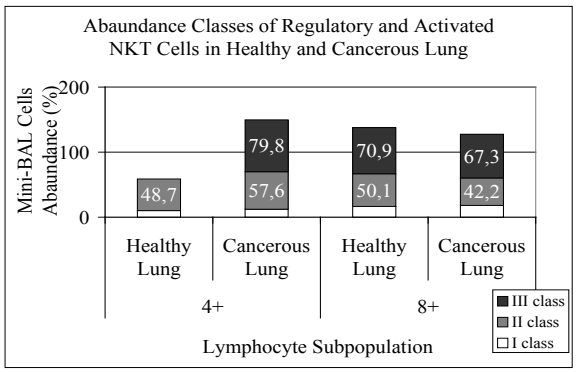
In Table 1. we can see the numbers of subjects enlisted in the individual parts of studies on CD4+, CD8+, NK and NKT cells.

4. DISCUSSION

Men are still significantly more likely to suffer from lung cancer than women (2). In our study, the ratio of patients was around 2,67:1 in favour of males, which corresponds to most literature data. In literature, the mean age of patients with lung cancer is about 60 years (2). In our study, the mean age was 68.31 ± 8.59 years. The mean age of women suffering from lung cancer in the world is slightly higher than those in men, but in the last decade there is a trend of continuous reduction in average age of women. (2, 13). In our study, average age of men and women also differ significantly, and the men were on average about 5 years younger than women. In Europe, the most common lung cancer is adenocarcinoma, followed in frequency with squamous cell carcinoma (13). In this study their



Graph 6. shows the average proportional representation of activated and regulatory forms of NK cells in regard to low, moderate and classes with high lymphocyte abundance



Graph 7. The average proportional representation of activated and regulatory forms of NKT cells in regard to low, moderate and classes with high lymphocyte abundance

representation is still reverse. Traditionally the most common pathohistological type of lung cancer was squamous cell cancer. But currently, the implementation of rigorous expressed antismoking campaigns has produced a sudden increase in adenocarcinoma of the lung, especially in women. This increase is attributed to the reduction in incidence of squamous cell carcinoma, because this type of lung cancer has the strongest relationship with tobacco smoke (2, 14). Current global and European statistical analyses show that currently, at the time of diagnosis, approximately 50% of patients with lung cancer are in the III or IV clinical stage (14). Similar results were obtained, and in our study.

In this study, we wanted to examine the composition and the extent of T-immunological events in the lung affected with cancer compared to healthy lung of the same patients. It was showed that in lung cancer a local immune response is characterized by immature helper and cytotoxic T lymphocytes and regulatory NK and NKT cells.

Today, many studies indicate the existence of a correlation between the number of tumor-infiltrating lymphocytes in the analyzed tissue with the histological grade of the lung disease, tumor size, vascular invasion and survival (1, 15). In our study, it was observed that the number of CD4+ and CD8+ T-lymphocytes in lung with primary cancer was significantly higher than in healthy lungs of the same patients. From this we can conclude that the helper and cytotoxic component of the local T-lymphocyte response to the lung cancer is markedly stimulated. The results correspond to the majority of literature data (16, 17, 18, 19), and can be explained by the fact that the healthy tissue in most cases corresponds to the cancerous process within it, by mobilizing virtually all the components of the immune system. Local immune response in lung cancer arises due to postopstructional pneumonitis, but it is also activated in order to destroy the tumor, and limit its development. Chen et al. in 2005 analyzed the cellular composition of aspirated pleural fluid of malignant etiology, pleural fluid of non-malignant etiology and leukocyte composition of peripheral blood in both groups. Their study showed that there were increased numbers of regulatory CD4+CD25+ T cells in malignant pleural effusion from patients with lung cancer compared with pleural lavage from patients with lung cancer without pleural effusion, and that these cells had constitutive high-level expression of Foxp3 and cytotoxic lymphocyte-associated antigen-4. CD4+CD25+ T cells inhibited the proliferative response of CD4+CD25- T cells. Anticytotoxic lymphocyte-associated antigen-4 monoclonal antibody reduced the inhibitory activity of CD4+CD25+ T cells.

Since differences in the ratio of mature and immature forms and CD4+ and CD8+ T-lymphocyte subpopulations in the site of the primary lung carcinoma and healthy lung of the same patients were not statistically significant, we searched for differences in subpopulations of patients with more pronounced inflammatory reaction. Therefore we have divided the proportional representation of mature and immature forms and CD4+ and CD8+ T-lymphocytes, into 3 classes as described in the Materials and methods. A similar division can be found with histopathological quantification of the expression of hormone receptors in breast cancer (20). The authors of this study have divided the intensity of expression of receptors into 4 classes, while in our study due to relatively small total number of subjects involved in the study; we have used 3 classes of proportional representation of lymphocyte populations. By forming the classes of proportional representation of T-lymphocyte subpopulations, the greatest importance, was given for the results in the class of moderate and high representation.

By comparing the average percentage of mature and immature subpopulations and CD4+ and

	Class of low representation	Class of mild representation	Class of high representation	Total
CD4+27+28+	17	24	14	55
CD4+27-28-	30	17	8	55
CD8+27+28+	42	11	2	55
CD8+27-28-	4	24	27	55
CD3-4+56+	21	22	3	46
CD3-8+ 56+	27	16	3	46
CD3+4+56+	24	21	1	46
CD3+8+56+	9	32	5	46

Table 1. lists the numbers of subjects enlisted in the individual parts of studies on CD4+, CD8+, NK and NKT cells

CD8+ T-lymphocytes of healthy and diseased lungs, we can see that the percentage of mature forms is substantially higher in healthy lungs, and that percentage of immature forms is much higher in the lung with primary cancer. The host organism is trying to resist the rapid changes in genotype and phenotype markings of tumor cells, with accelerated activation of the immune response. Because the first line of defence from malignancy is comprised from mostly immature T cells, they will be the ones to proliferate in the first phase of tumor tissue formation (5). However, the duplication time of induced lymphocytes is substantially longer than the same period of malignant cells, especially in poorly differentiated and undifferentiated carcinoma. In other words, the immature cells proliferate at lower rates than the speed of clonall expansion of tumor tissue (5). Furthermore, the high rate of cancer cell division is not followed by the adequate speed of mature lymphocyte formation, capable of recognizing end lysing the tumor cells. For effector CD4+ or CD8+ T-cells to achieve their anti-tumor effect, they first must be specifically sensitized. Lung cancer is rapidly evolves, and uses different mechanisms to avoid the anti-tumor response, and does not produce specific reactions in the tissue in which it is found. Therefore, in the vicinity of tumor, and in the tumor tissue itself, almost always there is an increased number of lymphocytes, but in their immature, effector insufficient form.

In the making of this study there were several limitations:

* During the presentation of mini-BAL volume obtained by BAL was not measured, and the cells were concentrated only in the volume of one specimen (test tube). So the samples of cells concentrated in 1ml from different amounts of BAL were compared. However, if we assume that the sample of 55 patients is large enough, and that these errors are equally distributed in samples of healthy and diseased lungs, then the results of this study may be accepted while consciously taking into account certain level of relative error.

* This study did not take into account the level of influence that postopstructional pneumonitis had on local immune response.

* There is a substantial possibility of systemic effects of tumor on the diseased lung. But if we assume that the systemic effects bring the same changes to the healthy lung, then we could compare them and attribute the difference to the local reaction to malignancy.

* The study included a small and heterogeneous sample of respondents in terms of multiple histological types of lung cancer, various degrees of pathohistological differentiation and various clinical stages of disease.

* This study did not analyze the function of lymphocytes, but only their morphology. In spite of these limitations, this study, which is primarily of basic nature, suggests a new angle on the cancerous process of the lungs and pose a realistic basis for further research in this direction.

5. CONCLUSION

At the site of primary lung cancer local immune response was markedly more stimulated on the account of immature forms of T helper and cytotoxic lymphocytes and regulatory forms of NK and NKT cells.

ABBREVIATIONS

[1] *BAL* – Bronchoalveolar lavage; miniBAL – done usually in experimental studies (installing of 20 ml 0,9% NaCl solution)

[1] *event* – number of lymphocytes in 1 µl material analysed in flow-cytometer

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