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Apoptotic Damage of DNA in Human Leukaemic HL-60 Cells Treated with C2-Ceramide was Detected after G1 Blockade of the Cell Cycle

E. BÁRTOVÁ[1], A. ŠPANOVÁ[2], L. JANÁKOVÁ[3], M. BOBKOVÁ[1], B. RITTICH[4]

[1]Institute of Pathological Physiology, Medical Faculty, Masaryk University, [2]Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, [3]Masaryk Memorial Cancer Institute and [4]Faculty of Chemistry, Technical University, Brno, Czech Republic

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Summary

Apoptosis was induced by treatment of HL-60 cells with C2-ceramide. Apoptotic damage of DNA was detected according to the sub-G1 peak on a flow cytometer, according to the typical morphology and according to the DNA fragmentation "ladder" after gel electrophoresis. It was shown that the apoptotic cleavage followed after G1 blockade of the cell cycle. A high correlation coefficient (r_S=0.957) was found between the percentages of G1 blocked cells and apoptotic cells. This high correlation together with the appearance of the sub-G1 peak suggests that the G1 blocked HL-60 cells were subject to apoptotic death. It is deduced that the mechanisms leading to G1 blockade of the cell cycle and activation of apoptosis in HL-60 cells are interconnected.

Key words
HL-60 cells – C2-ceramide – Apoptosis – Cell cycle

Introduction

Living cells can die by one of two processes: by active self-destruction (apoptosis) or by passive necrosis (Kerr *et al.* 1972, Williams *et al.* 1992). Great attention has been given to the study of apoptosis since it has been known that the process of apoptosis is genetically controlled. Detailed knowledge of apoptotic pathways make it possible to directly affect cell survival or deterioration of cell status.

Some apoptotic death pathways can be activated in cancer cells. For instance, apoptosis can be activated in human promyelocytic HL-60 cells by treatment with different agents (Allen *et al.* 1993, Musilová *et al.* 1995).

Recently, some information has appeared about a product of sphingomyelin metabolism – ceramide (Kim *et al.* 1991, Mathias *et al.* 1991, Obeid *et al.* 1993, Raines *et al.* 1993, Jarvis *et al.* 1994, Wolf *et al.* 1994, Hannun and Obeid 1995). It is hypothesized to be a critical second messenger in the pathway involving *ras* activation, which in turn directly or indirectly provides a positive signal for the pathway which results in apoptosis. The nature of the apoptotic signal is currently not defined (Gulbins *et al.* 1995). Here we show that the apoptotic signal is activated by impairment of the G1 phase of the cell cycle after C2-ceramide treatment of HL-60 cells.

Methods

Cell lines

The human leukaemic promyelocytic permanent cell line HL-60 was obtained from EC ACC Porton Down Salisbury, U.K. The cells were cultivated in RPMI 1640 medium with 10 % foetal calf serum complemented with glutamine (2 mM) and gentamicin (50 mg/ml) at 37 °C in 5 % CO_2/95 % air atmosphere. The cells were passaged twice a week at a density of $2x10^5$ cells/ml. The passages 15 to 45 were used in these experiments.

Reagents

C2-ceramide (N-acetyl sphingosine) (Sigma St. Louis, MO, U.S.A.) was used in a concentration of 5 μM.

Flow cytometric measurements

For the flow cytometric detection of the cell cycle $2x10^5$ cells were used. The cells were washed twice in an ice-cold phosphate buffer solution (0.17 M NaCl, 3 mM KCl, 4 mM $Na_2HPO_4.12\ H_2O$, 2 mM KH_2PO_4, pH 7.5). One millilitre of Vindel's solution (1 mM Tris, pH 8.0, 1 mM NaCl, 0.1 % Triton X100, 0.7 % RNase A) with 10 μg of ethidium bromide was added after washing and the mixture was incubated at 37 °C for 30 min. Fluorescence intensity was measured with a flow cytometer COULTER^R EPICS^R argon ion laser for excitation at 488 nm. Using flow cytometry, 10^4 cells were detected and relative cell counts in G1, S and G2/M phases were estimated. Apoptotic cells were fixed in 70 % ethanol and washed in phosphate-citric acid buffer (Gong *et al.* 1994) before being dissolved in Vindel's solution with ethidium bromide and used for flow cytometric analysis.

Detection of apoptosis by DNA fragmentation test

The DNA was prepared according to Musilová *et al.* (1995). DNA fragmentation into oligo- and mononucleosomal fragments was detected on 1.8 % agarose gel and visualized under UV light after staining with ethidium bromide (1 μg/ml) following gel electrophoresis. Gel electrophoresis was performed in TBE buffer at 5V/cm according to Maniatis *et al.* (1982).

Morphological determination of apoptosis

Apoptotic cells (apoptotic bodies) were stained by standard methods: May-Grünwald and Giemsa-Romanowski. Cell viability was assessed by the ability to exclude eosin. Two hundred cells were scored for each experiment.

Statistical analysis

The percentage of apoptotic cells detected morphologically and the percentages of cells in cell cycle phases were expressed as means ± S.E.M. The correlation between the percentage of apoptotic cells and G1 blocked cells was counted with the aid of the Spearman correlation coefficient (Benedik 1989).

Results

The addition of extracellular C2-ceramide (N-acetyl sphingosine) at a concentration of 5 μM to the HL-60 cells influenced the growth of cells. A difference in the cell distribution in the individual phases of cell cycle was detected in comparison with the controls after 12–24 h of treatment using flow cytometry (Table 1). On the average, the increase of C2-ceramide treated cells in the G1 phase of the cell cycle was 13.5 %, number of cells in the S phase decreased by 6.7%.

Table 1
Cell distribution in cell cycle phases of control HL-60 cells and HL-60 cells treated with C2-ceramide estimated in three independent experiments

Cell cycle phase	G1	S	G2/M
Control cells (%)	45.0	27.1	22.1
	47.1	23.1	19.7
	45.1	21.0	22.4
C2-ceramide treated cells (%)			
	53.2	17.0	19.1
	64.8	14.4	18.6
	57.6	19.6	22.3

Apoptosis in HL-60 cells treated with C2-ceramide was assessed by three different methods.

1) Apoptosis was detected using flow cytometry according to the detection of the sub-G1 peak which appeared on the histogram before the G1 peak of treated cells (Fig. 1B). This sub-G1 peak could not be detected on the DNA histogram of control cells (Fig. 1A).
2) Apoptosis was determined according to typical apoptotic morphology by microscopic counting of the apoptotic cells. We detected 0.1 % and 8.9 % apoptotic HL-60 cells after their cultivation without and with C2-ceramide, respectively. The typical control and apoptotic morphologies are shown in Fig. 2.
3) Apoptosis was detected according to the "DNA ladder" which appeared after gel electrophoresis of apoptotic DNA (Fig. 1B, right). The ladder corresponded to the number of apoptotic cells estimated morphologically. The ladder formed by DNA fragments of about 200 bp in length (mononucleosomes) and their multiples could be visualized on the agarose gel if the percentage of apoptotic cells in the sample was greater than 4 % (our experience). Therefore no DNA ladder was detected in control cells (Fig. 1A right).

The relationship between the percentage of apoptotic cells in the population and the percentage of blocked cells in the G1 phase of the cell cycle was estimated statistically using the Spearman correlation coefficient. A high correlation $r_s=0.957$ was found ($P<0.05$).

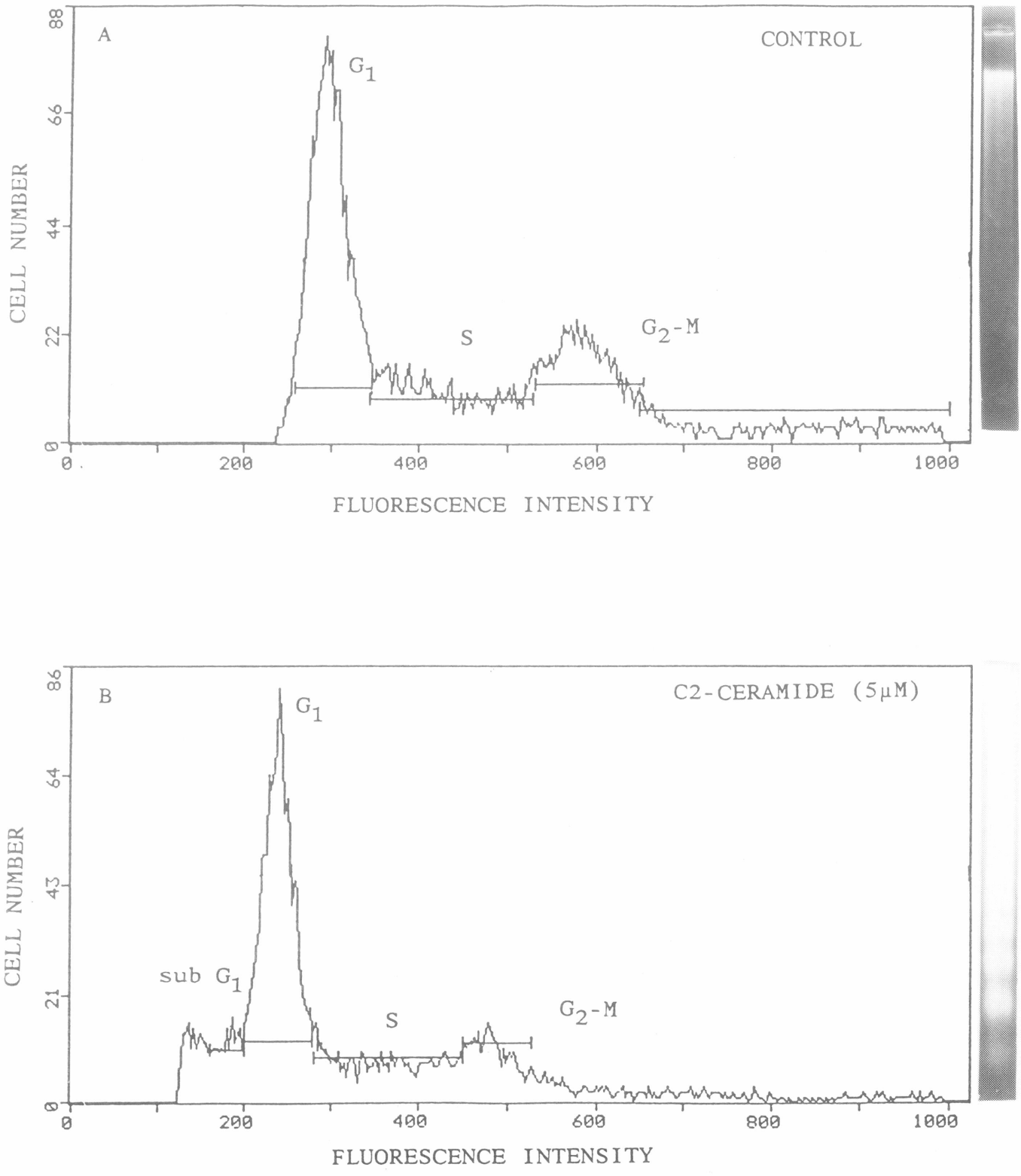

Fig. 1. *Flow cytometric estimation of the cell cycle and gel electrophoresis of DNA. A: Control HL-60 cells with no signs of apoptosis. A piece of agarose gel with DNA without apoptotic DNA fragmentation is shown on the right. B: HL−60 cells treated with C2-ceramide showing apoptotic sub-G1 peak. Apoptotic DNA ladder is shown on the right.*

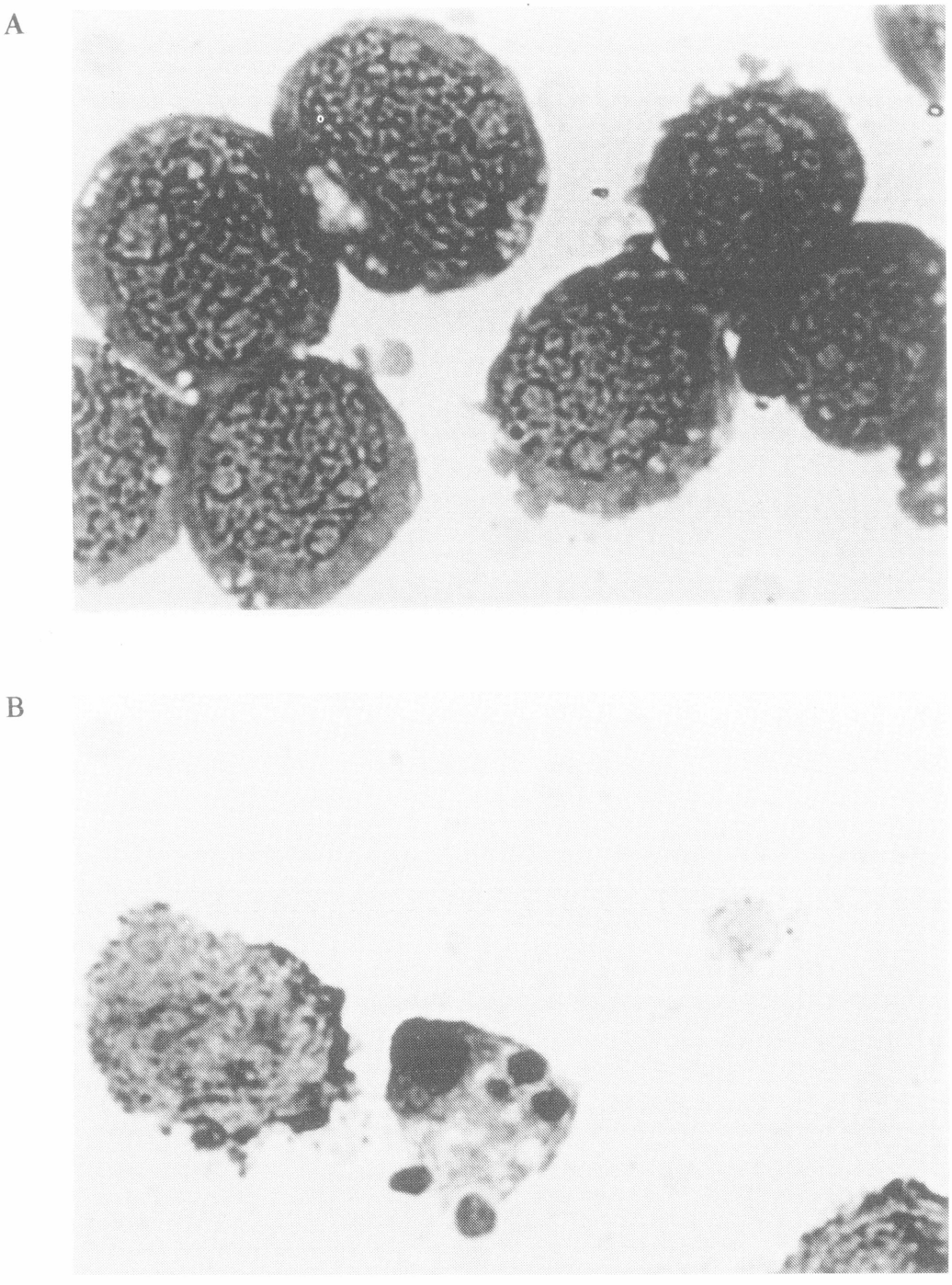

Fig. 2. *The typical control (upper panel) and apoptotic (lower panel) morphology of HL60 cells.*

Discussion

We have shown here that the addition of C2-ceramide to the HL-60 cells leads to G1 blockade of the cell cycle and to enhanced apoptosis. Previously, we reported that the addition of PMA to the HL-60 cells leads to G2/M blockade of the cell cycle without the activation of apoptosis. Both C2-ceramide as well as PMA treatment activate the *ras* oncogene (Gulbins *et al.* 1995) and downregulate the *c-myc* oncogene in HL-60 cells (Wolff *et al.* 1994) with a different impairment of the cell cycle and apoptosis. This may help to reveal the connection of the mechanisms leading to the blockade of the G1 phase of the cell

cycle and activation of apoptosis in HL-60 cells. We assume that the *c-myc* down-regulation may be accompanied by possible inhibition of cycline B expression (Milner et al. 1995) and blockade in the G2/M phase after PMA treatment. In this case, no activation of apoptosis was detected (Musilová *et al.* 1995). On the contrary, the supplementation of HL-60 cells with C2-ceramide led to the G1 arrest of the cell cycle and to the activation of apoptosis.

Apparently apoptosis appeared in cells blocked in G1. This is suggested here by the high correlation coefficient between the number of blocked and apoptotic cells. Due to the asychronic cell culture the apoptotic program is activated in a part of cells only. The results given above confirm that *ras* activation in itself is necessary but not sufficient for induction of apoptosis and that activation of apoptosis depends on the second signal which is an adequate blockade of the cell cycle.

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References

ALLEN P.D., BUSTIN S.A., MACEY M.G., JOHNSTON D.H., WILLIAMS N.S., NEWLAND A.C.: Programmed cell death (apoptosis) in immunity and haematological neoplasia. *Br. J. Biomed. Sci.* **50:** 135–149, 1993.

BENEDIK J.: In: *Biostatistik*, Brno, 1989, p. 172–174.

GONG J., TRAGANOS E., DARZYNKIEWICZ Z.: A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. *Anal. Biochem.* **218:** 314–319, 1994.

GULBINS E., BISSONNETTE R., MAHBOUBI A., MARTIN S., NISHIOKA W., BRUNNER T., BAIER G., BAIER-BITTERLICH G., BYRD C., LANG F., KOLESNICK R.N., ALTMAN A., GREEN D.: FAS-induced apoptosis is mediated via a ceramide-initiated ras signalling pathway. *Immunity* **2:** 341–351, 1995.

HANNUN Y.A., OBEID L.M.: Ceramide: an intracellular signal for apoptosis. *TIBS* **20:** 73–77, 1995.

JARVIS D.W., KOLESNICK R.N., FORNARI F.A., TRAYLOR R.S., GEWIRTZ D.A., GRANT S.: Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway. *Proc. Natl. Acad. Sci. USA* **91:** 73–77, 1994.

KERR J.R.F., WYLLIE A.M., CURRIE A.R.: Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br. J. Cancer* **26:** 239–257, 1972.

KIM M.–Y., LINARDIC C.M., OBEID L.M., HANNUN Y.A.: Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor α and γ-interferon. *J. Biol. Chem.* **266:** 484–488, 1991.

MANIATIS T., FRITSCH E.F., SAMBROOKE J.: *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory, 1982.

MATHIAS S., DRESSLER K.A., KOLESNICK R.N.: Characterization of a ceramide-activated protein kinase: stimulation by tumor necrosis factor α. *Proc. Natl. Acad. Sci. USA* **88:** 10009–10013, 1991.

MILNER A.E., GRAND R.J.A., GREGORY C.D.: Repression of apoptosis in Burkitt lymphoma cells by down–regulation of Myc protein: a common mechanism of bcl-2 dependent and independent survival. *Abstracts of the 9th International Congress of Immunology,* 23–29 July, San Francisco, Abstract No. 4402, 1995, p. 742.

MUSILOVÁ E., ŠPANOVÁ A., HOLÁ J., KOZUBÍK A.: Changes in the cell cycle induced by phorbolesters, retinoic acid, dimethyl sulfoxide, transforming growth factor-1 and starvation can influence the response of promyelocytic HL-60 cells to apoptosis after γ-irradiation. In: *Proc. Symp. Rad Biol. Appl. Space Res.* S. KOZUBEK, G. HORNECK (eds), Brno, 1995, pp. 50–58.

OBEID L.M., LINARDIC C.M., KAROLAK L.A., HANNUN Y.A.: Programmed cell death induced by ceramide. *Science* **259:** 1769–1771, 1993.

RAINES M.A., KOLESNICK R.N., GOLDE D.W.: Sphingomyelinase and ceramide activate mitogen-activated protein kinase in myeloid HL-60 cells. *J. Biol. Chem.* **268:** 14572–14575, 1993.

WILLIAMS G.T., SMITH C.A., MCCARTY N.J., GRIMES E.A.: Apoptosis: final control point in cell biology. *TICB* **2:** 263–267, 1992.

WOLF D., ROTTER V.: Major deletion in the gene encoding the p53 tumor antigen causes lack of p53 expression in HL-60 cells. *Proc. Natl. Acad. Sci. U.S.A.* **82:** 790–794, 1985.

WOLFF R.A., DOBROWSKY R.T., BIELAWSKA A., OBEID L.M., HANNUN Y.A.: Role of ceramide-activated protein phosphatase in ceramide–mediated signal transduction. *J. Biol. Chem.* 269: 19605–19609, 1994.

Reprint requests
Dr. E. Bártová, Institute of Pathological Physiology, Faculty of Medicine, Masaryk University, Komenského nám. 2, 662 43 Brno, Czech Republic.