

HUMAN CYTOMEGALOVIRUS DNA LEVEL IN PATIENTS WITH IDIOPATHIC PULMONARY FIBROSIS

S. DWORNICZAK¹, D. ZIORA¹, M. KAPRAL², U. MAZUREK², G. NIEPSUJ¹,
R. RAUER¹, T. WILCZOK², J. KOZIELSKI¹

¹Department of Lung Diseases and Tuberculosis, Medical University of Silesia, Zabrze and

²Department of Molecular Biology, Biochemistry and Biopharmacy, Medical University
of Silesia, Sosnowiec, Poland

The objectives of the study were to estimate human cytomegalovirus (HCMV) DNA copy number in broncho-alveolar lavage cells, blood leukocytes, and serum of patients with idiopathic pulmonary fibrosis (IPF). The study groups consisted of 16 patients, newly diagnosed with IPF and never treated, (mean age 40.9 ± 11.0 yr; F/M-7/9) and in 16 adult healthy volunteers (mean age 36.8 ± 6.4 yr; F/M-4/12) used as controls. The HCMV DNA copy number was calculated by a Q-PCR method using TaqMan ABI PRISM™7700. We found that the prevalence of the HCMV DNA positive subjects in the patient group (75%) did not differ significantly from that in the control group (69%). We also found that in both patient and control groups the mean HCMV DNA copy number in BAL cells was significantly higher than that in blood leukocytes ($\log_{10}=2.7$ vs. 1.2 for patients and 2.8 vs. 0.9 for controls, respectively). However, a higher HCMV DNA copy number in blood serum was observed in IPF patients than in controls ($\log_{10}=3.2$ vs. 2.0, respectively). We conclude that the lungs play an important role in the human pathobiology of cytomegalovirus sustenance.

Key words: broncho-alveolar lavage, human cytomegalovirus, idiopathic pulmonary fibrosis, quantitative polymerase chain reaction

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a specific form of chronic interstitial pneumonia of unknown etiology limited to the lung with the histopathological features of usual interstitial pneumonia (UIP). Recently, the knowledge about

interstitial lung diseases has improved and diagnostic and imaging methods, such as a high resolution computed tomography (HRCT) and broncho-alveolar lavage (BAL) are well recognized and the histological forms are described (1, 2). Despite the progress, the disease still remains one of the most poorly understood interstitial lung diseases.

The etiologic agents leading to pulmonary fibrosis are not fully understood. Some findings suggest that the persistent imbalance in the expression of Th2 vs. Th1 cytokines in the lung is a mechanism for progression of pulmonary fibrosis. Another factor that can play a role in the appearing or progression of lung fibrosis is infectious agents. In recent studies, it has been postulated that viral infections (latent or active), for instance caused by human cytomegalovirus (HCMV), can be associated with IPF (3, 4). Particularly, in immunocompromised patients the HCMV-associated interstitial pneumonia is the most common life-threatening infection (5).

The intention of this study was to estimate the human HCMV DNA copy number in different compartments (BAL cells, blood leukocytes, serum) in patients with IPF by using a real-time Q-PCR. The results gave us the opportunity to evaluate the distribution and probable contribution of human HCMV in the etiopathogenesis of interstitial lung diseases.

MATERIAL AND METHODS

Patients

The study consisted of a patient group and a control group of subjects. There were 16 patients of the mean age 40.9 ± 11.0 yr (7 females, 9 males) admitted to the Department of Phthisiopneumology of the Medical University of Silesia, Zabrze, because of persistent cough, shortness of breath, exercise dyspnea, and diffuse changes in X-ray. None of them had ever been treated from pulmonary pathology or underwent immunosuppression. In 6 cases, final diagnosis was established on the basis of HRCT images and histopathological findings, typical for UIP (6), obtained from lung biopsy performed under video-assisted thoracoscopy. In another 10 cases, final diagnosis was based on the HRCT images combined with the presence of specific clinical symptoms (7). The control group consisted of 16 subjects, adult volunteers, of the mean age 36.8 ± 6.4 yr (4 females, 12 males). Routine spirometry was performed in accordance with recommended standards (8). FVC and FEV1 were obtained from maximal expiratory flow-volume curves (Transferscreen II Jaeger, Würzburg, Germany). Values are expressed as the percentage of predicted values.

Clinical material

Blood samples were collected and broncho-alveolar lavage was performed in patients and in control subjects after informed consent had been signed. The experimental procedures were accepted by a local Ethics Committee (permit no. NN-013-167/00). BAL was performed according to the general principles of the methodology approved by the European Respiratory Society (8). In brief, after local anaesthesia of the upper respiratory tract and premedication, simultaneous double BAL from a bronchus of the middle lobe was performed in all subjects of the study. A solution of 0.9% NaCl (pre-warmed to 37°C) in portions of 20 ml (120 ml in total) was instilled through a bronchoscope. Subsequently, the lavage was retrieved by a gentle suction into a siliconed bottle on

ice. The first 20 ml portion of the aspirate was discharged as bronchial fraction, while aspirate from the remaining 100 ml was treated as alveolar fraction. The alveolar fraction of aspirate was centrifuged at 500g for 10 min. The cellular pellet, after washing with a buffered Hanks solution, was counted in a Bürker chamber. An aliquot of the suspension was then diluted to a concentration of 1×10^6 cell/ml. From a portion of the suspension, microscopic preparations were prepared and after staining with the May-Grünwald-Giemsa method (Hemacolor, Merck, Darmstadt, Germany), they were assessed cytologically for the percentage of lymphocytes, macrophages, neutrophils, and eosinophils. At least 500 cells were counted in any preparation. The remaining part of the cell suspension of alveolar fractions was divided into small portions and frozen to -70°C for molecular assays. After BAL had been performed, 10 ml of a patient's blood were taken: 5 ml on potassium ethylene diamine tetracetic acid (K2-EDTA) for the buffy coat harvesting and another 5 ml on cloth for serum completion. The obtained materials also were frozen to -70°C for molecular assays.

A portion of sediments was cultured and tested against most common and uncommon pathogens: fast growing bacteria, Mycobacteria, moulds, Pneumocystis carini, Mycoplasma, Chlamydia, Legionella, and common respiratory tract viruses. In 5 cases, the tests were positive and those patients were removed from the study group, so that only were the patients who tested negatively included.

Nucleic acid amplification

The real-time quantitative polymerase chain reaction (9, 10) was carried out, using primers and probes for a TaqMan assay, designed and determined by Primer Express™ Version 1.0, ABI PRISM software (Applied Biosystems, Foster City, CA) on the basis of a nucleotide sequence of immediate early protein (IE4) DNA coding region of the laboratory standard CMV strain AD169 (GenBank accession number X17403). Applying Internet Base "BLAST", the proposed primers and probes were tested for their complementarities and uniqueness for the human herpes virus-5. Finally, one pair of primers (FCMV123; RCMV123): FCMV1235'GCgggAgATgTggATggCTTgT3'; RCMV1235'TgACTgCagCCATTggTggTCTT3' and one probe: SCMV*FAM-TgCATgATgTgAgCAAgggCgCCg-TAMRA were selected. The ends of the constructed probe were labeled with fluorescent dyes. Genomic DNA was extracted from the BAL cell suspension, buffy-coat of blood leukocytes, and serum by using GENOMIC DNA PREP PLUS (A & A Biotechnology, Poland, Gdansk), according to the manufacturer's recommendations.

The amplification reaction mixture (total volume of 50 µl) consisted of TaqMan-buffer A (Applied Biosystems, Foster City, CA), 3 mM MgCl₂, 200 µM dNTP mix, 0.5 U Tth polymerase, an amplification reaction enhancer, 900 nM primers forward and reverse, 200 nM of the probe, and 1.3 µl of the DNA extract. The real-time quantitative polymerase chain reaction was carried out in a sequence detection system TaqMan ABI PRISM™7700 (Applied Biosystems, Foster City, CA). The PCR conditions were optimized as follows: 95°C for 5 s, 95°C for 10 min, and 45 cycles each of 95°C for 30 s, 60°C for 60 s, and 72°C for 10 min. The HCMV copy number was calculated with respect to the TaqMan PCR standard curve obtained by using commercially available DNA copy number standards of β -actin gene (Applied Biosystems, Foster City, CA). The PCR results were calculated as HCMV DNA copy number per 10^6 BAL cells, per 10^6 blood leukocytes, and per 1 ml of serum.

Evaluation of specificity, sensitivity, and reproducibility of HCMV copy number determination by a quantitative real-time polymerase chain reaction

Each reaction was performed in triplicate in the two independent assays. Average coefficients of intra-assay and inter-assay variability were 4.1% (range 3.3-5.2%) and 4.9% (range 3.1-5.9%), respectively. The specificity of the real-time Q-PCR assay was confirmed by the amplification of DNA isolated from the leukocytes buffy coat from patients after orthotopic heart transplantation

with HCMV viremia confirmed by a pp65 antigenemia assay by indirect immunofluorescence method (Biotest AG, Germany). The sensitivity of the above mentioned real-time PCR method was established by others at 10 viral DNA copies ($\log_{10} >1$) per 10^6 cells and 1 ml of serum (11, 12) and by the manufacturer (Applied Biosystems, Foster City, CA).

Exclusion of cross reactivity

Respiratory tract specimens that were positive for Influenza and Parainfluenza, Adenovirus, RSV (Respiratory screen kit, Dako, Denmark) were chosen to study Q-PCR specificity. No cross reaction was observed. Also, three samples of the blood from patients with mononucleosis were tested for a possible cross reaction. The results were negative in each case.

Enzyme immunoassay

Enzyme immunoassay detection of antibodies against recombinant antigens of HCMV in IgG and IgM classes was made with Biotest ELISA tests (Biotest, Germany) by using a Microplate reader ELX 800 (Bio-Tek Instruments Inc., Vermont, USA).

Statistical analysis

All calculations were performed with the aid of STATISTICA software (StatSoft, Poland, Cracow). Data concerning the HCMV DNA copy number were expressed as mean $\log_{10} \pm \text{SE}$. Differences between the means were examined using a non-parametric Mann-Whitney U test for two groups of variables. The prevalence of HCMV infection in the studied populations was compared by using a two-table Chi square test. Friedman ANOVA analysis of variance was used for within group differences. Correlations were calculated by using Spearman's rank order test. The level of confidence was established at $P < 0.05$.

RESULTS

All basic data characterizing the study and control groups are summarized in *Table 1*.

Prevalence of HCMV infection in the studied populations

The HCMV DNA copies were detected in both the study and the control group. The copy numbers were comparable in both groups with respect to the proportion of subjects from whom HCMV DNA could be detected (in all tested compartments); being 75% in the study ($n=12$) and 69% in the control group ($n=11$). Likewise, the number of subjects negative for HCMV DNA in all three compartments was almost equal in the control and study groups (31%; $n=5$ and 25%; $n=4$, respectively). The prevalence of HCMV infection, as confirmed by serological tests (data not shown), did not differ in both groups either.

Differentiated HCMV DNA copy number in the control and study groups

Comparing the BAL cell and blood leukocyte compartments, the higher HCMV DNA copy number was observed in the BAL cells in both IPF and

Table 1. Baseline characteristics of subjects.

Variables	IPF (n=16)	Control (n=16)	P Value
Age, yr	40.8 (10.9)	36.7 (6.4)	ns
Gender	F/M - 9/7	F/M -4/12	ns
Smoking habit	6 smokers/10 non-smokers	8 smokers/8 non-smokers	ns
FVC (% of predicted)	81.0 (3.7)	95.7 (3.0)	P<0.01
FEV1 (% of predicted)	82.2 (3.6)	97.0 (4.6)	P<0.01
DLCO (% of predicted)	66 (7.7)	88 (5.5)	P<0.05
BAL M ϕ (%)	58.1 (3.5)	78.4 (4.1)	P<0.001
BAL lymphocytes (%)	20.5 (3.5)	16.2 (2.7)	P<0.05
BAL neutrophils (%)	15.6 (2.4)	4.3 (2.1)	P<0.01
BAL eosinophils (%)	3.7 (2.1)	1.5 (0.5)	P<0.05
BAL cells [1 mln] viral load (log10)	2.7 (0.4)	2.8 (0.4)	ns
Blood leukocytes [1 mln] viral load (log10)	1.2 (0.1)	0.9 (0.1)	ns
Serum [1 ml] viral load (log10)	3.2 (0.4)	2.0 (0.4)	ns

Data are represented as mean (SE); ns= not significant; M ϕ - macrophages

control subjects. The difference in the HCMV DNA copy number between the BAL cells and the blood leukocytes was significant in either group of subjects (*Fig. 1*). In the blood serum compartment, a higher mean HCMV DNA copy number was observed in the IPF patients than in the control subjects, although the difference did not assume statistical significance (*Fig. 2*). Comparison of the HCMV DNA positive and negative IPF patients revealed only one statistically significant difference: the percentage of BAL macrophages was higher in the HCMV DNA positive patients ($63.6 \pm 15.0\%$ and $41.4 \pm 14.4\%$, respectively).

BAL cells, blood leukocytes and serum HCMV DNA copy number correlation

In the control group, the serum HCMV DNA viral load correlated positively with the blood leukocyte viral load ($r=0.43$) and the BAL macrophage percentage ($r=0.51$). In the same group, BAL cells HCMV viral load correlated positively with the blood leukocyte HCMV viral load ($r=0.50$). In the IPF patients, the serum HCMV DNA copy number correlated positively with the blood leukocyte and BAL cells HCMV viral loads ($r=0.64$ and $r=0.52$, respectively) and also with the BAL macrophage percentage ($r=0.44$). The blood leukocyte HCMV viral load correlated positively with BAL cells HCMV viral load ($r=0.40$) and the BAL macrophage percentage ($r=0.58$).

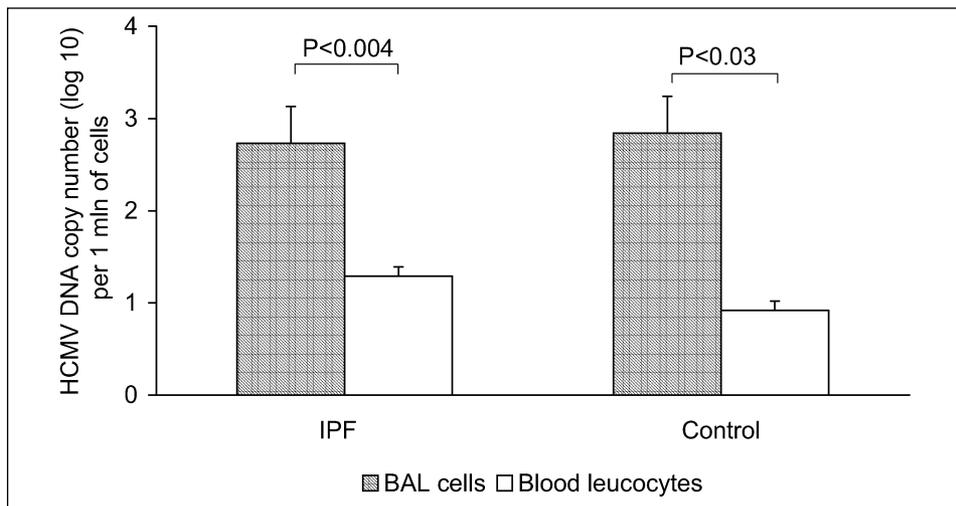


Fig. 1. Comparison of cellular compartment HCMV DNA copy numbers in the IPF (n=16) and control subjects (n=16). Data are means \pm SE. There were significant differences in the HCMV DNA copy number between the two compartments in either group of subjects, but no differences in either compartment between the two groups.

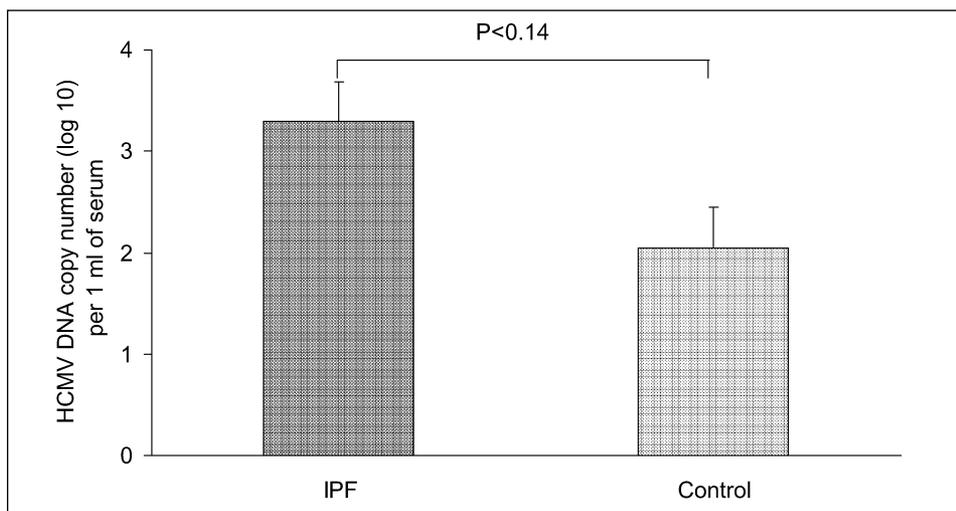


Fig. 2. Comparison of serum viral load in the IPF (n=16) and control groups (n=16). Data are means \pm SE. The difference did not assume statistical significance.

DISCUSSION

The prevalence of HCMV infection in the study group did not differ from the control group. This is in concordance with our previous results describing the

HCMV serological status in patients with interstitial lung changes, which suggested no higher frequency of past or active HCMV infection in IPF patients' population compared with control subjects (13). However, our data confirmed considerably widespread HCMV infection in the population. The mean HCMV DNA copy number in IPF patients was higher in both blood leukocytes and serum compared with control subjects, but the differences were not statistically significant. No correlations were observed between the HCMV DNA copy number and the serological status in both analyzed groups (data not published).

It is assumed that the most important marker of HCMV infection activity is the blood leukocyte HCMV DNA copy number (14, 15). Blood monocytes are the main location of virus carriage (16, 17). The probable source of HCMV DNA copies during latency are bone marrow cells (stem cells) that transfer viral DNA to monocytes during their development (18, 19). Circulation of monocytes and their natural ability to settle in different organs, especially in lung parenchyma and alveoli as organ specific macrophages, lead to viral spread. Such a mechanism was indirectly confirmed in the present study by showing the correlation between the BAL macrophage percentage and viral load in the analyzed compartments. Differentiation of blood monocytes to organ specific macrophages results in derepression of HCMV immediate early genes, being previously dumb in these cells (20). The HCMV DNA copy number in serum and blood leukocytes in IPF patients positively correlated with the BAL cells viral load and BAL macrophages percentage, which confirms that the inflammation process recruits blood monocytes to lung parenchyma (21, 22) and that their differentiation to the alveolar macrophage phenotype leads to replication of HCMV immediate early genes. Consistent with these results are the following: the significantly higher HCMV DNA copy number in BAL cells compared with that in blood leukocytes in both analyzed groups and a higher percentage of BAL macrophages in the IPF HCMV DNA positive patients compared with that in HCMV DNA negative patients. These observations underline a positive correlation between the blood leukocyte and BAL cell viral loads in both IPF and control groups. These data put the lungs as the prevalent place of latency or persistency of HCMV, compared with blood leukocytes and serum in humans (23, 24). This was also confirmed by others (25) in a report in which the highest murine cytomegalovirus DNA burden in lung cells reflects the organ specific site of latency and the place of the highest risk of infection recurrence. In humans, higher HCMV DNA positivity of BAL fluid compared with that of blood leukocytes has also been observed (26). A triggered inflammation process comprising alveolar macrophages could result in derepression of HCMV genes naturally transfected to these cells (due to primary infection) (27), which in the case of immunological permission, can reactivate latent infection. An active viral infection up-regulates the inflammation and a vicious circle closes (28). The potential role of the family of herpes viruses as cofactors modulating the

development and progression of interstitial lung disease in an animal model was confirmed by recently published data (29).

In conclusion, our results suggest a crucial role of the lungs in the human cytomegalovirus pathobiology. The contribution of HCMV infection in IPF patients should be taken into account during immunosuppressant treatment and in the case of progression, despite the continued steroid therapy.

Acknowledgments: We are grateful to our colleague Dr. D. Jastrzębski for his comments and support during the preparation of the manuscript. This work was supported by Medical University of Silesia grant NN-4-120/02.

REFERENCES

1. Katzenstein AL, Myers JL. Idiopathic pulmonary fibrosis: clinical relevance of pathologic classification. *Am J Respir Crit Care Med* 1998; 157: 1301-1315.
2. Rennard SI, Aalbers R, Bleecker E et al. Bronchoalveolar lavage: performance, sampling procedure, processing and assessment. *Eur Respir J* 1998; 11 (Suppl 26): 13S-15S.
3. du Bois RM, Wells AU. Cryptogenic fibrosing alveolitis/idiopathic pulmonary fibrosis. *Eur Respir J* 2001; 18 (Suppl 32): 43S-55S.
4. Lok SS, Egan JJ. Viruses and idiopathic pulmonary fibrosis. *Monaldi Arch Chest Dis* 2000; 55: 146-150.
5. Gutierrez CA, Chaparro C, Krajden M, Winton T, Kesten S. Cytomegalovirus viremia in lung transplant recipients receiving ganciclovir and immune globulin. *Chest* 1998;113:924-932.
6. Katzenstein AL, Myers JL. Idiopathic pulmonary fibrosis: clinical relevance of pathologic classification. *Am J Respir Crit Care Med* 1998; 157: 1301-1315.
7. American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS): *Am J Respir Crit Care Med* 2000; 161: 646-664.
8. Hankinson JL, Gardner RM: Standardization of spirometry. *Am Rev Respir Dis* 1988; 137: 493-495.
9. Kearns AM, Guiver M, James V, King J. Development and evaluation of a real-time quantitative PCR for the detection of human cytomegalovirus. *J Virol Methods* 2001; 95: 121-131.
10. Nitsche A, Steuer N, Schmidt CA et al. Detection of human cytomegalovirus DNA by real-time quantitative PCR. *J Clin Microbiol* 2000; 38: 2734-2737.
11. Kearns AM, Guiver M, James V, King J. Development and evaluation of a real-time quantitative PCR for the detection of human cytomegalovirus. *J Virol Methods* 2001; 95: 121-131.
12. Yun Z, Lewensohn-Fuchs I, Ljungman P, Vahlne A. Real-time monitoring of cytomegalovirus infections after stem cell transplantation using the TaqMan polymerase chain reaction assays. *Transplantation* 2000; 69: 1733-1736.
13. Dworniczak S, Ziora D, Basta L et al. Human Cytomegalovirus serological status in patient with interstitial lung diseases. *Przegl Epidemiol* 2003; 57: 431-437.
14. Gerna G, Zavattoni M, Baldanti F et al. Human cytomegalovirus (HCMV) leukodnaemia correlates more closely with clinical symptoms than antigenemia and viremia in heart and heart-lung transplant recipients with primary HCMV infection. *Transplantation* 1998; 65: 1378-1385.

15. Larsson S, Soderberg-Naucler C, Wang FZ, Moller E. Cytomegalovirus DNA can be detected in peripheral blood mononuclear cells from all seropositive and most seronegative healthy blood donors over time. *Transfusion* 1998; 38: 271-278.
16. Soderberg C, Larsson S, Bergstedt-Lindqvist S, Moller E. Definition of a subset of human peripheral blood mononuclear cells that are permissive to human cytomegalovirus infection. *J Virol* 1993; 67: 3166-3175.
17. Taylor-Wiedeman J, Sissons JG, Borysiewicz LK, Sinclair JH. Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. *J Gen Virol* 1991; 72: 2059-2064.
18. Maciejewski JP, St Jeor SC. Human cytomegalovirus infection of human hematopoietic progenitor cells. *Leuk Lymphoma* 1999; 33: 1-13.
19. Kondo K, Xu J, Mocarski ES. Human cytomegalovirus latent gene expression in granulocyte-macrophage progenitors in culture and in seropositive individuals. *Proc Natl Acad Sci USA* 1996; 93: 11137-11142.
20. Taylor-Wiedeman J, Sissons P, Sinclair J. Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers. *J Virol* 1994; 68: 1597-1604.
21. Bitterman PB, Saltzman LE, Adelberg S, Ferrans VJ, Crystal RG. Alveolar macrophage replication. One mechanism for the expansion of the mononuclear phagocyte population in the chronically inflamed lung. *J Clin Invest* 1984; 74: 460-469.
22. Craigen JL, Grundy JE. Cytomegalovirus induced up-regulation of LFA-3 (CD58) and ICAM-1 (CD54) is a direct viral effect that is not prevented by ganciclovir or foscarnet treatment. *Transplantation* 1996; 62: 1102-1108.
23. Toorkey CB, Carrigan DR. Immunohistochemical detection of an immediate early antigen of human cytomegalovirus in normal tissues. *J Infect Dis* 1989; 160: 741-751.
24. Fajac A, Vidaud M, Lebargy F et al. Evaluation of human cytomegalovirus latency in alveolar macrophages. *Am J Respir Crit Care Med* 1994; 149: 495-499.
25. Balthesen M, Messerle M, Reddehase MJ. Lungs are a major organ site of cytomegalovirus latency and recurrence. *J Virol* 1993; 67: 5360-5366.
26. Takemoto Y, Takatsuka H, Wada H et al. Evaluation of CMV/human herpes virus-6 positivity in bronchoalveolar lavage fluids as early detection of acute GVHD following BMT: evidence of a significant relationship. *Bone Marrow Transplant* 2000; 26: 77-81.
27. Kondo K, Xu J, Mocarski ES. Human cytomegalovirus latent gene expression in granulocyte-macrophage progenitors in culture and in seropositive individuals. *Proc Natl Acad Sci USA* 93: 11137-11142.
28. Geist LJ, Monick MM, Stinski MF, Hunninghake GW. The immediate early genes of human cytomegalovirus upregulate expression of the interleukin-2 and interleukin-2 receptor genes. *Am J Respir Cell Mol Biol* 1991; 5: 292-296.
29. Lok SS, Haider Y, Howell D, Stewart J, Hasleton PS, Egan J. Murine gammaherpes virus as a cofactor in the development of pulmonary fibrosis in bleomycin resistant mice. *Eur Respir J* 2002; 20: 1228-1232.

Author's address: S. Dworniczak, 1 Koziółka ST., PL-41-803 Zabrze, Poland; tel/fax +48 32 2745664; e-mail: sdworniczak@slam.katowice.pl