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Expression of Genes for Certain Enzymes of Pyrimidine and Purine Salvage Pathway in Peripheral Blood Leukocytes Collected From Patients With Graves' or Hashimoto's Disease

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Abstract Increased activities of some enzymes, which participate in pyrimidine and purine salvage pathway, were found in blood fractions of patients suffering from different autoimmunological diseases, thyroid diseases included. The aim of the study was to estimate the expression of genes, specific for deoxycytidine kinase (dCK, EC 3.7.1.74), thymidine kinase 1 (TK1; EC 2.7.1.21), and adenosine deaminase (ADA, EC 3.5.4.4) in blood leukocytes, collected from patients with autoimmunological thyroid diseases (AITD), i.e., Graves' or Hashimoto's disease. The total mRNA was isolated from peripheral blood leukocytes and, afterwards, submitted to reverse transcription (RT), with the following amplification of genes encoding for particular examined enzymes and β-actin, as a supervisory gene [RT-polymerase chain reaction (RT-PCR)]; ADA gene was amplified with the use of three different primer pairs (ADA3, ADA4, and ADA5). PCR products were electrophoresed in 8% polyacrylamide gel and then, submitted to densitometric analysis. The levels of expression of all the examined genes in leukocytes from patients with either Graves' or Hashimoto's disease were significantly increased when compared to those in controls; above a twofold elevation of expression of *TK1*, *ADA4*, and *ADA5* genes was observed. In conclusion, the changes of activities of salvage enzymes in patients with AITD occur likely at transcription level; the measurement of gene expression for purine and pyrimidyne salvage enzymes may likely help explain the mechanism of autoimmune diseases, being also significant in the diagnostics and/or monitoring of AITD. J. Cell. Biochem. 89: 550–555, 2003.

Key words: pyrimidine; purine; salvage pathway; Graves' disease; Hashimoto's disease

Increased activities of some enzymes, involved in nucleoside salvage metabolism, have been observed in blood fractions in patients suffering from different autoimmune disease [Hoshino et al., 1994; Hitoglou et al., 2001; Köse et al., 2001], autoimmunological thyroid disorders (AITD) included [Covas et al., 1996; Karbownik et al., 2002].

Deoxycytidine kinase (dCK, EC 2.7.1.74) is an enzyme with a broad substrate specific activity, catalyzing phosphorylation of natural deoxynucleosides (i.e., deoxycytidine, deoxyadenosine, and deoxyguanosine) and a number of analogues of purine nucleosides [Carlucci et al., 1997].

Thymidine kinase (TK, EC 2.7.1.21) catalyses the phosphorylation of thymidine (deoxythymidine) to deoxythymidine monophosphate. Thymidine kinase 1 (TK1) is a cytosol isoform of TK; its activity and gene expression are cell cycle-regulated and increase during the S phase [Hengstschläger et al., 1994].

Adenosine deaminase (ADA, EC 3.5.4.4) is an enzyme, which irreversibly catalyzes the deamination of adenosine (Ado) and of 2′-deoxyadenosine (dAdo) to inosine and 2′-deoxyinosine. This enzyme plays an important role in the maturation and function of lymphocytes of both T and B cell lineages [Goday et al., 1985].

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Among AITD, Graves' and Hashimoto's diseases are the most important ones. Although endocrinologists-clinicians distinguish between Graves' and Hashimoto's disease, there is a view that these two diseases constitute two poles of one autoimmunological thyroid disease.

Under conditions of the decreased activity of immunological system (diminished cellular and humoral immune response), a decrease/absence of some salvage enzymes was found [Markert, 1991; Hershfield and Mitchell, 1995]; thus, it seemed plausible for us to widen characteristics of these enzymes in autoimmunological disorders.

The aim of the study was to estimate the expression of genes specific for dCK, TK1, and ADA in blood leukocytes collected from patients with AITD, i.e., Graves' or Hashimoto's disease.

MATERIALS AND METHODS

The procedures, used in the study, were approved by the Ethical Committee of the Medical University of Łódź. Forty eight adults were involved in the study, including 20 patients with Hashimoto's thyroiditis (nine males and 11 females, mean age: 48.8 ± 11.8), 12 patients with Graves' disease (five males and seven females, mean age: 56.5 ± 9.6), diagnosed on the basis of clinical symptoms, hormonal and immunological tests and—in some cases of Hashimoto's thyroiditis—of cytological examination of the thyroid. In all the AITD patients, increased concentrations of antithyroid antibodies were observed. The AITD patients were euthyroid, resulting from, usually, long-lasting appropriate treatment (Hashimoto's patients—with levothyroxine in substitutive doses, and Graves' patients—with methimazole). Sixteen healthy euthyroid subjects (eight males and eight females, mean age: 49.2 ± 10.1), without any features of AITD, served as Controls.

RNA Isolation

Total RNA was extracted from peripheral blood leukocytes in guanidinium isothiocyanate, by the use of a commercially prepared kit [Total RNA Prep Plus (A&A Biotechnology, Gdynala, Poland)].

RNA concentration and purity in the final preparations was spectrophotometrically quantified by measuring absorbance at 260 and 280 nm (Spectrophotometer DU 640, Beckman, Fullerton, CA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT was performed in a reaction mixture (20 μl), containing: 5 ng of total RNA, 0.3 μl of Random Primer Oligonucleotides (3 μg/μl) (Gibco, Pasley, Scotland), 2 μl of 0.1 M dithiothreitol (DTT, Gibco), 4 μl of 5× First-Strand Buffer) (250 mM Tris-HCl, pH 8.3; 375 mM of KCl; 15 mM of $MgCl_2$) (Gibco), 1 μl of Reverse Transcriptase M-MLV (Moloney Murine Leukemia Virus Reverse Transcriptase) (200 U/μl) (Gibco), 1.6 μl of dNTP (2,500 μM) (Gibco), and 0.3 μl of Inhibitor RNAse (10 U/μl) (Gibco).

Amplification was performed on a TRIO-Thermoblock thermocycler (Biometra, Göttingen, Germany) in a total volume of 25 μl, containing: 1,000 ng of cDNA (RT product), 2.5 μl of 10× PCR buffer (10 mM of Tris-HCl, pH 8.8; 150 mM of KCl, 1.5 mM of $MgCl_2$, 0.1% Triton X-100) (Finnzymes OY, P.O. Box 148, FIN-02201, ESPOO), 2.5 μl of dNTP (200 μM of each) (Gibco), 1 μl of *Taq* polymerase (DyNAzyme II DNA polymerase) (2 U/μl) (Finnzymes), and 25 pmol of each, i.e., 3′- and 5′-PCR primer.

The sequences of the synthetic oligonucleotide primers (TIB Mol-BIOL 27/2, Libelt St., 61-707 Poznan), used for PCR amplification (and size of their products), are shown in Table I. The reaction conditions, under which the amplifications were carried out, are defined in Table II. The gene for ADA was amplified with the use of three different primer pairs designated as ADA3, ADA4, ADA5; similarly, ADA gene is designated in the current study as *ADA3*, *ADA4*, *ADA5*, depending on the primer used. The amplification of control housekeeping gene, β-actin, served as internal standard.

Analysis RT-PCR Products

RT-PCR products were identified, using 8% polyacrylamide–Tris-borate-ethylenediamine tetraacetate (TBE) gels and were visualized by ethidium bromide staining. Densitometric analyses were conducted, using the Image Master system (VDF Version 3.0, Pharmacia Biotech, Vienna, Austria). Results are expressed as relative optical density (ROD) (intensity of the band) in arbitrary units.

Statistical Analysis

The data were statistically analyzed, using a one-way analysis of variance (ANOVA), followed by a Newman–Keuls test. Statistical signifi-

TABLE I. Nucleotide Sequences of Polymerase Chain Reaction (PCR) Primers and Size of Products

Gene	Sequence	Band size (bp)
dCK		
Sense	5′-CTT CTC TAA ATA TTC AAG AG-3′	300
Antisense	5′-CAG CTT GCC TCT CTG AAT GG-3′	
TK1		
Sense	5′-GCG TGG TGA AGC TGA CGG CG-3′	320
Antisense	5′-CAG TAG GCG GCA GTG GCA GG-3′	
ADA3		
Sense	5′-GAG GAG AGG GAT CGC CCT CC-3′	123
Antisense	5′-GCG ATA GCA GGC ATG TAG TA-3′	
ADA4		
Sense	5′-GGG CTG CCG GGA GGC TAT CA-3′	134
Antisense	5′-TCA GCC TGG TTC CAG GGG AT-3′	
ADA5		
Sense	5′-AGG GGA CCT CAC CCC AGA CG-3′	113
Antisense	5′-TGG GCT GGT GGC GCA TGC AG-3′	
β-Actin		
Sense	5′-TGA CCC AGA TCA TGT TTG AGA-3′	484
Antisense	5′-ACT CCA TGC CCA GGA AGG A-3′	

cance was determined at the level $P < 0.05$. The results are presented as mean ± SEM.

RESULTS

RNA expression levels were significantly higher for all the examined enzymes of peripheral blood leukocytes from patients with either Hashimoto's or Graves' disease than those in the Controls (Figs. 1–5).

The expression of mRNA encoding for dCK increased by 58.3% in leukocytes from the patients with Hashimoto's disease, and by 77.5% in leukocytes from the patients with Graves' disease, when compared to that observed in Controls (Fig. 1).

Above a twofold increase of RNA expression of TK1 was observed in leukocytes collected from patients with both autoimmunological diseases, in case of Hashimoto's disease, *TK1* gene expression was higher by 170.3% and, in case of Graves', disease gene expression of that enzyme was higher by 130.5% than that in Controls (Fig. 2).

RNA expression encoding for ADA was evaluated by the use of three different primer pairs; similar results were obtained for ADA gene expression, regardless of the primer used. The expression of ADA3 increased by 64.9% in leukocytes from the subjects with Hashimoto's disease, and by 57.9% in leukocytes from the patients with Graves' disease (Fig. 3). About a twofold increase of RNA expression was found when ADA4 and ADA5 primers were used; the

TABLE II. Conditions of Amplification Reactions

PCR primers	PCR conditions
TK1	Initial denaturation at 95°C for 5 min, 40 cycles
ADA5	Denaturation at 95°C for 1 min Annealing at 68°C for 1 min Elongation at 72°C for 1 min Final elongation at 72°C for 10 min
ADA3	Initial denaturation at 95°C for 5 min, 40 cycles
ADA4	Denaturation at 95°C for 1 min
dCK	Annealing at 66°C for 1 min Elongation at 72°C for 1 min Final elongation at 72°C for 10 min
β-Actin	Initial denaturation at 94°C for 5 min, 40 cycles Denaturation at 95°C for 1 min Annealing at 60°C for 1 min Elongation at 72°C for 1 min Final elongation at 72°C for 10 min

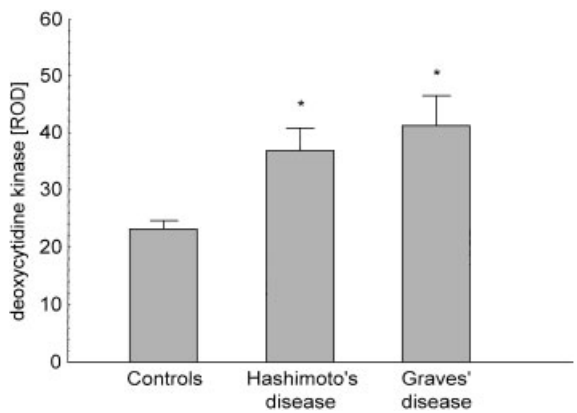

Fig. 1. The expression of mRNA encoding for deoxycytidine kinase (dCK) in peripheral blood leukocytes from patients with Hashimoto's disease (n = 20) or Graves' disease (n = 12) and in Controls (n = 16). Gene expression was expressed as the amount of amplified cDNA. Results are expressed as relative optical density (ROD) in arbitrary units. Bars represent the mean ± SEM. *$P < 0.05$ versus Controls.

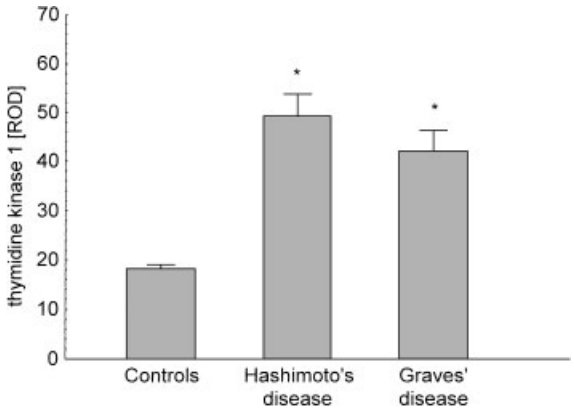

Fig. 2. The expression of mRNA encoding for thymidine kinase 1 (TK1) in peripheral blood leukocytes from patients with Hashimoto's disease (n = 20) or Graves' disease (n = 12) and in Controls (n = 16). Gene expression was expressed as the amount of amplified cDNA. Results are expressed as ROD in arbitrary units. Bars represent the mean ± SEM. *$P < 0.05$ versus Controls.

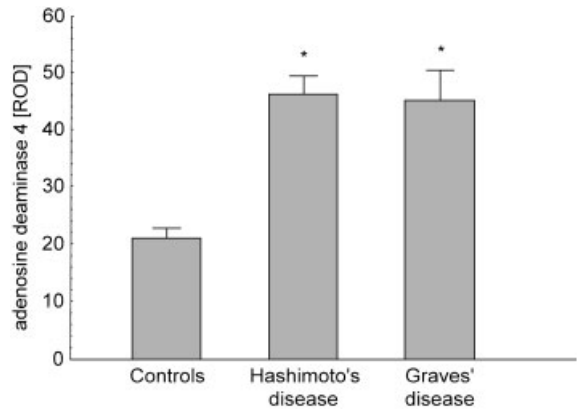

Fig. 4. The expression of mRNA encoding for ADA, amplified by the use of primer pair designated as ADA4, in peripheral blood leukocytes from patients with Hashimoto's disease (n = 20) or Graves' disease (n = 12) and in Controls (n = 16). ADA gene is designated in the current study as ADA3. Gene expression was expressed as the amount of amplified cDNA. Results are expressed as ROD in arbitrary units. Bars represent the mean ± SEM. *$P < 0.05$ versus Controls.

expression of RNA specific for ADA4 and ADA5 was higher by 119.3 and 98.4%, respectively, in Hashimoto's disease, and by 114.5 and 115%, respectively, in Graves' disease, comparing to the values observed in control leukocytes (Figs. 4 and 5).

DISCUSSION

It should be considered whether the observed increased expressions of gene for dCK, TK, and ADA in blood leukocytes from patients with AITD are involved in the pathomechanism of these disorders or whether they are a consequence of primary immunological disturbances.

Both ADA and dCK prevent cells from overproduction of Ado and dAdo. Destructive effects of these nucleosides in elevated intracellular concentrations were found for different cell types [Wakade et al., 1995; Kulkarni and Wakade, 1996]. In turn, the increased expression of ADA mRNA has been found in male mouse testes early after delivery [Meng et al., 1997]; the authors suggested that the role of

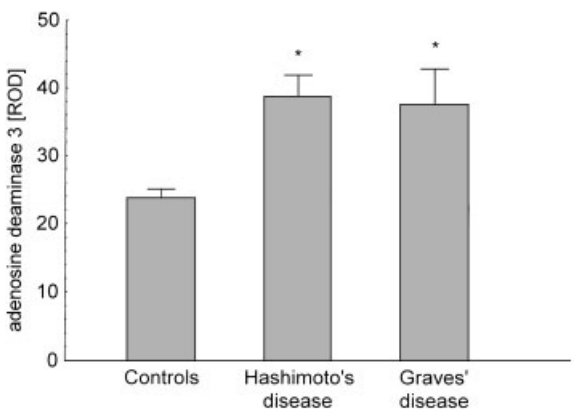

Fig. 3. The expression of mRNA encoding for adenosine deaminase (ADA), amplified by the use of primer pair designated as ADA3, in peripheral blood leukocytes from patients with Hashimoto's disease (n = 20) or Graves' disease (n = 12) and in Controls (n = 16). ADA gene is designated in the current study as *ADA3*. Gene expression was expressed as the amount of amplified cDNA. Results are expressed as ROD in arbitrary units. Bars represent the mean ± SEM. *$P < 0.05$ versus Controls.

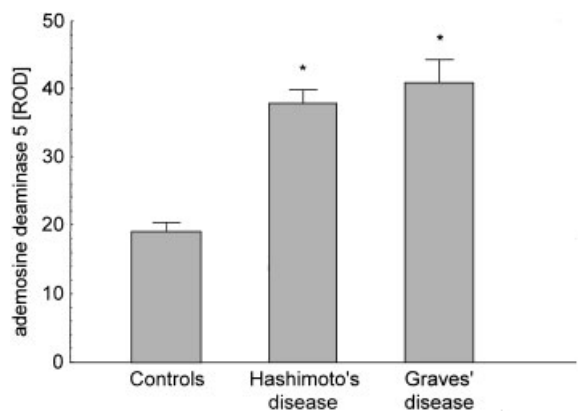

Fig. 5. The expression of mRNA encoding for ADA, amplified by the use of primer pair designated as ADA5, in peripheral blood leukocytes from patients with Hashimoto's disease (n = 20) or Graves' disease (n = 12) and in Controls (n = 16). ADA gene is designated in the current study as ADA3. Gene expression was expressed as the amount of amplified cDNA. Results are expressed as ROD in arbitrary units. Bars represent the mean ± SEM. *$P < 0.05$ versus Controls.

ADA, specific for male gonads, could be the protection against toxic actions of Ado and dAdo during cell proliferation and differentiation. Similarly, the increased ADA and dCK mRNA expressions observed in the course of AITD in the present study, could constitute protective mechanisms against toxic effects of Ado and dAdo.

However, another consequence of decreased amounts of intracellular Ado and dAdo should also be considered. It is known that Ado and dAdo exert immunosuppresive effects [Birch and Polmar, 1986; Prabhakar et al., 1995] and that Ado can participate in physiological processes of T-cell elimination and, thus, preventing autoimmunological processes [Szondy, 1994]. In contrast, increased dCK and ADA activities (found in the present study) and, consequently, decreased concentration of Ado and dAdo within the cell, could participate in the induction of symptoms of any autoimmunological disorder, for example, Graves' or Hashimoto's disease.

Autoimmunological processes could be induced as a consequence of certain properties of salvage enzymes. ADA is considered to be a marker of T-cell activation [Khosla et al., 1992; Chiang et al., 1994], and human dCK is said to function as an antigen, which reveals immunogenic properties [Kawamura et al., 2000], what suggests that ADA and dCK could participate in the pathogenesis of autoimmunological diseases.

Thus, increased dCK and ADA gene expressions, followed by increased activities of the enzymes in question, could—directly or indirectly—induce autoimmunological process and, in this way, participate in the pathomechanism of autoimmunological diseases.

It is not excluded that the increased activity of some purine and pyrimidine salvage enzymes is characteristic for all the autoimmunological disturbances. Much evidence has been gathered for the existence of this relation. For example, an increased ADA activity was found in skin and blood plasma of psoriatic patients [Köse et al., 2001]. Increased activities of some isoforms of ADA were found in peripheral blood serum or lymphocytes, collected from patients with juvenile rheumatoid arthritis and systemic lupus erythematosus [Hitoglou et al., 2001]. Also, in diabetic patients (in both—diabetes type 1 and type 2), an increased ADA activity was found in blood serum [Hoshino et al., 1994].

As regards AITD, an increased ADA [Nishikawa et al., 1995; Covas et al., 1996; Karbownik et al., 2002] and dCK [Karbownik et al., 2002] activities were found in different blood fractions in patients suffering from Hashimoto's or Graves' disease. The increased expression of RNA for salvage enzymes in AITD speaks in favor of the assumption that in patients suffering from autoimmunological thyroid diseases the modification of these enzymes occurs at transcription level.

The aforementioned phenomenon—the increased activities or RNA expression for salvage enzymes—seems to be in contradiction with the observation made in patients with severe combined immunodeficiency disease, resulting from inherited ADA deficiency and characterized by diminished cellular and humoral immune responses [Hershfield and Mitchell, 1995].

It should also be taken into consideration that the increased gene expression for some purine and pyrimidyne salvage enzymes, as observed in the present study, may be a consequence of autoimmunological disturbances. Unfortunately, no studies have been performed, so far, on the influence of antithyroid or of any other organ-specific antibodies on the purine and pyrimidine metabolism. It may be reasonable to examine the activities of the enzymes in question in patients predisposed to autoimmunological diseases, in whom the clinical symptoms of the disease would definitely be induced in the future. However, according to the current knowledge, it is impossible to select such patients in advance.

In conclusion, the changes of activities of salvage enzymes in patients with AITD occur likely at transcription level. The measurement of expression of genes for purine and pyrimidyne salvage enzymes may help explain the mechanism of autoimmune diseases and be significant in the diagnostics and/or of monitoring of AITD.

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