Biochemical Markers of Lymphocyte Maturation

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Abstract

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We studied maturation-associated changes of cell membrane Na^+, K^+- and Mg^{2+}-ATPase activities and gamma-glutamyltranspeptidase activity (GGT) during prenatal and postnatal development of lymphocytes in pig thymus and lymph nodes, i.e. primary or secondary lymphoid organs. Furthermore, we analyzed the developmental changes in expression of alpha subunits (Gs, Gq/11) of membrane heterotrimeric GTP-binding proteins.

We demonstrated marked developmental decrease of both Na^+, K^+- and Mg^{2+}-ATPase activities in thymus and lymph node lymphocytes. On the other hand, we found elevated membrane gamma-glutamyltranspeptidase (GGT) activity as a function of age in lymph node lymphocytes in contrast to declined GGT in thymus. These findings are probably associated with antibody formation related to antigenic pressure during postnatal development.

Finally, we assayed developmental changes of G alpha (s) and G alpha q/11 subunits in thymus and lymph nodes. There were ontogenetic specifities in expression of G alpha (s) or G alpha q/11 and appropriate effector enzyme systems generating of 2nd messengers cAMP or 1,4,5 IP_3, respectively, in cell signal transduction.

The results of this study contribute to our understanding of the dynamic changes of both enzymes and G alpha subunit profiles at the level lymphocyte cell surface linked to maturation events in ontogenetic development.

Pig, ontogeny, thymus, lymph nodes, lymphocytes, ATPases, gamma-glutamyltranspeptidase, heterotrimeric GTP-binding protein, G-protein

It is generally accepted that T and B lymphocytes and their subpopulations are characterized by morphological and phenotypic markers, including surface antigens or receptors. Their cell surface enzyme equipment can be different as well. These enzymes belong to various groups, e.g. phosphatases, nucleotidases, kinases, peptidases and glycosidases with possible functions in cell activation, maturation, differentiation, molecules transducing cell signal and adhesive structures (Naquet and Pierres 1991; Benrezzak et al. 1999).

Adenosine triphosphate phosphohydrolases (ATPases), catalysing hydrolysis of ATP to ADP and inorganic phosphate, are ubiquitous transmembrane enzymes in higher eukaryotes, (Masat et al. 1996; Anner and Volet 1999). When catalytic activity of ecto-ATPases is inhibited, e.g. Mg^{2+}, Ca^{2+}- ATPase by irreversible antagonists, then T-, B- and NK cell effector functions are influenced, e.g. antigen-induced cytokine secretion and cytolytic activity, antibody formation and spontaneous proliferation (Barankiewicz et al. 1988; Dombrowski et al. 1998).

Na^+, K^+-ATPase is a fundamental enzyme system dependent on composition and properties of phospholipid microenvironment and it consists of alpha and beta subunit (Webb et al. 1995; Anner and Volet 1999). Various structural changes in enzyme

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molecule, such a loss of beta-1 subunit, are associated with maturation states in T and B lymphocytes and subsets in (Masat et al.1996).

Besides cell surface ATPases from lipid-dependent regulatory group, we focused our attention to gamma-glutamyltranspeptidase (GGT), an enzyme without lipid microenvironment influence. This enzyme catalyses glutamyl residue transfer from gamma-glutamylpeptides and their derivatives to a cceptors. GGT plays a role in glutathione metabolism and transport of amino acids and small peptides across cell membrane (Lisý and Lodin 1977; Kovářů 1992; Lawrence et al. 2000). Higher GGT activity can be related to differentiation stages of normal and neoplastic cells. Immunoregulatory role of GGT and glutathione *in vivo* was studied using GGT knockout mice or GGT participation in effector T cells (Lawrence et al. 2000; Roozendaal et al. 2001).

The above-mentioned enzymes are a part of modulation of cell signal transduction. Important role in transmembrane cell signalling is played by membrane heterotrimetric GTP-binding proteins (G proteins). Heptahelical transmembrane receptors for external signal molecules (drugs, neurotransmitters, hormones, cytokines etc.) are coupled to G proteins, composed of, and chains that act as cell signal transducers and amplifiers modulating more effector systems and second messenger pathways (Malbon 1997). Functionally important G subunits differ in effects on effectors: Gs and Gi (stimulation and inhibition of adenylyl cyclase, etc.), Gq/11 (influence of phospholipase C) and Go (ion channel action), (Milligan 1988; Malbon 1997).

Molecular physiology mechanisms underlying B- and T-cell development are poorly understood as yet. Differential G protein expression in B- and T-cell development including G alpha s and G alpha q/11 were studied (Grant et al. 1997). Role of G protein-coupled receptor in T cell development is suggested, including development of T helper cell immune responses (Zabel et al. 1999). T lymphocyte activation with engagement of T cell receptor-CD3 complex is modulated by heterotrimeric G proteins, including G alpha q/11 family (Tsoukas et al. 2000). Modulation in expression of main type G alpha subunits of natural killer lymphocytes was investigated (Kovářů et al. 2001).

We focused our attention on more detailed analyzes of spontaneous developmental changes of cell membrane Na^+, K^+- and Mg^{2+}- ATPase activities during prenatal and postnatal development of pig thymus and lymph nodes, primary and secondary lymphoid organs. Furthermore, we studied changes of gamma-glutamyltranspeptidase activity (GGT). We also analyzed the developmental levels of G alpha subunit (Gs, Gq/11) in pig thymus and lymph nodes. This study is expected to contribute to information about dynamic changes of both enzyme and G alpha subunit profiles at the level lymphocyte cell surface linked to maturation events.

Materials and Methods

Experimental animals

We used fetuses and piglets (Czech Large White; 8 for each age) of three litters. Optimal halothan-oxygen-N$_2$O anaesthesia was used during experimental hysterotomy and other experimental procedures (Kovářů et al. 1971; Kovářů et al. this volume). Fetuses FD 90 and FD 110, which were used as newborn, were obtained by experimental hysterotomy. Piglets of this age (FD 110) are routinely used e.g. in gnotobiology as germ-free animals and their organ systems are fully functionally and morphologically developed. Postnatal piglets were reared under physiological conditions and fed maternal milk. Starting day 5 to day 30, ČOS-1 commercial feed mixture was provided. On day 30 (weaning), a stepwise change for ČOS-2 commercial diet was performed and this diet was used until day 60. Experimental animal care was provided according to recommendation of FELASA and European Community directives.

Preparation of thymus or lymph node lymphocytes

Thymus or lymph node tissue fragments were dissociated in Potter-Elvehjem homogenizer in MEM medium, 10 mM HEPES, pH 7.3, spleen lymphocyte were purified by elimination of erythrocytes by hypotonic lysis and cells of reticula by clumping in Ca^{2+}, Mg^{2+} - free buffer. Cells were kept in 1% BSA containing MEM medium, 10 mM HEPES, pH 7.3 at 4 °C overnight (for other details, see Kovářů et al. 1997).

Enzyme activities

ATPase activities were measured by spectrophotometric semimicromethod of released inorganic phosphate. Enzyme buffers were of various compositions in mM: Na^+, K^+- ATPase - 100 NaCl, 10 KCl, 3 $MgCl_2$, with 30 Tris-HCl, pH 7.3 +/- 0.3 mM ouabain, 1mM ATP (Serva, synthetic), Mg^{2+}- ATPase activity was estimated as difference between total ATPase and ouabain sensitive Na^+, K^+- ATPase, For other details see preceding papers (Kovářů et al. 1997).

GGT activity

Enzyme activity was estimated by using gamma-glutamyl-p-nitroanilide as the donor of the gamma-glutamyl residue in presence glycylglycine as the acceptor (Lisý and Lodin 1977).

G alpha subunit estimation

G alpha changes in cholate membrane extracts were analyzed by ELISA technique with our rabbit antibodies against synthetic C-terminal dekapeptides of alpha chains of Gs and Gq/11 (Milligan 1988). No cross reaction between our anti G alpha - antisera and initial peptides for immunization was detected. ELISA method of competitive inhibiton was performed, modified by us with use of Maxisorp microtitration plates (NUNC) for noncovalent peptide binding. Immunochemical staining alkaline phosphatase conjugated with goat antirabbit IgG and p-nitrophenylphosphate as substrate was used. Results were confirmed by Western immunoblotting. For other details see Kovářů et al. 1998.

Statistical analysis

The data are expressed as mean values +/- S.D. The differences between experimental samples were evaluated by unpaired Student´s *t*-test.

Results and Discussion

Spontaneous ontogenetic development of both ATPase activities, Na^+, K^+- and Mg^{2+}- ATPases, was characterized as an age-dependent decrease in thymus and lymph node lymphocytes (Tab. 1 and 2). In more detail, Mg^{2+}- ATPase activities were lower in thymus than in lymph nodes. This organ dependence of Mg^{2+}- ATPase activity is similar to the enzyme changes observed in other species, such as rabbit or mouse (Tandon et al. 1983; Hell et al. 1985; Kovářů 1992). We demonstrated age dependent changes in Mg^{2+}- ATPase activities, i.e. lower mesenterial lymph node - and higher peripheral lymph node lymphocyte - enzyme activity ($p < 0.01$). These characteristics can reflect effects of both developmental and environmental factors on both lymph node systems. The data indicate a relationship of Mg^{2+}- ATPase activity in lymphoid organs and maturation events (Hell et al. 1985; Barankiewicz et al. 1988; Benrezzak et al. 1999). This enzyme participates

Table 1
Development of ATPase and GGT activities in thymus. s.a.- specific activity in nmol Pi/10^6/ 1h

Age (days)	Na^+K^+-ATPase (s.a.)	Mg^{2+}-ATPase (s.a.)	GGT (s.a.)
15	1.8 ± 0.3	17.0 ± 1.2	33.1 ± 3.4
30	1.5 ± 0.2	15.1 ± 1.5	35.9 ± 0.5
60	1.3 ± 0.1	8.9 ± 0.6	23.7 ± 0.4
90	0.8 ± 0.6	15.5 ± 0.1	23.4 ± 0.3

Table 2
Development of ATPase and GGT activities in lymph nodes. For other details see Table 1.

Age (days)	Mesenteric			Peripheral		
	Na^+K^+-ATPase (s.a.)	Mg^{2+}-ATPase (s.a.)	GGT (s.a.)	Na^+K^+-ATPase (s.a.)	Mg^{2+}-ATPase (s.a.)	GGT (s.a.)
15	11.8 ± 1.5	48.61 ± 5.8	24.2 ± 3.0	32.8 ± 4.1	105.8 ± 9.1	40.0 ± 0.8
30	10.2 ± 1.2	35.18 ± 2.8	29.4 ± 0.6	16.9 ± 1.5	56.0 ± 2.6	40.7 ± 1.6
60	6.8 ± 1.2	22.10 ± 2.2	42.9 ± 4.5	12.6 ± 1.3	52.9 ± 3.0	57.9 ± 1.0
90	n.d.	n.d.	78.1 ± 3.0	10.7 ± 1.0	49.0 ± 1.5	91.5 ± 3.4

in many lymphocyte effector mechanisms in all components of immunity, i.e. natural, cell and humoral immune responses (Dombrowski et al. 1998).

We observed a markedly lower Na^+, K^+- ATPase activity than that of Mg^{2+}- ATPase in both thymus and lymph node lymphocytes at all tested intervals. Prenatal and postnatal changes of Na^+, K^+- ATPase were characterized by a sharp decline to 20% and 32% in thymus and lymph nodes, respectively (Fig. 1). Furthermore, we studied the relationship between Na^+, K^+- ATPase, membrane lipid fluidity and lymphocyte maturation (Kovářů 1992). We demonstrated also participation of Na^+, K^+- ATPase activity in mitogen lectin induced lymphocyte activation from quiescent state related to DNA synthesis and proliferation (Kovářů H. et al. 2003, in press).

Gamma-glutamyltranspeptidase (GGT) activity increased markedly both as a function of age in lymph node lymphocytes and as compared to declined thymus lymphocyte enzyme activity (Tab. 1 and 2). Postnatal lymph node GGT activity was increased to 257% (PD 90) compared to FD 80, whereas opposite changes were found in thymus, i.e. decrease to 51% on PD 90 (Fig. 2). The ratio of lymph node/thymus lymphocyte GGT on PD 60 and PD 90 was approximately 3 and 5 , respectively, and this range is to some extent similar to human B/T lymphocyte GGT ratio (Kovářů 1992; Lawrence et al. 2000). It is suggested that higher GGT activity in lymphocytes can indicate a relationship to synthesis of immunoglobulin and lymphokine secretion by these cells. We demonstrated that spontaneous developmental changes are probably consequence of B lymphocyte functional maturation with immunoglobulin synthesis as demonstrated by intrauterine immunization or postnatal immunization (Kovářů F 1992; Kovářů F et al., this volume).
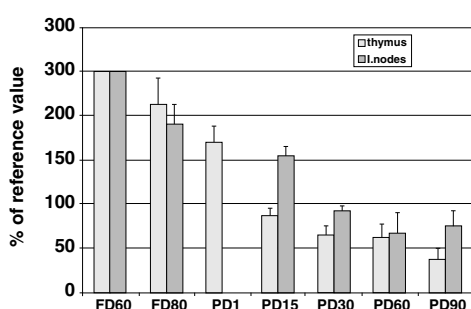

Fig. 1: Development of activity of Na^+, K^+- ATPase activity in thymus and lymph nodes (% of reference activity). Fetal days (FD) and postnatal days (PD), thymus (hatched columns), lymph nodes (dark columns)
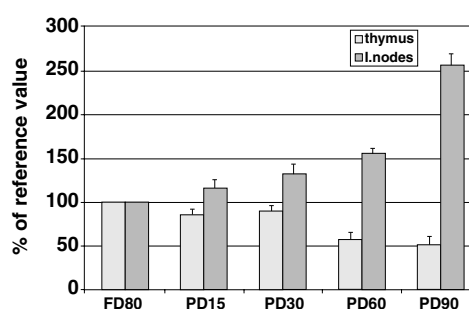
Fig. 2: Development of activity of GGT activity in thymus and lymph nodes. For other details see Fig. 1.

G-proteins. In thymus, the developmental role of G alpha q/11 subunit expression can be important because of its high levels in prenatal period compared to levels of Galpha q/11 in lymph nodes elevated from fetal to neonatal life ($p < 0.05$) (Fig. 3 ab). Level of G alpha (s) subunit was markedly increased as function of age from fetal to postnatal intervals in both thymus and lymph nodes ($p < 0.01$). All data indicate the role of main subtypes of G proteins in cell signal transduction to intracellular responses via both effector systems adenylyl cyclase, and phospholipase C generating cAMP or 1,4,5 IP_3 messenger.

The role of G protein coupled receptor in T cell development is suggested, including G alpha q family in B lymphocyte differentiation (Mapara et al. 1995; Zabel et al. 1999). Furthermore, G proteins in transmembrane cell signalling during lymphocyte activation with engagement of T cell receptor-CD3 complex were modulated by heterotrimeric G proteins, including G alpha q/11 family (Tsoukas et al. 2000). cAMP dependent cell-growth inhibition of T cell line, Jurkat cells was studied (Gouy et al. 1991). It is evident
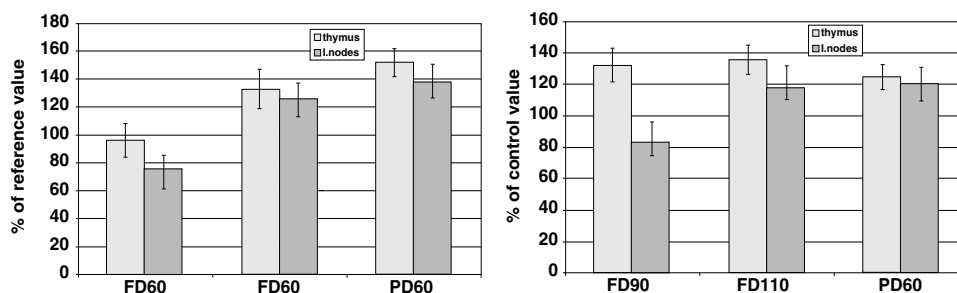
Fig. 3: Development of G alpha (s) (3a, left) a G alpha q/11 (3b, right) subunit levels in thymus (light columns) and lymph nodes (dark columns), G alpha q/11, G alpha (s) (% of reference level on PD 90)

that G proteins regulate a diverse range of biological responses including growth, proliferation and differentiation.

In this paper, we studied membrane enzymes and G proteins as probable markers involved in lymphocyte maturation of both thymus or lymph nodes. Our results indicate that further elucidation of the role of membrane systems - GGT, ATPases and G proteins will provide a valid basis for understanding their role in lymphocyte effector functions.

Biochemické znaky maturace lymfocytů

Zaměřili jsme se na studium maturačních dějů buněčného povrchu a studovali jsme aktivity Na^+, K^+- a Mg^{2+}- ATPasy a gama-glutamyltranspeptidasy (GGT) lymfocytů během prenatálního a postnatálního vývoje prasečího thymu a lymfatických uzlin, tj. primárního a sekundárního lymfatického orgánu. Dále jsme analyzovali vývojové změny exprese alfa subjednotek (Gs, Gq/11) membránových heterotrimerních GTP-vazebných proteinů.

Prokázali jsme vývojově pokles aktivit Na^+, K^+- a Mg^{2+}-ATPasy lymfocytů jak v thymu tak v mízních uzlinách. Na druhé straně jsme stanovili vzestup GGT aktivity v lymfocytech mízních uzlin na rozdíl od snížené aktivity enzymu. Tyto změny jsou patrně spojeny s tvorbou protilátek působením antigenního tlaku.

Dále jsme analyzovali změny vývojových profilů G alfa (s) a G q/11 subjednotky. Zjistili jsme ontogenetické specifity v obou lymfatických orgánech v expresi obou subjednotek, které ukazují roli efektorových enzymových systémů a příslušných druhých poslů cAMP a 1,4,5 IP_3 v transdukci membránového signálu.

Tato studie přispívá k pochopení dynamických změn jak enzymů tak profilů G alfa subjednotek na povrchu lymfocytů spojených s maturačními ději.

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