Special Issue

Cell suicide in starving hybridoma culture: survival-signal effect of some amino acids

František Franěk & Karolína Šrámková

Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Key words: apoptosis, hybridoma, amino acids, starvation

Abstract

Two mouse hybridoma cell lines cultured in different basal media with the iron-rich protein-free supplement were subjected to deliberate starvation by inoculation into media diluted with saline to 50% or less. In the diluted media the growth was markedly suppressed and a large fraction of cells died by apoptosis. The cells could be rescued from apoptotic death by individual additions of amino acids, such as glycine, L-alanine, L-serine, L-threonine, L-proline, L-asparagine, L-glutamine, L-histidine, D-serine, β -alanine or taurine. Amino acids with hydrophobic or charged side chains were without effect. The apoptosis preventing activity manifested itself even in extremely diluted media, down to 10% of the standard medium. The activity of L-alanine in the protection of cells starving in 20% medium was shown also in semicontinuous culture. In the presence of 2 mM L-alanine the steady-state viable cell density more than doubled, with respect to control, and the apoptotic index dropped from 37% in the control to 16%. It was concluded that the apoptosis-preventing amino acids acted as signal molecules, rather than nutrients, and that the signal had a character of a survival factor. The specificity of present results, obtained with two different hybridomas, supports our view (Franěk and Chládková-Šrámková, 1995) that the membrane transport macromolecules themselves may play the role of the recognition elements in a signal transduction pathway controlling the survival of hybridoma cells.

Introduction

The character of death in hybridoma cultures

The environment of transformed animal cells in bioreactors is rather remote from the physiological environment of their non-transformed ancestors. When the culture conditions deviate significantly from the physiological range, cultured cells tend to respond by entering the programmed way of cell death, called apoptosis. This is particularly true for cells of haemopoietic origin, that are prone to undergo a disciplined suicide, whenever the external signals indicate a disbalance between cell generation and cell deletion. This behavior reflects the essential difference between microbes and animal cell lines. The unicellular microbial organ-

ism struggles for maintaining its individual life. On the other hand, transformed animal cells retain, to various degrees, the features of social behavior of cells constituting the animal organism, for which the well-being of the organism is dominating over the fate of the individual cell. An intrinsic suicide program, pre-existing in most metazoan animal cell, is normally suppressed by survival signals (Raff *et al.*, 1993).

The fact that apoptotic mode of death occurs in hybridoma cultures has been revealed several years ago (Al-Rubeai *et al.*, 1990; Franěk and Dolníková, 1991). In further studies the kinetics of apoptosis onset and the correlation with the exhaustion of nutrients has been revealed (Franěk and Dolníková, 1991a; Franěk *et al.*, 1992; Vomastek and Franěk, 1993; Singh *et al.*, 1994; Mercille and Massie, 1994). However, the

character of external stimulus, or stimuli, leading to the triggering of apoptosis has still not been identified. The identification of external apoptotic stimuli is complicated by the fact that the triggering of apoptosis is generally of double character, inductive or protective. Apoptosis is induced, in some cases, by specific signaling molecules, in other cases by withdrawal, or exhaustion, of survival factors (Raff, 1992; Williams and Smith, 1993).

Strategy of identification of apoptosis repressors

Mouse hybridomas growing in iron-rich protein-free medium (Franěk and Dolníková, 1991a; Franěk *et al.*, 1993) were found to die by apoptosis at the late stationary and decline phases of the batch culture. The extent of spontaneous apoptosis was found to be inversely related to the initial level of nutrients (Franěk and Dolníková, 1991a).

In order to elucidate the possible protective role of the components of the nutrient medium, and to dissect the effect of nutrients from the effect of toxic metabolites we developed a strategy based on a deliberate dilution of nutrients (Table 1). If the culture in standard medium serves as a model experimental setup, the dissection of the role of individual determinants is almost impossible. This situation is nontransparent, because both the low nutrients and the high metabolites levels influence the cells in the same direction, i.e. toward enhanced apoptosis. On the other hand, the model based on deliberately reduced nutrients is evidently more simple, the toxic metabolites are largely diluted out, their influence may be neglected, and the presumed protective effect of individual nutrients, over the baseline of poorly growing apoptotic culture, may be studied.

A quantitative screening method was developed in which the apoptosis-preventing effect of individual substances or mixtures of substances was expressed as the relative cell density N_r . This value was calculated from the viable cell density in standard medium (N_{st}), in diluted medium (N_{dil}), and in diluted medium with added test substance (N_{add}), using the formula

$$N_r = [(N_{add} - N_{dil}) / (N_{st} - N_{dil})] \times 100.$$

Findings of exploratory experiments obtained using the strategy of diluted basal medium

A significant cessation of growth with concurrent

increase of the apoptotic index were observed in media diluted below 50%. The hybridoma culture on the verge of starvation-induced apoptotic death proved to be sensitive to additions of individual groups of substances constituting the medium. It was found that the culture might be rescued by balanced amino acid mixtures, but not with a vitamin mixture. Surprisingly, the mixture of nonessential amino acids displayed the highest effect. Optimum effects were achieved at amino acid concentrations five to ten times exceeding the conventional levels (Franěk, 1995).

Using a single model hybridoma cell line, and basal medium diluted to 45%, the protective effect of individual medium components was tested. Significant suppression of apoptosis in the starving culture, and concurrent resumption of growth, could be achieved by millimolar concentrations of the following L-amino acids: glycine, alanine, serine, threonine, proline, asparagine, glutamine, histidine. Some activity was observed also with β -alanine, taurine and 4-aminobutyric acid. The set of active amino acids did not fit to any of the conventional categories of essential and nonessential amino acids (Eagle, 1959). On the basis of this finding we suggested that some amino acids did not act solely as nutrients, but also as specific signal molecules. The specificity of the effect pointed to the involvement of adaptively regulated amino acid transport systems A and N (Franěk and Chládková-Šrámková, 1995).

The findings of the exploratory experiments provoke numerous questions. For instance, is the protective effect generally valid for all hybridomas? Is the effect transient or may it last in continuous cultures? What is the lowest basal medium dilution in which hybridoma cells still continue to grow, and, do the protective amino acids suppress apoptosis even in this extremely diluted media? Why the individual amino acids are most active at nonphysiologically high concentrations? In the present paper we attempt at answering some of these questions.

Material and methods

Mouse hybridoma TSH-5.07 produces an IgG1 monoclonal antibody to thyrotropin. Mouse hybridoma ME-750 is an IgG2a producer of antibody to a lymphocyte membrane antigen. The two hybridoma cell clones used in the present experiment were established in the Institute of Molecular Genetics, Czechoslovak Acad-

Table 1. Strategy of investigation of apoptosis repressors and inducers

Experimental situation analyzed	Determinants of apoptosis			
	Nutrients (repressors)		Toxic metabolites (inducers)	
	Level	Apoptosis enhancement	Level	Apoptosis enhancement
Culture in standard medium at the stationary phase	Low	Yes	High	Yes
Culture in medium with deliberately reduced nutrients	Low by definition	Yes	Low	No

emy of Science. The myeloma partner was the line Sp2/0-Ag14.

The hybridoma TSH-5.07 was cultured in DMEM/F12 (1:1) basal medium. The hybridoma ME-750 was cultured in RPMI 1640 medium fortified with MEM essential amino acids and MEM vitamins. The media were buffered with Hepes (3.75 g l^{-1}) and sodium bicarbonate (2.0 g l^{-1}), and supplemented with the iron-rich protein-free growth-promoting mixture containing $200 \mu\text{M}$ ferric citrate (Franěk *et al.*, 1992). The media for cell starvation were prepared by diluting the basal media with phosphate-buffered saline (pH 7.2) supplemented with the iron-rich growth-promoting mixture. All cultures were kept at 37°C in a humidified atmosphere containing 5% CO_2 .

The short-term assays of the apoptosis-preventing activity were arranged in 1-ml volumes placed in 24-well multidishes. Quadruplicate analyses were set up at viable cell densities of $(1.3 \pm 0.2) \times 10^5$ cells/ml, and media dilution to 45% (hybridoma TSH-5.07) or 50% (hybridoma ME-750). The effect of tested substances was evaluated after 48 h (hybridoma TSH-5.07) or after 72 h (hybridoma ME-750). Three to six independent experiments were carried out for obtaining each relative viable cell density value.

Semicontinuous cultures were set up in 25 cm^2 T-flasks. From the total culture volume of 6 ml a volume of 2 ml was withdrawn each day, and replaced by 2 ml of diluted medium or diluted medium containing the tested substance. The values of cell density and of the apoptotic index were recorded daily after establishment of steady state. At least two independent experiments were carried out and five steady-state values from each experiment were averaged.

The viable cell densities were counted in a haemocytometer or determined upon addition of MTT (Mossman, 1983). The apoptotic index, i.e. the percent-

age of apoptotic cells relative to total cell density was determined by microscopic counting of cells displaying apoptotic morphology, i.e. shrunken cells with ruffled membrane (Franěk, 1995).

Results

The finding of apoptosis-preventing effect of some amino acids, shown in exploratory experiments, was complemented by an analogous screening carried out with another hybridoma. The eight L-amino acids identified in the first study (Franěk and Chládková-Šrámková, 1995) displayed significant activities, i.e. $N_r > 10\%$, with both these hybridomas (Fig. 1). The maximum relative viable cell densities N_r achieved by additions of the most active amino acids were comparable to maximum N_r values, 60 to 80%, observed with amino acid mixtures (Franěk, 1995). Although the two cell lines were cultured in different basal media, the set of amino acids exhibiting the apoptosis-preventing and growth-resuming activity was identical, the differences being only of quantitative character. This finding suggests an existence of a general mechanism underlying the effect under study, independent of the amino acid balance in the specific basal media.

The concentration profiles of the activity were characterized by relatively broad optimum zones in the millimolar concentration range (Fig. 2). Similar concentration profiles were obtained with other active L-amino acids (not shown). Interestingly, D-serine reached approximately 70% of the activity of L-serine, while the activity of D-glutamine remained on an insignificant level.

The testing of several non-coded amino acids at 2 mM concentrations, carried out with the hybridoma TSH-5.07, revealed relatively high apoptosis-

Figure 1. Relative viable cell density N_r in diluted media supplemented with L-amino acids. Final concentration of each amino acid was 2 mM. Solid columns – hybridoma TSH-5.07 (medium dilution 45%); shaded columns – hybridoma ME-750 (medium dilution 50%).

preventing activity of β -alanine ($N_r = 47\%$) and taurine ($N_r = 45\%$). The activity of glycine betaine ($N_r = 25\%$) and 4-aminobutyric acid ($N_r = 17\%$) was low but still significant. Sarcosine and reduced glutathione were inactive. 2-Aminoisobutyric acid yielded a low positive result ($N_r = 20\%$) with the hybridoma TSH-5.07, but a negative result with the hybridoma ME-750.

The effects reported in preceeding paragraphs were observed in media diluted to the range of 45 to 50%. In this range of media dilutions the apoptotic index in the control experiment without any additive was relatively high, 60 to 70% (Fränk, 1995), but the extent of apoptosis was not overwhelming. Therefore, we decided to verify the existence of the apoptosis prevention effect at medium dilutions from 10 to 40% (Fig. 3). The effect was apparent even in 10% medium. Moreover, the relative effect increased up to dilution of 15% (L-serine) or 10% (L-glutamine). The behavior of cells at media dilutions between 0% and 10% was difficult to be examined for technical reasons. The viable cell

densities were extremely low and reproducibility of the data was poor.

The severe starvation conditions of a 20% basal medium were selected for the investigation of the long-term anti-apoptotic effect of a typical amino acid in semi-continuous culture. Steady state established after four days, and was stable for more than one week. The addition of 2 mM L-alanine led to more than doubling of the steady-state viable cell number (Table 2). The values of the apoptotic index mirrored the viable cell numbers and confirmed thus that higher viable cell density in L-alanine-supplemented medium was associated with suppressed rate of apoptotic death.

Discussion

General features of the phenomenon

The protein-free hybridoma culture used in this study,

Figure 2. Relative viable cell density N_r of hybridoma TSH-5.07 in medium diluted to 45% and supplemented with selected amino acids at various concentrations.

Table 2. Growth and apoptosis in semicontinuous cultures of hybridoma ME-750 starving in 20% medium

Added amino acid	Viable cell number ^a		Apoptotic index ^a	
	Cells ml ⁻¹ × 10 ⁻³	Relative to control	%	Relative to standard medium
None (control)	221±11	1.00	37±3	4.1
2 mM L-Alanine	486±22	2.20	16±2	1.8
Standard medium	1405±106	6.35	9±1	1.0

^a Mean and SEM of steady-state values.

Figure 3. Viable cell densities of hybridoma TSH-5.07 in highly diluted media supplemented with amino acids. Upper panel: viable cell density with added amino acid relative to viable cell density in the respective control without amino acid (N_{add}/N_{dil}). Lower panel: Viable cell density in diluted medium relative to viable cell density in standard medium (N_{dil}/N_{st}). Final concentration of each amino acid was 4 mM. Average SEM was $\pm 8\%$ of the mean value.

and in our previous work (Franěk and Dolníková, 1991a; Franěk *et al.*, 1992; Vomastek and Franěk, 1993; Franěk, 1995; Franěk and Chládková-Šrámková, 1995), is unique among the systems serving for the investigation of the signals for cell death. No external growth factors are present. Thus, only low-molecular nutrients, and possibly some autocrine substances, may act as signaling molecules possessing the character of survival factors that maintain the cell cycle running, and block the intrinsic suicide programme pre-existing

in every cell. The novel strategy employed in the preceding studies (Franěk 1995; Franěk and Chládková-Šrámková, 1995) and in this work is based on the assumption that the culture on the verge of starvation-induced death will discriminate the functions of individual medium components more sharply than the cultures conducted in standard nutrient media.

The present work suggests that the favorable effect of elevated amino acid levels on cell viability has very little to do with the nutritive values of amino acids.

Five members of the active amino acid group (Fig. 1) belong to the category of nonessential amino acids, while only three (glutamine, threonine, histidine) are essential from the point of view of animal cell nutrition (Eagle 1959).

Our present results revealed the following features of the phenomenon of protection against apoptosis in starving hybridoma cultures through addition of selected substances:

- (a) Individual substances manifest effects quantitatively comparable to the effects achieved with balanced L-amino acid mixtures.
- (b) The effect is selective. Significant apoptosis-preventing activity is a property of less than ten coded L-amino acids. On the other hand, this activity is not confined to coded L-amino acids solely.
- (c) The action of these substances is equivalent to the effect of a survival factor that maintains the progression of the cell cycle, even if the levels of other nutrients are critically low.

The fact that individual substances may mimic the effect of balanced nutrient mixtures becomes conceivable, if we assume an existence of sensors that process the signal represented by a high extracellular level of a single active substance in the same manner as the signal provided by a mixture of amino acids, each present at a moderate concentration. The clue to the identification of the putative sensors obviously lies in the specificity patterns of the phenomenon.

Analysis of specificity

Membrane macromolecules recognizing certain groups of amino acids are known in their function of transport systems. Indeed, when we compare the specificity pattern of the apoptosis-preventing effect with the specificities of known amino acid functional categories (Table 3), we find highest overlap with some transmembrane transport systems. An almost complete fit can be reached when combining the specificity patterns of the sodium-dependent transport systems A (amino acids with short linear side chain), N (amino acids with nitrogen-containing side chain), and SATT (alanine, serine, threonine). The activities of the two D-amino acids tested (Fig. 2) are in agreement with the known high stereospecificity of glutamine-transporting system N, and the moderate stereospecificity of the serine-transporting system A (Shotwell *et al.*, 1983). The participation of systems ASC and SATT cannot be ruled out. However, they are highly stereospecific and

could not have mediated the observed positive effect of D-serine.

Seven out of thirteen nutritionally essential amino acids (Eagle, 1959) are transported into the cell via system L. In spite of the obvious importance of this system, none of its substrates participates in the protection of starving cells against apoptosis. It is necessary to remark, that the sodium-independent system L differs in many respects from the sodium-dependent system A, e.g. in the changes during the cell cycle or in exchange properties (Oxender *et al.*, 1977; Tupper *et al.*, 1976).

Possible mechanisms of action

It has been shown that aminoacyl-tRNA synthetases may participate in some cases of amino acid-dependent gene expression (Kilberg *et al.*, 1994). However, the activities of D-serine and of β -amino acids observed in this work seem to rule out that these enzymes play a general role in the control of apoptosis prevention.

The distinct character of signals for apoptosis prevention and of nutritional demands are apparent from the fact that most of the amino acids endowed with the apoptosis-preventing capacity belong to nonessential amino acids and some of them are secreted by hybridoma cells instead of being consumed. Numerous data exist showing that alanine, glycine, serine, asparagine or proline are under most circumstances produced by cultured hybridoma cells (Hiller *et al.*, 1993; Bushell *et al.*, 1994). This makes improbable the explanation of the phenomenon on the basis of supposed utilization of these amino acids in the energetic metabolism.

The working hypothesis that some amino acids act as signals, and transport systems as the recognition elements of the signal transduction pathway, implies that certain amino acids act as survival factors capable of protecting the cell against apoptosis and maintaining the progression of the cell cycle. The unusually high concentrations of the active substances, that are required for an optimal effect, are not surprising in the light of the knowledge of the K_m values of the membrane transport processes of the relevant amino acids, i.e. 100 μ M to 1 mM (Kilberg *et al.*, 1980). The possibility of the involvement of systems A and N in a signal transduction pathway is supported by the fact, that these systems are known to undergo adaptive regulatory changes, including gene expression, increased or new protein synthesis (reviewed by Kilberg *et al.*, 1994). The present results suggest that the signal recognition function of members of this family

Table 3. Specificity patterns of functional categories of amino acids

Category																	Reference		
Apoptosis-preventing set	<u>Gly</u>	<u>Ala</u>	<u>Ser</u>	<u>Thr</u>	<u>Pro</u>	<u>Asn</u>	<u>Gln</u>	<u>His</u>										This paper	
Essential amino acids				<u>Thr</u>			<u>Gln</u>	<u>His</u>	Arg	Lys	Cys	Ile	Leu	Val	Met	Tyr	Phe	Trp	Eagle, 1959
Induction of DNA synthesis							<u>Gln</u>					Ile							Ley and Tobey, 1970
Survival at low density			<u>Ser</u>			<u>Asn</u>	<u>Gln</u>				Cys								Eagle and Piez, 1962
Transport systems:																			
A	<u>Gly</u>	<u>Ala</u>	<u>Ser</u>	<u>Thr</u>	<u>Pro</u>	<u>Asn</u>								Val	Met				Kilberg, 1986
ASC		<u>Ala</u>	<u>Ser</u>								Cys								Christensen <i>et al.</i> , 1967
SATT		<u>Ala</u>	<u>Ser</u>	<u>Thr</u>															Shafqat <i>et al.</i> , 1993
N						<u>Asn</u>	<u>Gln</u>	<u>His</u>											Kilberg <i>et al.</i> , 1980
L												Ile	Leu	Val	Met	Tyr	Phe	Trp	Shotwell <i>et al.</i> , 1982

Amino acids identical with the apoptosis-preventing set are underlined.

of transporters, as well as the linkage with adaptive gene expression, may take part in the control of cell cycle progression and programmed cell death.

Implications for hybridoma technology

The tendency to commit suicide long before the nutrients are exhausted represents an unpleasant property of hybridoma cells. To overcome this tendency sophisticated feeding strategies have been designed or, alternatively, attempts at transfecting these cells with apoptosis-suppressing genes, such as bcl-2, have been made.

Our findings promise a simple and rational way to oppose the hybridoma's suicidal tendency by increasing the levels of some amino acids or closely analogous substances. These additives are entirely harmless to the possible therapeutic product of the technology, because they represent components of common basal media.

The cultures conducted under reduced levels of nutrients approach the situation in high cell density cultures, in which the concentrations of nutrients become critically low. It is intended to develop further the strategy of 'deceiving' the sensors for amino acid concentrations, whether they are identical with membrane transporters or not, and to find generally useful remedies against premature suicide in hybridoma cultures.

Acknowledgements

The authors are indebted to Miss Jana Bimková for her skillful technical assistance. This work was supported in part by the Grant No. 503/93/2292 of the Grant

Agency of the Czech Republic, and the Grant No. I/68 957 of the Volkswagen Foundation.

References

- Al-Rubeai M, Mills D and Emery AN (1990) Electron microscopy of hybridoma cells with special regard to monoclonal antibody production. *Cytotechnology* 4: 13–28.
- Bushell ME, Bell SL, Scott MF, Spier RE, Wardell JN and Sanders PG (1994) Enhancement of monoclonal antibody yield by hybridoma fed-batch culture, resulting in extended maintenance of viable cell population. *Biotechnol Bioeng* 44: 1099–1106.
- Christensen HN, Liang M and Archer EG (1967) A distinct Na⁺-requiring transport system for alanine, serine, cysteine, and similar amino acids. *J Biol Chem* 242: 5237–5246.
- Eagle H (1959) Amino acid metabolism in mammalian cell cultures. *Science* 130: 432–437.
- Eagle H and Piez K (1962) The population-dependent requirement by cultured mammalian cells for metabolites which they can synthesize. *J Exp Med* 116: 29–43.
- Franěk F (1995) Starvation-induced programmed death of hybridoma cells: Prevention by amino acid mixtures. *Biotechnol Bioeng* 45: 86–90.
- Franěk F and Chládková-Šrámková K (1995) Apoptosis and nutrition: Involvement of amino acid transport system in repression of hybridoma cell death. *Cytotechnology* 18: 113–117.
- Franěk F and Dolníková J (1991) Nucleosomes occurring in protein-free hybridoma cell culture. Evidence for programmed cell death. *FEBS Letters* 284: 285–287.
- Franěk F and Dolníková J (1991a) Hybridoma growth and monoclonal antibody production in iron-rich protein-free medium: Effect of nutrient concentration. *Cytotechnology* 7: 33–38.
- Franěk F, Vomastek T and Dolníková J (1992) Fragmented DNA and apoptotic bodies document the programmed way of cell death in hybridoma cultures. *Cytotechnology* 9: 117–123.
- Hiller GW, Clark DS and Blanch HW (1993) Cell retention-chemostat studies of hybridoma cells – Analysis of hybridoma growth and metabolism in continuous suspension culture on serum-free medium. *Biotechnol Bioeng* 42: 185–195.

- Kilberg MS (1986) System A-mediated amino acid transport: metabolic control at the plasma membrane. *Trends Biochem Sci* 11: 183–186.
- Kilberg MS, Handlogten ME and Christensen HN (1980) Characteristics of an amino acid transport system in rat liver for glutamine, asparagine, histidine, and closely related analogs. *J Biol Chem* 255: 4011–4019.
- Kilberg MS, Hutson RG and Laine RO (1994) Amino-acid regulated gene expression in eukaryotic cells. *FASEB J* 8: 13–19.
- Ley KD and Tobey RA (1970) Regulation of initiation of DNA synthesis in Chinese hamster cells II. Induction of DNA synthesis and cell division by isoleucine and glutamine in G₁-arrested cells in suspension culture. *J Cell Biol* 47: 453–459.
- Mercille S and Massie B (1994) Induction of apoptosis in nutrient-deprived cultures of hybridoma and myeloma cells. *Biotechnol Bioeng* 44: 1140–1154.
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assay. *J Immunol Methods* 65: 55–63.
- Oxender DL, Lee M and Cecchini G (1977) Regulation of amino acid transport activity and growth rate of animal cells in culture. *J Biol Chem* 252: 2680–2683.
- Raff M (1992) Social controls on cell survival and cell death. *Nature* 356: 397–400.
- Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y and Jacobson MD (1993) Programmed cell death and the control of cell survival: Lessons from the nervous system. *Science* 262: 695–699.
- Shafqat S, Tamarappoo BK, Kilberg MS, Puranam RS, McNamara JO, Guadano-Ferraz A and Freneau RT Jr. (1993) Cloning and expression of a novel Na⁺-dependent neutral amino acid transporter structurally related to mammalian Na⁺/glutamate co-transporters. *J Biol Chem* 268: 15351–15355.
- Shotwell MA, Kilberg MS and Oxender DL (1983) The regulation of neutral amino acid transport in mammalian cells. *Biochim Biophys Acta* 737: 267–284.
- Shotwell MA, Mattes PM, Jayme DW and Oxender DL (1982) Regulation of amino acid transport system L in Chinese hamster ovary cells. *J Biol Chem* 257: 2974–2980.
- Singh RP, Al-Rubeai M, Gregory CD and Emery AN (1994) Cell death in bioreactors: A role for apoptosis. *Biotechnol Bioeng* 44: 720–726.
- Tupper JT, Mills B and Zorngiotti F (1976) Membrane transport in synchronized Ehrlich ascites tumor cells: Uptake of amino acids by the A and L system during the cell cycle. *J Cell Physiol* 88: 77–88.
- Vomastek T and Franěk F (1993) Kinetics of development of spontaneous apoptosis in B cell hybridoma cultures. *Immunol Letters* 35: 19–24.
- Williams GT and Smith CA (1993) Molecular regulation of apoptosis: Genetic controls on cell death. *Cell* 74: 777–779.

Address for correspondence: F. Franěk, Institute of Molecular Genetics, Vídeňská 1083, CZ-14220 Praha 4, Czech Republic.