Physiol. Res. 46: 137 – 144, 1997

Cell Surface Enzymes of Brain Cortex Cells or Lymphocytes During Early Allogeneic Reaction

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Received January 14, 1997
Accepted February 10, 1997

Summary

The aim was to study the role of major histocompatibility complex (MHC), in mice named H-2, during early allogeneic reactions (AR) of brain cortex cells or lymphocytes. We used neuronal and glial enriched perikarya, spleen and thymus lymphocytes or their subpopulations. Rat AR was also assayed between C-6 astrocytoma cells and spleen lymphocytes. We demonstrated that: 1) H-2 dependent stimulation of Na^+,K^+-ATPase and ouabain-sensitive K^+-dependent p-nitrophenylphosphatase (K^+-pNPPase) activities represented specific response in both AR of unseparated brain cells or lymphocytes. On the other hand, non-specific AR-induced stimulation of Ca^{2+}-ATPase activity was observed. 2) Allogeneic enriched glial fractions reacted similarly by the same enzyme activation in contrast to no change in AR between enriched neuronal fractions. Allorecognition ability of glial cells was confirmed by AR between C-6 astrocytoma cells and lymphocytes. 3) Mature thymus lymphocytes exerted alloreactivity by specific activation of Na^+,K^+-ATPase or K^+-pNPPase, in contrast to no change in AR between immature lymphocyte subpopulations. 4) MHC Class II monoclonal antibody inhibited Na^+,K^+-ATPase and K^+-pNPPase activities in brain cells as well as in thymus and spleen lymphocytes in a dose-dependent manner. Results support former studies about alloantigen-induced uncoupling in brain oxidative cortex metabolism (Kovářů *Med. Biol.* 58: 273, 1980) *via* Na^+,K^+-ATPase and K^+-pNPPase inhibition by mechanism which can mimic MHC restriction.

Key words

H-2 allorecognition – Astrocytes – C-6 glioma cells – Lymphocytes – Na^+,K^+-ATPase activation – Na^+,K^+-ATPase and MHC Class II antibody interaction

Introduction

There is now considerable information concerning neuroimmunological perspectives based on the concept of CNS cells participating in immune functions (for review see Fabry *et al.* 1994, Hart and Fabry 1995). CNS cells can act as antigen-presenting cells of non-professional, inductive type, expressing MHC Class I and Class II molecules. In human allogeneic reaction, adult glial cells ($GFAP^+$) or glioma cell lines with MHC Class II antigen positivity induced proliferation of lymphocytes after 7 days (Takiguchi *et al.* 1985). Murine astrocyte transplants into anterior eye chamber serve as antigen-presenting cells (Lublin *et al.* 1992). It is suggested that MHC Class II antigens are normal constituents of human cerebral microvasculature (Graeber *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).

It has been suggested that MHC antigens, in addition to their function in immune restriction-linked interactions, exert a non-immune activity by their association with surface receptors for insulin, glucagon, endorphin, and epidermal growth factor, thereby affecting their binding affinity for the specific peptide ligands (for review see Brams and Claesson 1989).

We have earlier demonstrated allorecognition-dependent metabolic paradox in interacting brain cortex cells related to K^+-oxidative metabolism uncoupling accompanied by O_2 uptake decrease and breakdown of ATP and creatine phosphate levels (Kovářů 1976, 1980, Kovářů and Lodin 1980). In preceding paper of this issue (Kovářů *et al.* 1997) alloantigen-induced increase of brain cortex Na^+,K^+-ATPase activity was linked to changes in conformation and allosteric properties of this regulatory enzyme complex (Sandermann 1983). Early allogeneic reaction of brain cells or lymphocytes is accompanied by other activation events, such as Ca^{2+}-ATPase stimulation and 5'-nucleotidase (CD 73) inhibition in contrast to unchanged Mg^{2+}-ATPase (Kovářů and Kovářů 1979, Kozáková *et al.* 1990, Kovářů 1992). Alloantigen-induced lymphocyte interaction is generally named as mixed lymphocyte reaction (MLR). We focused our attention on analyses of allorecognition-induced changes of Na^+,K^+-ATPase and its external part ouabain-sensitive K^+-pNPPase representing K^+-dependent dephosphorylation step (Robinson *et al.* 1983, Norby 1989). We tested alloantigen-induced Na^+,K^+-ATPase activation in astrocytes because they are not only antigen-presenting cells but could also be the cells participating in active response mimicking MHC-induced restriction. Murine glial AR was compared with those of thymus T lymphocytes, known for low occurrence of MHC Class II antigens (Pichler and Wyss-Coray 1994), and spleen lymphocytes as professional antigen-presenting cells. Idea of glial alloreactivity was also analysed in AR between C-6 glioma cells and spleen lymphocytes. Possibility of MHC Class II monoclonal antibody interaction with Na^+,K^+-ATPase and K^+-pNPPase of C-6 cells or lymphocytes was assayed.

Material and Methods

Animals

Two-month-old male mice of SPF inbred strains A/Ph and C3H/Cbi/BOM as well as conventional CBA/J strain and IVN inbred rats were purchased from Velaz (Šumice or Prague). Two- to three-month-old male mice of the congenic lines named B.10 D2, C57 BL/10 Sn and B10.LP/Sn Ph were kindly provided by Dr. Pěknicová from Institute of Molecular Genetics, AS CR, Prague.

Brain cortex cell suspension

Brain tissue fragments were dissociated by mechanical sieving through nylon sieves in polyvinylpyrrolidone-containing isolation medium, wash out into appropriate isotonic incubation media and immediately used in AR (for details see Kovářů 1980, Kovářů *et al.* 1997).

Preparation of enriched neuronal and glial fraction

Trypsinized and sieved brain cortex cell suspensions from 3–4 weeks old mice (Kovářů 1980) were prepared and washed. Sedimented cells (from 5 cortices) were resuspended in 20 ml of 30 % Percoll (Pharmacia) in 320 mM saccharose, 0.1 % BSA (Serva for tissue culture purity), 5 mM glucose, 10 mM HEPES, pH 7.3 and centrifuged in swing-out rotor (5,000 x *g* at 4 °C for 20 min). Cells floating between top layer (myelin, debris) and sediment (erythrocytes, nuclei, debris) were diluted to 20 % Percoll by the same solution and gradient in situ was performed in angle rotor (20, 000 x *g* at 4 °C for 30 min). Upper and lower part of floating perikarya was taken as enriched glial and neuronal fraction, respectively, upon morphological checking (Kovářů 1992).

Preparation of thymus or spleen lymphocytes

Thymus or spleen tissue fragments were dissociated in Potter-Elvehjem homogenizer in MEM medium, 10 mM HEPES, pH 7.3. Spleen lymphocytes were purified by elimination of erythrocytes by hypotonic lysis and cells of reticula by clumping in Ca^{2+}- and Mg^{2+}-free buffer (Kovářů and Kovářů 1979, Kovářů *et al.* 1979) Spleen cells were kept in MEM medium containing 1% BSA, 10 mM HEPES, pH 7.3 at 4 °C overnight, then centrifuged and diluted in appropriate buffers.

Preparation of thymus lymphocyte subpopulation

Fresh thymus cell suspension (1 x 10^8/ml) was resuspended in 20 ml of 85 % Percoll (Pharmacia) in 250 mM saccharose, 0.4 % BSA and 10 mM HEPES, pH 7.3. *In situ* Percoll gradient was performed at angle rotor at 32,000 x *g* for 30 min at 4 °C. Two subpopulations T_1 and T_2 (1.102 and 1.133 g/ml with density marker beads, Pharmacia) were separated. Cells were then washed three times in isotonic medium and resuspended in appropriate buffer for enzyme activity assays. T cell subpopulations were characterized by FITC-PNA labelling, morphometric analysis, and functional tests (lectin-induced proliferation). Mature T_1 subpopulation of small lymphocytes was FITC-PNA negative and represented 2.5 % of initial thymus lymphocyte suspension. Cells were kept in MEM medium containing 1 % BSA, 10 mM HEPES, pH 7.3 at 4 °C overnight.

C-6 glioma cells

Rat cells of Wistar strain origin (ATCC CLM 107, Rockville, MD) were cultured in MEM medium with 5 % bovine fetal medium and gentamycine for three days (for details see Giacometti *et al.* 1988). Cell suspension was prepared by mechanical dissociation without trypsinization. Cells were washed out and transferred into appropriate isotonic incubation medium and used immediately in AR.

Allogeneic cells

Two way ARs were performed in ratio 1:1 with stationary and dynamic incubation for 100 min (brain) or 120 min (lymphocytes). Initial cell amount were adequate to 7 mg of protein/ml (brain), 10^7 cells/ml (lymphocytes, C-6 cells + lymphocytes), (Kovářů and Kovářů 1979, Kovářů 1980, Kovářů *et al.* 1997).

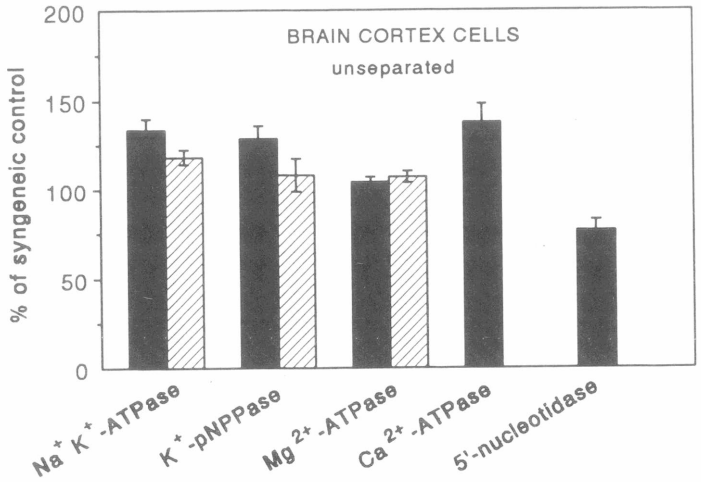

Fig. 1. *Allogeneic reactions of brain cortex cells from inbred strains. Results are means ± S.D. of 9–12 measurements. Full columns indicate allogeneic cells (A/Ph + C3H/Cbi/Bom; H2a + H2k haplotypes) whereas hatched columns represent syngeneic cells.*

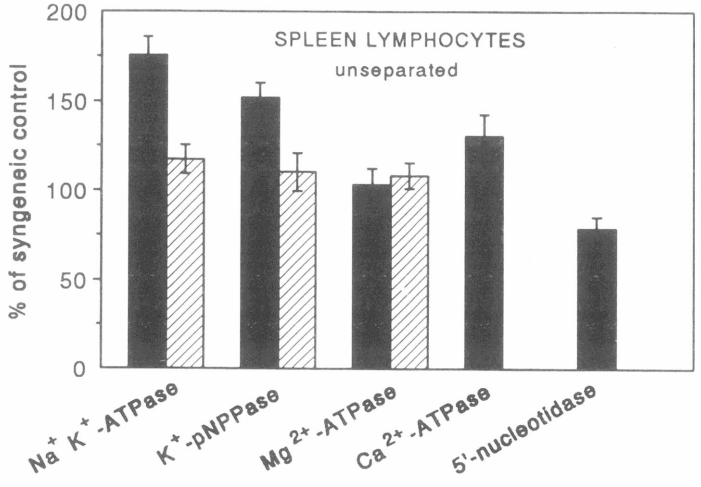

Fig. 2. *Allogeneic reactions of spleen lymphocytes from inbred strains. Results are means ± S.D. of 8–10 measurements. For other legend see Fig. 1.*

Enzyme activities

ATPase activities were estimated by spectrophotometric semimicromethod of released inorganic phosphate. Enzyme buffers were of various compositions in mM: Na^+,K^+-ATPase – 120 (100) NaCl, 20 (10) KCl, 4 (3) $MgCl_2$ for brain cells (lymphocytes), respectively, with 30 Tris-HCl, pH 7.3, ±0.3 mM ouabain, 1 mM ATP (Serva, synthetic). Mg^{2+}-ATPase activity was estimated as difference between total ATPase and ouabain-sensitive Na^+,K^+-ATPase. Ca^{2+}-ATPase – 1.5 mM $CaCl_2$, no $MgCl_2$, 1 mM ATP and other salts as for Na^+,K^+-ATPase. 5'-nucleotidase (in mM) – 100 NaCl, 20 KCl, 4 $MgCl_2$, 1.5 $CaCl_2$, 30 Tris-HCl, pH 7.3, 1 mM 5'-AMP for both cell types. The activity of pNPPase was estimated in buffer with 240 (200) mM D-mannitol (Serva) for brain cells (lymphocytes), respectively, instead of NaCl, other salt concentrations were the same as for Na^+,K^+-ATPase, ±0.3 mM ouabain, 1 mM p-nitrophenylphosphate, (Robinson *et al.* 1983, Kovářů

1992). For other details see preceding papers (Kovářů *et al.* 1997, Kozáková *et al.* 1990, 1997).

Cell incubation with monoclonal antibodies

We used antirat MHC Class II monoclonal antibody of P7/7 type (Serotec) and HAE9 monoclonal antibody against human erythroblasts as negative controls (gift of prof. Abelev from Oncological Centre, Academy of Medical Sciences, Moscow). Cells were incubated with indicated amount of monoclonal antibody in enzyme buffer at ice bath for 15 min. Cells were then washed and transferred into appropriate enzyme buffer and enzyme activity assays were performed.

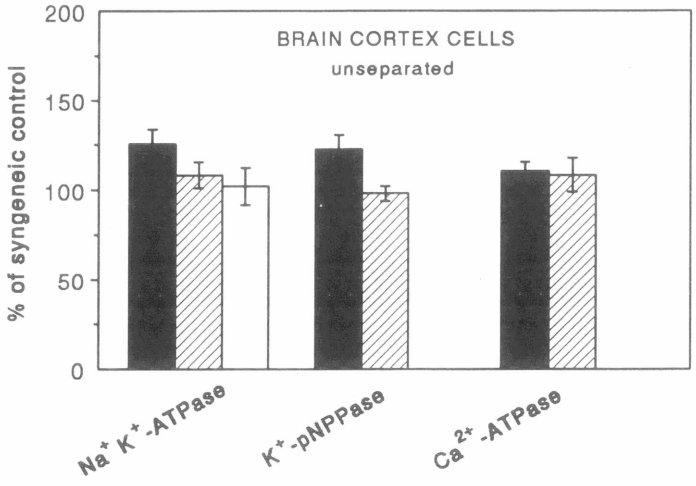

Fig. 3. *Allogeneic reactions of brain cortex cells from congenic lines. Results are means ± S.D. of 6–8 measurements. Full columns represent congenic lines differing in H-2 (B.10 D2 + C57 BL/10 Sn), hatched columns show congenic lines different in H-13 (C57 BL/10 Sn + B10.LP/SnPh) whereas open column demonstrates H-Y difference (males + females of B.10 D2 or C57 BL/10 Sn congenic lines).*

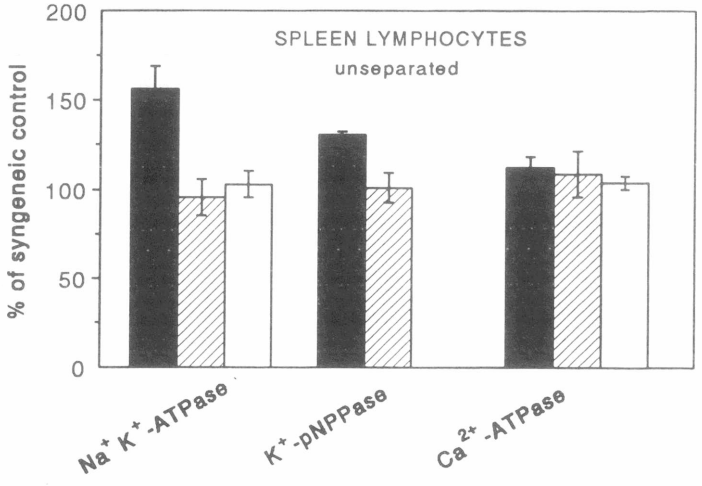

Fig. 4. *Allogeneic reactions of spleen lymphocytes from congenic lines. Results are means ± S.D. of 6–8 measurements. For other legend see Fig. 3.*

Results

We performed comparative study of enzyme activities in ARs between unseparated brain cells or lymphocytes. Qualitative similarities between both interacting systems were detected (Figs 1 and 2), i.e. activation of Na^+,K^+-ATPase, K^+-pNPPase and Ca^{2+}-ATPase, inhibition of ecto-5'-nucleotidase and unchanged Mg^{2+}-ATPase. Quantitative responses were smaller in brain AR in comparison with lymphocyte AR. Question arises if the alloantigen-induced changes in surface enzyme activities can be dependent on H-2 and/or non-H-2 differences. For allogeneic reactions we therefore used congenic lines with H-2 differences and with differences in H-13 or H-Y antigens (differences between males and females of the same congenic line) (Figs 3 and 4). We demonstrated that the stimulation of Na^+,K^+-ATPase

or K^+-NPPase represented a specific response, which was dependent on H-2 alloantigen differences in both brain or lymphocyte ARs. This was in contrast to non-specific changes in Ca^{2+}-ATPase dependent on both H-2 and non-H-2 differences. Further experiments were performed to assay which brain cortex main cell types (neuron-, glia-enriched fractions) or thymus lymphocyte T subpopulations participate in AR (Fig. 5). In brain AR, we observed alloantigen-induced glial-glial stimulation of Na^+,K^+-ATPase and K^+-pNPPase activities. Partial extent of alloantigen response was found between glial-enriched and neuronal-enriched fractions and no response between neuronal perikarya interaction. In AR between T lymphocyte subpopulations, we demonstrated Na^+,K^+-ATPase and K^+-pNPPase activation of mature T_1 subfractions. Combinations of T_1 + T_2 cell fractions in AR partially influenced enzyme activities whereas T_2 + T_2 AR did not affect them.

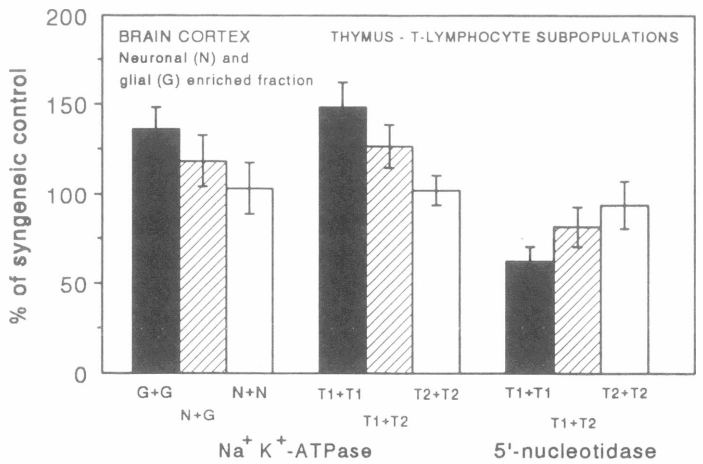

Fig. 5. *Allogeneic reactions of brain cortex cell fractions or thymus T-lymphocyte subpopulations from inbred strains (A/Ph + CBA/J; H2a + H2k haplotypes). Results are means ± S.D. of 5–7 measurements.*

Table 1. Enzyme activities during allogeneic reaction of rat C-6 glioma cells and spleen lymphocytes

Cells	Enzyme	% of controls
C-6 + lymphocytes	Na^+K^+-ATPase	172.4±12.8
	Ca^{2+}-ATPase	135.7±19.3
	5'-nucleotidase	92.0± 8.1

Basal enzyme activity (nmol $P_i/10^6$ cells/h) – Na^+,K^+-ATPase: 183 (C-6), 21 (Ly); Ca^{2+}-ATPase: 24 (C-6), 128 (Ly); 5'-nucleotidase: 7–8 (C-6, Ly). Control value was calculated as average from initial enzyme activity in C-6 cells and lymphocytes. Data are means ± S.D. of 8–10 measurements.

Possibility of alloantigen-induced response between rat glial cells and lymphocytes was assayed using rat AR between C-6 astrocytoma cells and spleen lymphocytes (Table 1). We demonstrated allorecognition of lymphocytes by C-6 cells with stimulation of Na^+,K^+-ATPase activity because of enzyme activity is nearly one order higher in C-6 cells in comparison with lymphocytes. Allogeneic reaction also comprised 5'-nucleotidase (CD 73) inhibition. We demonstrated dose-dependent inhibitory effects of antirat MHC Class II monoclonal antibody on Na^+,K^+-ATPase and K^+-pNPPase activities in rat spleen or thymus lymphocytes and C-6 cells . MHC Class II monoclonal antibody-induced inhibitory effects were comparable in all tested cells, i.e. in cells with low MHC Class II occurrence (thymus lymphocytes and C-6 glioma cells) as well as in high MHC Class II positive spleen lymphocytes. On the other hand, Mg^{2+}-ATPase, ouabain-resistant p-NPPase and Ca^{2+}-ATPase activities were not influenced by MHC Class II monoclonal antibody. No effect of human erythroblast HAE-9 monoclonal antibody as negative control was found (Tables 2, 3 and 4).

Table 2. Effect of monoclonal antibody against rat MHC Class II on enzyme activities of C-6 glioma cells

MHC Class II antibody μg/ml	Na^+K^+-ATPase	K^+-pNPPase ouabain-sensitive		pNPPase ouabain-resistant	Ca^{2+}-ATPase
		No K^+	20 mM K^+		
0	100	100	100	100	100
12	97.4±5.5	105.1±3.2	107.3±6.7	101.2±3.2	103.8±3.7
25	66.1±8.4	111.2±2.4	57.4±5.3	103.4±1.9	92.5±5.4
50	50.9±7.3	92.4±7.2	42.6±9.3	108.6±6.7	107.1±7.3

Data (in % of control enzyme activity) are means of 3–4 experiments ± S.D.

Table 3. Effect of HAE-9 monoclonal antibody against erythroblast on enzyme activities of C-6 glioma cells

HAE-9 antibody μg/ml	Na^+K^+-ATPase	K^+-pNPPase ouabain-sensitive	
		no K^+	20 mM K^+
0	100	100	100
12	108.7± 7.9	108.3±4.2	91.1±6.7
25	109.4± 8.5	102.6±6.6	102.8±7.9
50	92.3±11.1	88.4±2.2	115.3±5.6

Data (in % of control enzyme activity) are means of 3 experiments ± S.D.

Table 4. Effect of monoclonal antibody against rat MHC Class II on enzyme activities of rat thymus or spleen lymphocytes

MHC Class II antibody (μg/ml)	K^+-pNPPase ouabain-sensitive		pNPPase ouabain-resistant	
	thymus	spleen	thymus	spleen
0	100	100	100	100
12	102.3±10.1	83.4± 7.3	106.8± 6.7	98.3±8.4
25	64.6± 9.4	66.4±12.1	94.9± 9.1	109.2±6.1
50	37.1± 8.6	52.7± 5.8	98.4±10.6	94.1±5.2

Data (in % of control enzyme activity) are means of 3 experiments ± S.D.

Discussion

Data presented here describe the early H-2 allorecognition *via* non-conventional cell surface events in brain cortex cells or their astrocyte fraction. This was comparable with AR of spleen unseparated lymphocytes or allogeneic T_1 subpopulations of mature thymus lymphocyte. We demonstrated allorecognition-induced activation of Na^+,K^+-ATPase or its external part ouabain-sensitive K^+-dependent pNPPase. On the other hand, it is suggested that resting T lymphocytes, which express very little MHC Class II antigens, lack the ability to present antigen and to stimulate MLR (Pichler and Wyss-Coray 1994) with a special respect

to murine T cells. Our results indicated a possibility to use higher amounts (10^7/ml) of T_1 cells interacting in early AR (Kovářů 1992). In AR between mature T_1 lymphocytes, these subpopulations were highly enriched in comparison with 2.5 % of these cells in initial thymus cell suspension. Thus alloantigen-induced changes were measurable in T_1 AR in contrast to no change in AR between initial thymocyte cell suspension with the same cell concentration.

We demonstrated the ability of glial cells to switch on allorecognition but not with typical MHC-dependent mechanism under the defined *in vitro* conditions, and it is known inducible astrocyte activity as antigen presenting cell (for review see Hertz *et al.* 1990). Similar results were obtained in AR of C-6 astrocytoma cells with lymphocytes, indicating allorecognition reactivity of C-6 cells. We estimated MHC Class II antigen positivity by fluorescence microscopy in thymus lymphocytes and C-6 cells, which was lower than 5 % in contrast to nearly 18% positive spleen lymphocytes. On the other hand, fluorescence-activated cell sorter analysis of C-6 glioma cells labelled with antirat MHC Class II monoclonal antibody exhibited average 2.4 % positivity the whole population, but in minor subpopulation of C-6 cells, we estimated 16.7 % positive large granular cells (Kovářů, Kovářů and Zendulková – unpublished results). There is question if minor MHC Classs II positive subpopulation of C-6 cells is able to initiate enzyme changes in AR, or if glial AR induced enzyme changes can be based on other non-conventional mechanism.

Our results can be related to the demonstrated dose-dependent inhibitory effect of MHC Class II antibody on Na^+,K^+-ATPase activity or ouabain-sensitive K^+-dependent p-NPPase activity (Tables 2, 3 and 4). This finding is in agreement with our former experiments about pig or human lymphocyte Na^+,K^+-ATPase and K^+-pNPPase, which were inhibited by antihuman Class II monoclonal antibody (cross-reacting with pig lymphocytes) (Kovářů *et al.* 1988, Kovářů 1992). Furthermore, these assays demonstrated that pig and human lymphocyte K^+-pNPPase activity was not blocked by antiMHC Class II antibody when K^+ ions were omitted in the assay. Similar K^+-dependence was demonstrated for K^+-pNPPase of C-6 glioma cells.

It was reported that Na^+,K^+-ATPase can participate in phylogenetically conserved self vs non-self recognition events of immunocyte (granulocyte) of *Limulus polyphemus* based on GlcNAc/NANA saccharide specificity (Gupta *et al.* 1991) of highly glycosylated β chain of enzyme molecule with regulatory properties compared to α enzyme subunit with catalytic activity (Norby 1989). Adhesion events can also be included in recognition events with participation of astroglia (Kovářů 1976, 1980). It was proven that adhesion AMOG molecule in brain has 40 % amino acid homology with β-subunit of Na^+,K^+-ATPase (Schachner 1991, Isenmann *et al.* 1995). There are new data, indicating amino acid homology of β chain of Na^+,K^+-ATPase with MHC Class II associated invariant chain (Ii) peptide (Baum *et al.* 1996). Thus we can conclude that both allogeneic reaction of brain cells or lymphocytes *via* early activation of Na^+,K^+-ATPase or its external part ouabain-sensitive K^+-dependent p-NPPase possesses features of H-2 alloreactivity, probably with mimicry of MHC-dependent restriction.

Acknowledgement

Supported by grant 309/95/1121 (Grant Agency of Czech Republic). Estimation of 5'-nucleotidase activity was performed by Dr. H. Kozáková from Institute of Microbiology, Academy of Sciences, Prague.

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