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IMMUNE SYSTEM ALTERATIONS IN LUNG CANCER PATIENTS

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The immune system plays an important role in the defense against neoplastic disease and immune responses show temporal changes related to circadian variations of antibodies, total lymphocytes in the peripheral blood and cell mediated immune responses. In this study we evaluate. lymphocyte subpopulations and interleukin-2 (IL-2) serum levels in peripheral blood samples collected at four-hour intervals for 24-hours starting at 06.00h from ten healthy subjects aged 65-79 years (mean age ± S.E. 67.28 ± 3.11) and from ten subjects suffering from untreated non small cell lung cancer aged 65-78 years (mean age ± S.E. 68.57 ± 1.81). Areas under the curve, mean diurnal levels (mean of *06.00-10.00-14.00 h*) and mean nocturnal levels (mean of *18.00-22.00-02.00 h*) were calculated, and the presence of circadian rhythmicity was evaluate. When we compared AUC values there was a decrease in $CD8^{bright}$ (T suppressor subset) and an increase in CD16 (natural killer cells) and of IL-2 serum levels in cancer patients. When we compared mean diurnal levels, CD8 (T suppressor/cytotoxic subset) and $CD8^{bright}$ levels were lower, and CD16 levels were higher in cancer patients. When we compared mean nocturnal levels, CD16 and CD25 (T and B activated lymphocytes with expression of the a chain of IL-2 receptor) levels were higher, while CD8, $CD8^{bright}$, CD20 (total B-cells), TcRd1 (epitope of the constant domain of d chain of T-cell receptor 1) and dTcS1 (epitope of the variable domain of d chain of T-cell receptor1) levels were lower in cancer patients. A clear circadian rhythm was validated for the time-qualified changes in CD4, CD20, HLA-DR with acrophase at night, and CD8, $CD8^{bright}$, $CD8^{dim}$, CD16, TcRd1 and dTcS1 with acrophase in the morning in the control group. A clear circadian rhythm was validated for the time-qualified changes in CD4 with acrophase at night, in the group of cancer patients. Results obtained in our study show that lung cancer is associated with anomalies of proportion and circadian variations of lymphocyte subsets that must be considered when adoptive immunotherapy has to be planned.

Immune response is important in the natural history of neoplastic disease and lymphocytes are an essential component of this biological host reaction. Peripheral blood lymphocytes show that circadian variations of specific subpopulations (1-10), and abnormalities in the proportions of various lymphocyte subsets have been found in a number of tumors (11-13). Strategies to enhance immunological response, above all cellular immunity, in oncologic patients, rely on biological response modifiers and adoptive immunotherapy. It is important to identify immunological alterations in cancer patients in order to evaluate immunomodulatory effects. The aim of this study is to evaluate alterations in the immune system function expressed as modifications in the 24-hour pattern of IL-2 and lymphocyte subsets changes in patients suffering from lung cancer.

Key words: circadian rhythm, lymphocyte subpopulations, interleukin 2, immune response, lung cancer

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MATERIALS AND METHODS

The study was approved by the local Ethical and Scientific Committee. After obtaining of informed consent, peripheral blood samples were collected at four-hours intervals for 24 hours from ten healthy subjects aged 65-79 years (mean age±s.e. 67.28±3.11) and from ten subjects suffering from untreated non small cell lung cancer aged 65-78 years (mean age±s.e. 68.57±1.81). All subjects were studied in our department and were submitted to the same social routine (light/dark cycle and mealtimes). The extent of the tumor was evaluated by clinical examination, bronchoscopy, computed tomography (CT) of the brain, chest, and upper abdomen, and ultrasonography of the liver. Tumor cell type was determined by biopsy (5 non small cell lung cancer I-II stage and 5 non small cell lung cancer III-IV stage). An indwelling catheter, which kept patient with a slow infusion of 0.9% NaCl, was inserted in an antecubital vein, and the blood samples were drawn at 4-hour intervals for 24 hours starting at 06.00h. In each blood sample we measured serum IL-2 level, and we analyzed lymphocyte subpopulations (CD2, CD4, CD8, CD8 bright, CD8 dim, CD16, CD20, CD25, HLA-DR, TcRd1, dTcS1) in peripheral blood anticoagulated with sodium ethylenediamine tetraacetic acid (EDTA). To measure serum IL-2 concentrations, blood samples were centrifuged immediately after collection and frozen at -20°C for later determination. All samples were analyzed in duplicate in a single assay; the intrassay and interassay coefficients of variation were below 5% and 7% for IL 2. Standard curves were run with every assay and the experimental values were derived from the curves. We measured IL-2 by immunoenzymatic assay (IL-2 EIA,Technogenetics). Analyses of lymphocyte subpopulations were performed on unfixed cell preparations with a fluorescence activated cell sorter (FACScan, Becton-Dickinson FACS Systems, Sunnyvale, California) and a panel of monoclonal antibodies to lymphocyte surface antigens (Ortho Diagnostic Systems: OKT11, OKT4, OKT8, OK-NK, OKB20, OKT26a, OK-DR; Medical Systems : TcRd1 and dTcS1). Additionally, mAbs were directly conjugated with phycoerythrin (PE) and 10 ml mAbs were added to 100 ml EDTA blood. After a 15-min incubation, the erythrocites were disintegrated, and after centrifugation, the pellets were washed were washed with PBS. Non-lymphocytic cells contaminating the preparations were excluded from analysis using scatter gates set on the 90∞ light scatter profile. The number of fluorescent cells was expressed as a percentage of the total lymphocytes.

Statistical analysis

The results were statistically evaluated by non-inferential descriptive biometric analysis (student's *t* test and Mann-Whitney rank sum test, as indicated, on mean diurnal and nocturnal levels and on areas under the curve, calculated according to the trapezoidal method). Inferential temporal descriptive biometric analysis was also employed, using the Single Cosinor and Population Mean Cosinor methods. These methods fit the best sinusoid to individual and group data, testing the occurence (whether the zero-amplitude assumption is rejected at a probability level $p<0.05$) and quantifing the parameters MESOR (Midline Estimating Statistic of Rhythm), amplitude half of the total variability from rhythm adjusted mean) and acrophase (timing of circadian crest referred to local midnight) of the circadian rhythm (14-15).

RESULTS

Fig. 1, 2 and 3 report 24-hour profiles of CD4/CD8 ratio, and of percentages of lymphocyte subsets in peripheral blood and IL-2 serum levels in healthy controls and lung cancer patients (mean±s.e.). Tab. I reports integrated time-qualified percentage values of lymphocyte subpopulations expressed as AUC ± S.E. Tab. II reports chronobiological data derived from best fitting sine curves.

When we compared AUC values there was a decrease in $CD8^{bright}$ (T suppressor subset) ($p<0.05$) and an increase in CD16 (natural killer cells) ($p<0.01$) and in IL-2 serum levels ($p<0.01$) in cancer patients. There were no statistically significant differences between the groups in the AUC values of CD2 (total T cells), CD4 (T helper/inducer subset), CD8 (T suppressor/cytotoxic subset), $CD8^{dim}$ (T cytotoxic subset), CD4/CD8 ratio, HLA-DR (B cells and activated T cells), CD20 (total B cells), CD25 (T and B activated lymphocytes with expression of the a chain of IL-2 receptor), TcRd1 (epitope of the constant domain of d chain of T-cell receptor1) and dTcS1 (epitope of the variable domain of d chain of T-cell receptor1).

When we compared mean diurnal levels (mean of *06.00-10.00-14.00 h*), CD8 and $CD8^{bright}$ levels were lower (p=0.04 and p=0.01 respectively) and

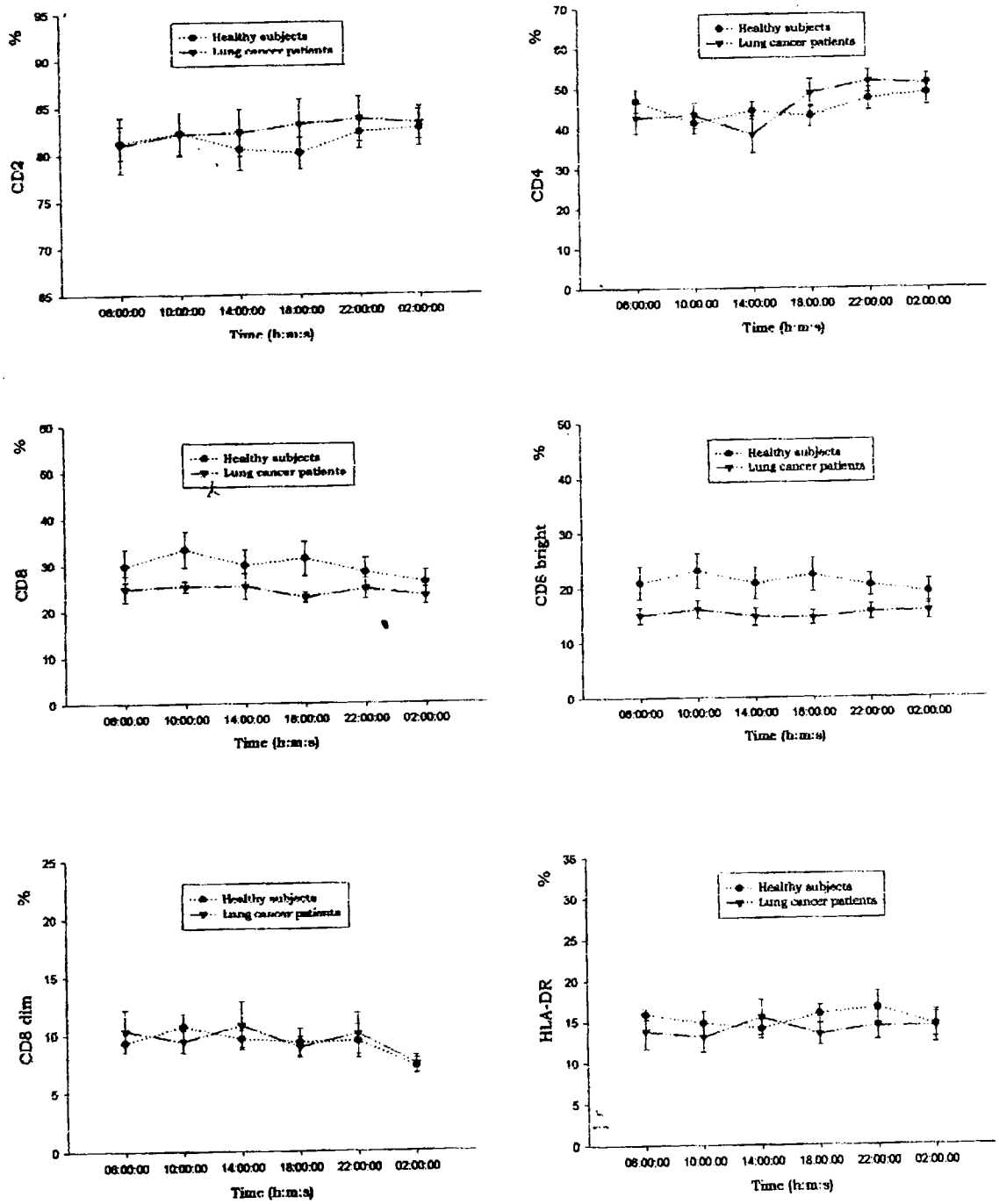

Fig. 1. *Twenty four hours profiles of CD2, CD4, CD8, CD8 Bright, CD8 Dim, HLA-Dr lymphocyte subsets on peripheral blood in healthy controls and lung cancer patients (mean ± S.E.).*

CD16 levels were higher (p=0.004) in cancer patients. When we compared mean nocturnal levels (mean of *18.00-22.00-02.00 h*), CD16 (p<0.0001) and CD25 (p=0.02) levels were higher, while CD8 (p=0.01), $CD8^{bright}$ (p=0.002) , CD20 (p=0.02) , TcRd1 (p=0.002) and dTcS1 (p=0.04) levels were lower in cancer patients.

A clear circadian rhythm was validated for the time-qualified changes of CD2, CD4, CD20, and HLA-DR with acrophase at night and CD8, $CD8^{bright}$, $CD8^{dim}$, CD16, TcRd1, and dTcS1 with acrophase in the morning in the control group. A clear circadian rhythm was validated for the time-qualified changes in CD4 with acrophase at night in the group of cancer patients.

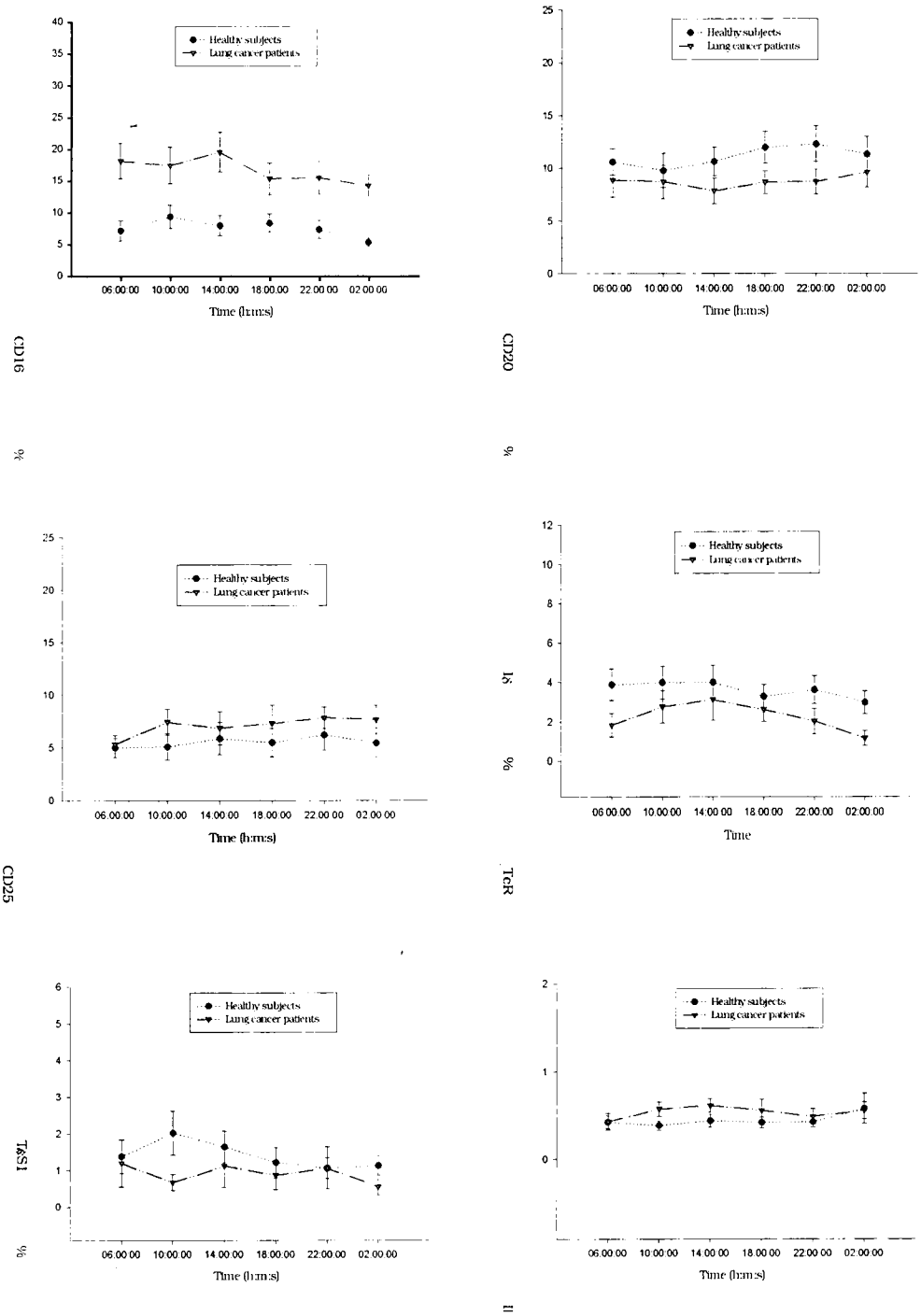

Fig. 2. *Twenty four hours profiles of CD16, CD20, CD25, TcRδ1 and δTcS1 lymphocyte subsets on peripheral blood and IL-2 serum levels in healthy controls and lung cancer patients (mean±s.e.)*

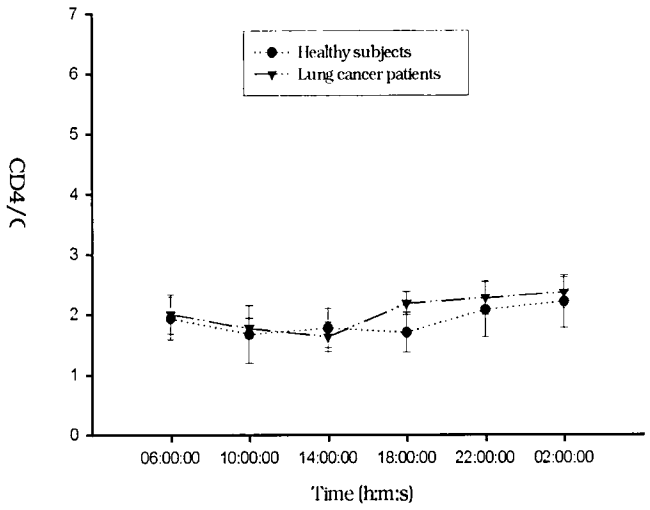

Fig. 3. *Twenty four hours profile of CD4/CD8 ratio in healthy controls and lung cancer patients (mean ± S.E.).*

	Healthy old subjects	Lung cancer patients
CD2	1628.26±37.83	1645.66±48.78
CD4	890.29±47.79	909.43±53.88
CD8	608.80±68.19	486.79±33.20
CD4/CD8 ratio	36.62±7.59	40.31±3.93
CD8 Bright	429.21±54.47	301.06±24.97•
CD8 Dim	190.54±17.06	193.23±24.64
CD16	156.12±28.61	340.27±50.26•
CD20	219.62±26.94	172.75±25.27
CD25	108.05±22.92	136.09±25.30
HLA-DR	306.48±19.41	279.09±30.5
TcRδ1	74.50±13.70	47.95±12.81
δTcS1	29.12±7.97	19.13±8.93
IL-2	8.34±0.98	11.71±0.82•

Tab. I. *Integrated time-qualified 24-hours values expressed as AUC ± s.e.*
Units: % for lymphocyte subpopulations, IU/ml for IL-2; all parameters analyzed in all the subjects; P<0.05•

		Healthy old subjects		
Factor	***p***	**MESOR±s.e.**	**Amplitude±s.e.**	**Acrophase±s.e. (°)**
CD2	0.042	81.50±0.39	0.97±0.55	-49.3±32.8
CD4	0.032	45.16±0.73	3.15±1.04	-18.8±18.9
CD8	0.022	29.63±0.77	2.61±1.09	-192.3±23.9
CD8 Bright	0.040	20.94±054	1.36±077	-205.4±32.2
CD8 Dim	0.026	9.26±0.40	1.09±0.56	-189.6±29.6
CD16	0.013	7.51±0.41	1.43±0.59	-200.6±23.4
CD20	0.005	11.11±0.09	1.21±0.12	-320.7±5.8
CD25	0.241	5.51±0.15	0.47±0.21	-296.5±26.3
HLA-DR	0.035	15.30±0.43	0.69±0.61	-338.9±50.5
TcRδ1	0.027	3.62±0.14	0.41±0.20	-167.1±28.1
δTcS1	0.037	1.41±0.06	0.44±0.09	-162.8±11.7
IL-2	0.427	0.54±0.03	0.06±0.04	-221.3±37.9
		Lung cancer patients		
Factor	***P***	**MESOR±s.e.**	**Amplitude±s.e.**	**Acrophase±s.e. (°)**
CD2	0.136	82.55±0.27	1.09±0.38	-311.5±19.8
CD4	0.036	45.85±1.17	6.16±1.66	-347.6±15.4
CD8	0.533	24.18±0.47	0.82±0.66	-171.8±45.8
CD8 Bright	0.594	15.22±0.27	0.43±0.39	-71.3±51.4
CD8 Dim	0.628	9.47±0.55	0.81±0.78	-192.6±54.9
CD16	0.185	16.64±0.60	2.11±0.85	-170.0±22.9
CD20	0.128	8.74±0.15	0.64±0.21	-34.7±19.3
CD25	0.533	7.07±0.39	0.70±0.56	-301.0±45.9
HLA-DR	0.897	14.09±0.43	0.29±0.61	-282.6±120.4
TcRδ1	0.080	2.25±0.07	0.91±0.11	-211.1±6.7
δTcS1	0.924	0.92±0.13	0.08±0.19	-223.3±142.7
IL-2	0.632	0.41±0.01	0.02±0.01	-254.6±55.3

Tab. II.
Chronobiological data derived from best fitting sine curves (fitted period: 24 hours = 360°)

DISCUSSION

The host immune defense plays an important role, especially in earlier phases of neoplastic disease. Lymphocytes are an essential component of specific immune responses wich produce tumour (tumor) rejection. Effector cells that exert antitumoral effects include tumor infiltrating lymphocytes (TIL, populations of antigen-specific major histocompatibility complex restricted T-cells, usually CD8 cytotoxic T-cells, whose response may be stimulated by T-helper 1 cytokine milieu including IFN-γ, IL-2 and IL12), natural killer (NK) cells (large granular lymphocytes that express neither α/β or γ/δ T-cell receptor nor CD3 on their surface, can lyse a number of different tumour cells and may be stimulated by IFN-γ, IL-2, IL12 and IL18) also exert antitumoral effects. Additionally, lymphokine-activated killer cells (LAK, a mixed population of peripheral blood lymphocytes that develop non-major histocompatibility complex restricted lytic activity for malignant, but not normal cells, after culture in vitro with high concentrations of IL-2) have an antitumoral effect (16-19). Cancer can alter immunity through direct invasion and replacement of normal lymphoid tissue, through the production of humoral factors, which interfere with immune function, or by causing cachexia and malnutrition, which increase the severity of the immunodeficiency. Futhermore, important alterations of the immune system are known to occurr during aging. These are characterized by a decreased response to exogenous antigens associated with an increased frequency of autoimmune phenomena primarily affecting T cells. The most striking age-related changes include increased numbers of T lymphocytes with an activated phenotype (HLA-DR and CD25) and levels found in our healthy volunteers are comparable to values obtained by precedent studies (20-23). As shown by results obtained in our study, there are different circadian variations in the total number of circulating immune cells and in specific lymphocyte subpopulations. The T-helper/inducer and the T-suppressor/cytotoxic populations in the peripheral blood change with circadian rhythmicity but in an opposite phase, showing a temporal organization of lymphocyte functions. The different changes of lymphocyte subsets may be responsible for time-dependent variations of magnitude and/or expression of immune responses. They may also explain circadian variations of some biological phenomena, such as allograft survival (more prolonged if the transplant is performed at night) or response to intradermal purified protein derivative in tuberculin-sensitive subjects highest in the morning) (24-26). The total number of circulating T- and B-cells changes with circadian rhythmicity, with acrophase at night. This rhythm of variation is recognizable for the changes in total T-cells, T-helper/inducer subset, B-cells and activated T-cells, and total B-cells. Changes in serum levels of IL-2 do not show circadian periodicity . The T-suppressor/cytotoxic lymphocytes, natural killer cells and the levels of TcRd1 and dTcS1 are higher in the morning and show a clear circadian rhythmicity, suggesting that T-cell receptor g/d complex is mainly expressed at the cell surface of cytotoxic lymphocytes. This complex is involved in T-cell activation, and human γ/δ T-cells are a subgroup of the peripheral blood mononucleocytes comprising less than 10% of peripheral blood T-cells (27-29) The current study shows that total B-cells, CD8 lymphocytes, and in particular, the T-suppressor subset are diminished in lung cancer patients, with loss of normal circadian rhythmicity. The decrease in T-suppressor subpopulation has been observed in other types of cancer (gastric cancer, colorectal carcinoma and urological cancer) (30-32) and may represent a marker of immunological disregulation. NK cells and T- and B-activated lymphocytes with expression of the a chain of IL-2 receptor are increased in our patients and this may be related to increased IL-2 serum levels found in the cancer group. NK cells have the ability to kill tumor cells spontaneously without the need for prior activation or MHC class I restriction. They can contribute to immune surveillance of cancer controlling metastatic formation through the bloodstream, are readily responsive to biologic response modifiers. IL-2 activated NK cells could also be effective against established solid tissue metastases. In a study conducted in mice, NK cells have been essential in the response to adjuvant treatment of superficial bladder cancer with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG). The model of this local immunotherapy indicates that NK cells, CD4 and CD8 lymphocytes are required for the BCG-induced

control of bladder tumor growth. The interplay of accessory cells (macrophages and dendritic cells) and different lymphocyte subpopulations is determinant for immune activation and control of tumor growth: NK cells could become activated by cytokines secreted by macrophages/dendritic cells (e.g. IL12) as well as CD4 and CD8 cells (e.g. IL-2 , IFN-γ). After activation, these NK cells would then contribute to the anti-tumor effect as producers of IFN-γ and more likely as cytotoxic effector cells, which directly lyse bladder tumor target cells (33) . Expression of the constant and variable domains of d chain of T-cell receptor 1 is decreased in cancer patients. This may be an important finding ; activated γ/δ expressing cells frequently exhibit cytotoxic activity against multiple target cell lines including neoplastic cells, and the number of γ/δ T cells is elevated in patients with infectious diseases, such as tuberculosis, typhoid fever, tularemia, or leprosy, and in hospital workers in close contact with tuberculosis patients. There is some speculation that γ/δ T-cells may be specialized for mycobacterial immunity or destruction of 'stressed' autologous cells which show increased expression of heat shock proteins and extracts from *Mycobacterium* species. A series of non-peptide antigens can also activate human γ/δ T-cells. The presence of g/d expressing cells exhibiting in vitro lymphokine activated killer activity against autologous acute leukemia cells has recently been demonstrated and in vitro analysis showed that γ/δ T cells produce IFN-γ, TNF-α and TNF-β, and have a markedly potent cytotoxic effect on tumor cells (lung cancer, renal cell carcinoma) (34-36).

In conclusion, our results have indicated that lung cancer is associated with anomalies of proportion and nyctohemeral variations of lymphocyte subsets, probably expression of an altered immune function in front of advancing neoplastic disease. This may impair the interplay of accessory cells and different lymphocyte subpopulations crucial for an effective immune response and must be taken into account when it is necessary to evaluate immunomodulatory effects determined by biological response modifiers and adoptive immunotherapy.

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