

In vitro Early Allogeneic Reaction of Murine Brain Cortex Cells

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Summary

An allogeneic reaction among brain cortex cells (mixed reaction) was demonstrated previously by H-2 alloantigen-induced uncoupling of oxidative metabolism (Kovářů *Med. Biol.* 58: 273, 1980). In the present study we have demonstrated that alloantigen already increased cell surface Na⁺,K⁺-ATPase activity after 100 min when the enzyme activation was highest at Mg²⁺/ATP ratio 4:1. The allogeneic cell reaction was accompanied by an elevation of membrane lipid fluidity and probably also by a thermotropic lipid phase transition which might influence the membrane lipid-dependent Na⁺,K⁺-ATPase activity, while Mg²⁺-ATPase remained unaffected. Furthermore, the effects of proteins and peptides released into the supernatant during the allogeneic reaction were analyzed in brain cortex cells. One of the isolated active peptide fractions, F_A (m.w. lower than 2.5 kD), was able to enhance Na⁺,K⁺-ATPase activity as well as to block K⁺-evoked O₂ uptake by brain cortex cells. Thus the F_A fraction simulated primary allorecognition events. The data indicate that various brain cell surface domains were influenced by a regulatory peptide fraction of the cytokine type during the early phase of allogeneic reaction. Allorecognition among brain cortex cells is directed against functionally important metabolic reactions.

Key words

Brain cells – Allorecognition – Surface Na⁺,K⁺-ATPase – Cytokine peptide fraction

Introduction

It is known that interaction of allogeneic lymphocytes differing in the major histocompatibility complex (MHC) caused a subsequent proliferation in a mixed lymphocyte reaction *in vitro* (MLR) (Janis and Bach 1970). This reaction represents an *in vitro* model of the graft vs host reaction with generation of cytotoxic T lymphocytes and multiple cytokines. Generally, lymphocyte stimulation is a two signal phenomenon. T cell receptor ligation, termed as signal 1, is followed by a costimulatory signal(s), termed as signal 2 (for review see Zier *et al.* 1996). Cytokine

cross-talk participates very intensively in signal 2 transduction (Toungouz *et al.* 1995). The earliest event in lymphocyte allorecognition is manifested by increased protein tyrosine phosphorylation accompanied by phospholipid hydrolysis *via* phospholipase C and changes in intracellular Ca²⁺ level (Isakov *et al.* 1994). Other events, such as crosslinking and/or adhesion, participate in triggering lymphocyte activation, e.g. T cell activation includes HLA class II molecules linked to protein tyrosine kinase and protein kinase C stimulation and adhesion with the participation of CD 11-a molecule (Odum *et al.* 1992).

Results were preliminary presented at "CNS – Advances in Research of Normal and Neoplastic Cells" which was held in Brno (April 25, 1996) as the satellite minisymposium of the 42nd International Congress of the European Tissue Culture Society (Kovářů *et al.* 1996).

Term "activation" refers to a highly pleiotropic set of cellular responses including cell cycle entry (G0 to G1 phase transition) during a 2 to 4 h period (for review see June 1991). The activation phase is accompanied by a number of stimulatory and costimulatory events involving changes of ion regulation with important participation of Na^+ , K^+ -ATPase (Na^+ , K^+ pump), Na^+ / H^+ exchangers, voltage-gated channels, ATP-driven Ca^{2+} pump, Ca^{2+} -sensitive K^+ -channels (Grinstein and Dixon 1989, Tedder and Engel 1994). Activation of Na^+ , K^+ -ATPase is dependent on the membrane phospholipid microenvironment, which influences membrane lipid fluidity. The allosteric properties of the Na^+ , K^+ -ATPase complex are regulated by its β subunit with a highly glycosylated terminal part which is oriented externally (Ferber *et al.* 1976, Kovářů *et al.* 1982a, Resch *et al.* 1983, Norby 1989). Pleiotropic effects of Na^+ , K^+ -ATPase are also associated with receptor-like properties (Anner 1985). For example, an inhibitory action of hypothalamic factor on enzyme activity from the outer cell surface was described by Carilli *et al.* (1985).

As mentioned above, lymphocyte stimulation switched on by signal 2 is dependent on post-receptor cytokine signalling accompanied by stimulatory and costimulatory changes, such as tyrosine kinases (Janus kinases) (Ihle 1995). A stimulatory effect of interleukin-2 on the activity of Na^+ , K^+ -ATPase was also demonstrated (Redondo *et al.* 1986).

It is generally accepted that cytokines affect cell surface through their receptors (Ihle 1995, Rothwell and Hopkins 1995). These authors assumed that the diversity of cytokine responses reflects multiple sites of action or involvement of different receptors or their subtypes. Similar mechanisms appear to be involved in regulating lineage commitment and cellular division, maturation or differentiation of the nervous and haematopoietic systems. Cytokines IL-5, IL-7, IL-9 and IL-11 could influence the development of ion channels and action potentials of cultured brain neurones (for review see Rothwell and Hopkins 1995). On the other hand, IL-4, IL-6, IL-7, IL-8 and tumour necrosis factor- α (TNF α) are able to enhance cell growth and survival of cultured neurones. Other cytokine effects are known, e.g. IL-2 is not only an immunoregulatory molecule but also an analgesic molecule. Pain threshold was increased after injecting human IL-2 into the lateral cerebral ventricle of rats (Jiang *et al.* 1995).

The CNS was considered to be an immunologically privileged organ not participating in cell-mediated immune reactions. According to new data, T lymphocytes infiltrate into the brain under some pathological conditions (viral encephalitis, multiple sclerosis, etc.) (Fabry *et al.* 1994) and antigen presentation is provided astrocytes, oligodendrocytes or

microglia as non-professional antigen-presenting cells (Hertz *et al.* 1990). Spontaneous occurrence of MHC Class II antigens is low in the CNS but their expression can be enhanced e.g. by interferon γ (Rothwell and Hopkins 1995). Furthermore, the ability of cultured glial or glioma cells to stimulate clones of allospecific T cells and to induce a mixed lymphocyte reaction *in vitro* was found (Takiguchi *et al.* 1985, Birnbaum *et al.* 1986).

Allorecognition of brain cortex cells was manifested by uncoupling of oxidative metabolism which was dependent on H-2 alloantigen differences as was demonstrated by using congenic lines (Kovářů 1980). These findings indicate that a metabolic paradox occurs during allogeneic brain cortex cell interaction. Blockade of high K^+ -induced O_2 uptake by interacting cells was accompanied by a marked breakdown of energy-rich ATP and creatine phosphate. This was in contrast to syngeneic controls in which a high K^+ -effect, (*in vitro* simulating depolarization), is associated with stimulated O_2 uptake coupled to decrease of energy-rich phosphates as a consequence of stimulation of Na^+ , K^+ -ATPase activity. These K^+ -dependent changes in normal brain cortex cells are well known (for review see Tsacopoulos and Magistretti 1996).

We studied the requirements needed for demonstration of alloantigen-evoked changes in Na^+ , K^+ -ATPase, such as the Mg^{2+} /ATP ratio, possibility of thermotropic lipid phase transition, and alteration of membrane lipid fluidity. We analysed cell-free supernatants of the allogeneic reaction and the effects of protein and peptide fractions on Na^+ , K^+ -ATPase activity, Mg^{2+} -ATPase and O_2 uptake by brain cortex cells to verify the possibility of participation of the supernatant fraction on the primary allogeneic reaction in a cytokine-like manner.

Material and Methods

Animals

Specific pathogen-free 2-month-old male A/Ph and C3H/Cbi/BOM inbred strains and conventional CBA/J mice (Velaz Farm Šumice and Prague, Czech Republic) were used.

Preparation of brain cells

Brain cortex cell suspensions from each strain were isolated separately by a mechanical sieving procedure of brain cortex fragments through nylon sieves (Seidengasfabrik, Thal-St. Gallen, Switzerland) in the isolation medium which consisted of 120 mM NaCl, 3.8 mM KCl, 1.5 mM CaCl_2 , 1.5 mM MgSO_4 , 1.2 mM KH_2PO_4 (all chemicals from Aristar) and 30 mM Tris-HCl (Sigma), pH 7.3 with 7.5 % polyvinylpyrrolidone (PVP, Fluka). The cell suspension was then washed repeatedly in the same medium

without PVP and resuspended in the appropriate volume. Cell suspensions were examined under phase contrast microscope. The samples consisted of approximately 70–80 % reasonably intact perikarya (for details see Kovářů and Lodin 1975).

Allogeneic reaction

Allogeneic cell suspensions consisted of equal parts of syngeneic brain cortex cells of A/Ola and C3H/Cbi/BOM strains with protein content of 1.5 mg/0.2 ml of final volume of the incubation medium. Two steps of cell incubation involved an stationary incubation in an ice-cold bath for 30 min with subsequent addition of 1 ml 100 mM D-glucose (Serva) followed by dynamic incubation in a shaking water bath at 37 °C for 60 min. The allogeneic reaction was stopped by cooling of samples to 21 °C for 5 min in ice-cold bath with the dilution (Kovářů 1980) to final 800 µg protein/ml. Enzyme activity was estimated immediately. Syngeneic cells, incubated under the same conditions, were used as control. Instead of enzyme assays served allogeneic cells as a source of cell-free supernatants (see below).

Estimation of ATPase activity

Samples of cell suspensions in a concentration of 80 µg of protein in 0.1 ml were resuspended in a buffer: 100 mM NaCl, 20 or 50 mM KCl, 4 mM MgCl₂, 25 mM Tris-HCl, pH 7.3 with 1 mM ATP (Serva), if not indicated otherwise. Some of samples contained 0.3 mM ouabain (Sigma). The activity of Na⁺,K⁺-ATPase was calculated as the difference between the ouabain-resistant Mg²⁺-ATPase and total ATPase activities (for further details see Kovářů and Lodin 1980).

Fluorescence anisotropy

Alloantigen-activated cells were incubated with lipophilic fluorescent probes N-phenyl-naphthylamine (NPN) and 1,6-diphenyl-1,3,5-hexatriene (DPH) for 30 min at 37 °C in a final concentration of 6 x 10⁻⁶ M and measured immediately (for details see Kovářů *et al.* 1982a).

Fractionation of supernatants

Incubated allogeneic cells were centrifuged at 12,000 x g for 15 min at 4 °C and the supernatants were immediately frozen at -25 °C. The thawed supernatants were pressure-dialysed in an Amicon ultrafiltration cell with UM-10 Diaflo membrane (Amicon Co.) at 4 °C. The high molecular portion (HMW) (>10 kD) was resuspended in an incubation medium to the initial volume and was tested immediately. Low molecular part (LMW) of the supernatants (<10 kD) was lyophilized. The powder, dissolved in an eluant was applied to the column of Biogel P-2, particle diameter from 75 to 150 µm (BioRad Labs) with 1 M acetic acid as eluant. The absorbance of each fraction was measured at 280 nm and 254 nm and collected A, B, C fractions were lyophilized and stored until use at 4 °C. Supernatants and their fractions were tested in quantities proportional to initial supernatants volumes and number of cells.

Protein determination

Protein concentrations in brain cell suspensions were determined according to modified biuret method (Kovářů and Lodin 1980). For further details see Kozáková *et al.* (1990, 1997).

Table 1. Effect of Mg²⁺/ATP ratio on allogeneic response of brain cortex cells

Reaction mixture		Cells	N	Na ⁺ ,K ⁺ -ATPase	Mg ²⁺ -ATPase
Mg ²⁺ (mM)	ATP (mM)			∑µmol Pi/mg protein/h	
3	0.4	syngeneic	7	2.31±0.21	4.33±0.17
		allogeneic	9	2.84±0.09	3.91±0.19
4	1.0	syngeneic	8	4.50±0.16	4.52±0.48
		allogeneic	10	5.91±0.61	4.13±0.22
1.5	1.2	syngeneic	8	4.88±0.18	3.53±0.11
		allogeneic	9	4.67±0.24	3.62±0.22

Data are means ± S.D. Cell incubation: 60 min with 5 mM K⁺ + 10 min with 50 mM K⁺ (37 °C). Substrate: 10 mM glucose.

Table 2
Effect of temperature on allogeneic response of brain cortex cells

Cooling	Cells	Na ⁺ ,K ⁺ -ATPase (% of control)	Mg ²⁺ -ATPase (% of control)
Direct 37 °C-->4 °C	allogeneic	89.9±3.8	105.3±3.9
	syngeneic	95.3±4.6	112.8±5.5
Stepwise 37 °C-->21 °C-->4 °C	allogeneic	141.1±6.6	103.1±8.0
	syngeneic	108.2±3.0	102.2±4.2

Results are means of 4 separate experiments ± S.D.

Table 3
Fluorescence anisotropy in membranes of allogeneic brain cortex cells

Cells	N	NPN	%	DPH	%
Syngeneic	4	0.147±0.013	100.0	0.280±0.010	100.0
Allogeneic	6	0.141±0.012	95.9	0.271±0.012	96.8

Data are means ± S.D. Fluorescence probes: NPN - *N*-phenyl-1-naphthylamine, DPH - 1,6-diphenyl-1,3,5-hexatriene.

Results

We analysed the effects of various Mg²⁺/ATP ratios on Na⁺,K⁺-ATPase and Mg²⁺-ATPase activities during the allogeneic reaction (Table 1). It is evident that the 4:1 ratio induced a very high Na⁺,K⁺-ATPase elevation in the allogeneic response without an Mg²⁺-ATPase change.

A temperature-dependence at the end of the dynamic phase of allogeneic cell incubation was analysed and their ATPase activities were also estimated. As shown in Table 2, when the cells were cooled immediately from 37 °C to 4 °C, both Na⁺,K⁺-ATPase and Mg²⁺-ATPase activities of allogeneic combination of cells were not changed as compared to the syngeneic controls. Stepwise cooling from 37 °C via 21 °C to 4 °C greatly enhanced Na⁺,K⁺-ATPase activity of mixed cells. No changes were found in Mg²⁺-ATPase activity.

Table 3 summarizes the estimations of fluorescence lipid anisotropy, which was decreased in allogeneic cells (i.e. membrane fluidity was increased) in comparison to the control using both lipophilic fluorescent probes *N*-phenyl-naphthylamine (NPN) and 1,6-diphenyl-1,3,5-hexatriene (DPH).

K⁺-induced changes in both enzyme activities of allogeneic and control cells were estimated (Fig. 1). The incubated cells served as sources of enzyme activities in the enzyme buffer with 50 mM K⁺ (Fig. 1a) or 20 mM K⁺ (Fig. 1b). Na⁺,K⁺-ATPase activity of allogeneic cells, assayed in the presence of non-stimulating 20 mM K⁺ concentration, was elevated very rapidly (3 min) as compared to the controls (Fig. 1b). No influence of Mg²⁺-ATPase activity was observed either in allogeneic or control cells (Fig. 1c,d).

In other experiments, supernatants from allogeneic cells were ultrafiltrated and the portion lower than 10 kD was fractionated on Biogel P-2 (Fig. 2a). Furthermore, its absorbance spectrum has maxima at 215 and 310 nm (Fig. 2b).

The results summarized in Table 4 indicate activity of individual supernatants and their fractions on O₂ uptake (measured for 30 min) and on enzyme activities in secondary cells. It is evident that the FA peptide fraction evoked changes that were comparable with alloantigen-induced alteration.

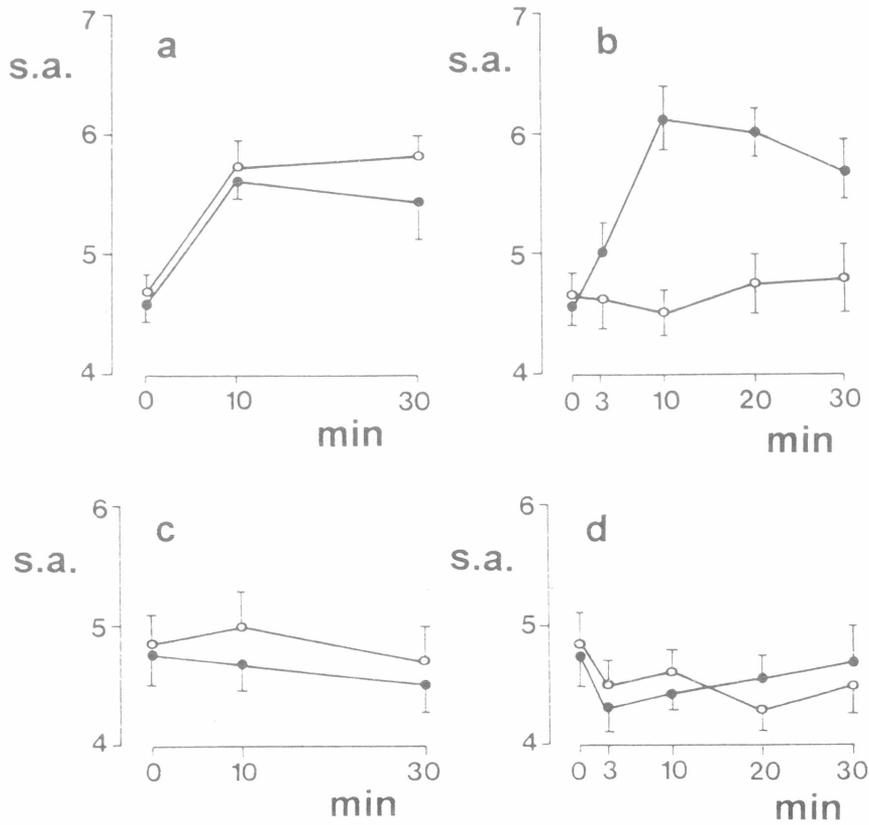


Fig. 1. Effect of K^+ ions on ATPase activities of brain cells. Na^+,K^+ -ATPase activity with 50 mM K^+ (panel a), 20 mM K^+ (panel b), Mg^{2+} -ATPase activity with 50 mM K^+ (panel c) or 20 mM K^+ (panel d). Full circles - allogeneic cells, open circles - syngeneic controls, s.a. - specific activity (mol P_i /mg protein/h). Data are means of 3 experiments \pm S.D.

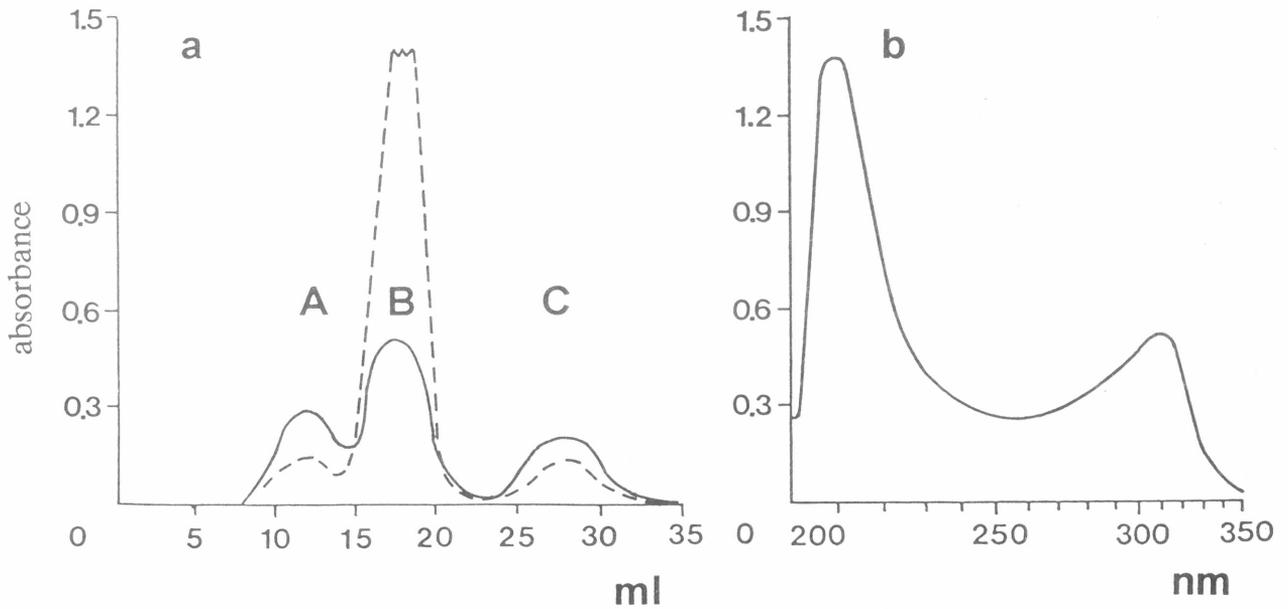


Fig. 2. Elution profile of LMW-S2 supernatant from Biogel P-2 gel chromatography (panel a), solid line - absorbance at 280 nm, dashed line at 254 nm. Absorbance profile of Biogel P-2 separated A fraction (F_A) of LMW-S2 supernatant (panel b).

We also compared intensity and time course of Na^+, K^+ -ATPase activation and K^+ -induced O_2 uptake by brain cells evoked by alloantigen reaction or F_A peptide fraction. Alloantigen-evoked stimulation of enzyme activity was 121 %, 142 % and 118% at 3, 10 and 30 min, respectively (Fig. 1b). Similar F_A fraction-induced increase of brain cell Na^+, K^+ -ATPase activity by 117 %, 134 % and 112 % was observed at the same incubation intervals (Kovářů 1992). Alloantigen reaction-evoked K^+ -induced O_2 uptake was 118 %, 105 %, and 92 % at 10, 20 and 30 min (Kovářů 1980).

Similarly, the F_A fraction affected K^+ -induced O_2 uptake by 113 %, 80 % and 71 % in comparison with 146 %, 133 % and 129 % in controls, respectively (Kovářů 1992). It is evident that the F_A peptide fraction was able to mimic a K^+ -induced O_2 uptake blockade as well as stimulation of Na^+, K^+ -ATPase activity in secondary cells in which a metabolic paradox was similar to that observed in primary allogeneic cell reaction. Furthermore, alloantigen- or F_A peptide fraction-evoked Na^+, K^+ -ATPase activation preceded a stepwise decrease of O_2 uptake.

Table 4

Effect of AR-induced supernatant and its components on secondary brain cortex cell response

Supernatant portion	O_2 uptake		$\text{Na}^+ \text{K}^+$ -ATPase
	5 mM K^+ $\mu\text{mol O}_2/\text{mg protein/h}$	50 mM K^+ % of initial values	s.a. $\mu\text{mol Pi}/\text{mg protein/h}$
None	55.9±1.9	39.1±3.6	4.36±0.21
S ₁	63.6±2.0	35.3±2.0	n.d.
S ₂	66.2±3.4	6.1±2.0	5.62±0.41
MW-S ₂	57.5±1.8	43.6±0.8	4.32±0.52
LMW-S ₂	68.4±1.5	9.4±1.9	5.27±0.47
LMW-S ₂ -F _A	64.1±2.7	-13.9±5.0	5.47±0.22
LMW-S ₂ -F _B	56.8±2.3	45.3±4.6	4.16±0.37
LMW-S ₂ -F _C	53.4±2.7	42.5±4.0	4.74±0.62

S₁ – supernatant after stationary incubation (Kovářů 1980)

S₂ – supernatant after stationary and dynamic incubation

HMW, LMW – high m.w. (> 10 kD) and low m.w. (< 10 kD) portion of S₂ supernatant

F_A, F_B, F_C – fractions eluted from Biogel P-2 column (see Fig. 2a)

Data are means ± S.D. of 7–9 estimations.

Discussion

In this paper we demonstrated the ability of adult murine brain cortex cells to switch on an allorecognition reaction and to respond to alloantigens. This is in agreement with our former studies (Kovářů 1980) and with the first demonstration of allogeneic reaction among brain cortex cells (Kovářů *et al.* 1972). Our findings have demonstrated an allogeneic brain cortex cell interaction as a metabolic paradox based on high K^+ -evoked oxidative metabolism blockade (Kovářů 1976, 1980). The allogeneic reaction, an unusual event among brain cortex cells (Kovářů and Kovářů 1979, Kozáková *et al.* 1990), depends on several critical conditions, such as the number of cells, the duration and type of stationary or dynamic incubation.

Tight cell-to-cell contact, important for self vs non-self recognition, was provided during the short stationary incubation. When this incubation was omitted or when cell-to-cell contact was inhibited by polyvinylpyrrolidone, no metabolic response was observed in the mixed reaction (Kovářů 1980, 1992). Similar conditions must be fulfilled for triggering of the allogeneic lymphocyte reaction (Janis and Bach 1970, Kovářů and Kovářů 1979).

The presented results indicate that alloantigen-induced changes of Na^+, K^+ -ATPase can be linked to an optimal $\text{Mg}^{2+}/\text{ATP}$ ratio (Table 1) and possible repeating conformational changes of the phosphorylated/dephosphorylated enzyme complex (Norby 1989) could also be involved. The temperature-dependent step during buffer dilution of allogeneic

cells accompanying cooling from 37 °C to 4 °C with an intermediate step at 21 °C (Table 2), indicated that probably thermotropic lipid transition linked to lipid-dependent regulatory Na⁺,K⁺-ATPase activity is involved. The critical temperature in the range of 19–22 °C for brain tissue related to lipid phase transition was estimated according to Arrhenius plots (Nemat-Gorgani and Meisami 1979, Sandermann 1983). The allosteric Na⁺,K⁺-ATPase complex is functionally dependent on phosphatidylserine or phosphatidylcholine in the membrane phospholipid microenvironment (Ferber *et al.* 1976, Resch *et al.* 1983, Oishi *et al.* 1990, Kovářů *et al.* 1982a, 1990). The allogeneic reaction of brain cortex cells was also accompanied by higher membrane lipid fluidity, measured with lipophilic fluorescence probes, mainly reflecting changes in the hydrophobic core of the lipid bilayer. Thus the alloantigen-induced Na⁺,K⁺-ATPase activity indicate changes in the relationship to the phospholipid microenvironment which is in agreement with increased membrane lipid fluidity in allogeneic lymphocytes (Halliday *et al.* 1979). Other lipid-dependent regulatory enzymes such as protein kinase C and 5'-nucleotidase (CD 73) are involved in lymphocyte stimulation (Szamel *et al.* 1989, Kozáková *et al.* 1990).

It is known that the stimulation of lymphocytes with the usually studied mitogenic lectin is associated with changes in membrane ion channel activity and other changes regulating ion transport, including

Na⁺,K⁺-ATPase, which participate in the initial steps of stimulated lymphocyte proliferation (Kaplan 1978, Kovářů and Kovářů 1979, Pieri *et al.* 1989, Grinstein and Dixon 1989).

The isolated F_A fraction (Fig. 2) is of peptide nature as is evident from UV spectra and other characteristics (Kozáková *et al.* 1997). Absorbance at 310 nm can indicate the presence of a metal ion, such as Zn²⁺, similarly as in the immunosuppressive peptide thymulin or serum thymic factor (FTS) (Kobayashi *et al.* 1990).

The results indicate that the release of allorecognition-induced peptide fraction participate in the mechanisms of the observed uncoupling of oxidative metabolism. The demonstrated changes in the allogeneic brain cortex reaction are directed against functionally important physiological specificities of CNS. Thus, this cytokine peptide can operate as a physiological messenger during a model allogeneic reaction in agreement with a more general mechanism of cytokine pleiotropic actions (Rothwell and Hopkins 1995, Jiang *et al.* 1995). More detailed cytokine effects are analysed in the following paper of this issue (Kozáková *et al.* 1997).

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