



5'-Nucleotidase and adenosine deaminase in patients with rheumatoid arthritis

5'-nukleotidaza i adenzin dezaminaza kod bolesnika sa reumatoidnim artritisom

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Abstract

Background/Aim. The essence of rheumatoid arthritis (RA) pathogenesis is inflammation, modification of immune system and cell damage. 5'-nucleotidase (5'-NT) and adenosine deaminase (ADA) have a significant role in the process of inflammation-caused tissue damage. The aim of the study is to define 5'-nucleotidase and adenosine deaminase activity in serum of patients with RA treated with methotrexate (MTX) and patients with RA who were not treated with methotrexate, as well as to determine the correlation between the enzymes' activities and the disease activity. **Methods.** The study included 160 patients suffering from RA, 60 of them were not treated with methotrexate (average age 56.8 years; 68.3% female) and 100 patients were treated with methotrexate (average age 59.8 years; 88% female), as well as 60 healthy controls (average age 58.8; 66.6% female). Patients suffering from chronic inflammatory diseases, chronic respiratory, cardiac and kidney insufficiency, severe acute diseases and other diseases which might modify inflammatory response were not included in the study. **Results.** There was no

statistically significant difference in 5'-NT values among groups. ADA values were significantly different in all tested groups. Post-hoc analysis (Dunnett's T3 test) showed that ADA activity in RA groups was significantly higher as compared to that in the control group ($p < 0.001$), and that ADA activity in the RA group with MTX was significantly smaller as compared to RA group without MTX ($p < 0.001$). There was not significant correlation between the disease activity and activities of tested enzymes. **Conclusion.** We concluded that adenosine deaminase activity was increased in patients with rheumatoid arthritis, as well as that the application of methotrexate led to the decrease of this enzyme activity in the serum of patients with rheumatoid arthritis. The activity of 5'-nucleotidase is not increased in patients with rheumatoid arthritis and did not depend on methotrexate treatment. Serum adenosine deaminase and 5'-nucleotidase activities are not good indicators of rheumatoid arthritis activity.

Key words:
 arthritis, rheumatoid; adenosine deaminase;
 5'-nucleotidase.

Apstrakt

Uvod/Cilj. U patogenetskoj osnovi reumatoidnog artritisa (RA) su inflamacija, promena imunog sistema i oštećenje ćelija. Značajnu ulogu u procesu oštećenja tkiva posredovanog inflamacijom imaju 5'-nukleotidaza (5'-NT) i adenzin dezaminaza (ADA). Cilj rada bio je određivanje aktivnosti adenzin dezaminaze i 5'-nukleotidaze u serumima obolelih od RA lečenih uz pomoć ili bez terapije metotretksatom (MTX), kao i utvrđivanje povezanosti ovih enzima sa aktivnošću bolesti. **Metode.** Ispitivanjem je bilo obuhvaćeno 160 bolesnika obolelih od RA, od kojih 60 nije bilo lečeno MTX (prosečne starosti 56,8 godina; 68,3% žena) i 100 bolesnika na terapiji MTX (prosečne starosti 59,8 godina, 88% žena), kao i 60 ispitanika kontrolne grupe

(prosečne starosti 58,8 godina; 66,6% žena). Bolesnici sa hroničnim inflamatornim oboljenjima, hroničnom respiratornom, srčanom i bubrežnom insuficijencijom, težim akutnim oboljenjima, i drugim bolestima od značaja koje bi mogle da modifikuju inflamatorni odgovor bili su isključeni iz istraživanja. **Rezultati.** Nije zabeležena razlika u aktivnosti 5'-NT između ispitivanih grupa. Vrednosti ADA su se značajno razlikovale između ispitivanih grupa. *Post-hoc* analizom (Dunnett-ov T3 test) pokazano je da je aktivnost ADA u grupama značajno viša u odnosu na kontrolnu grupu bolesnika ($p < 0,001$), kao i da je aktivnost ADA u grupi bolesnika sa RA lečenih MTX bila značajno niža u odnosu na aktivnost u grupi bolesnika sa RA koji nisu primili MTX ($p < 0,001$). Nije bilo statistički značajne korelacije između DAS28 skora i aktivnosti ispitivanih enzi-

ma. **Zaključak.** Aktivnost adenzin dezaminaze povećana je kod bolesnika sa reumatoidnim artritismom. Primena metotreksata dovodi do smanjenja aktivnosti ovog enzima u serumu bolesnika sa reumatoidnim artritismom. Aktivnost 5'-nukleotidaze nije povećana kod bolesnika sa reumatoidnim artritismom i ne zavisi od terapije metotreksatom. Akti-

vnosti adenzin dezaminaze i 5'-nukleotidaze u serumu nisu dobri pokazatelji aktivnosti reumatoidnog artritisa.

Ključne reči:
artritis, reumatoidni; adenzin dezaminaza; 5'-nukleotidaza.

Introduction

Rheumatoid arthritis (RA) is a frequent chronic inflammatory arthropathy which is present in 1% of the world population. Even though the etiology of the disease is not well known the present information indicates that RA is a result of the simultaneous influence of genetic risk factors, hormone factors, immunology and external factors¹. The disease is characterized by the proliferation of autoreactive clones of T and B lymphocytes with the proliferation of synovial cells, the formation of pannus, activation of chondrocytes and metalloproteinases, which leads to the destruction of joint cartilage, bones and the surrounding structures². The main characteristics of early immune reaction in RA are T lymphocytes, especially CD4 cells which initiate the entire sequence of further events.

Rheumatoid arthritis is followed by the increased activity of enzymes which take part in nucleic acid metabolism³. Adenosine deaminase (ADA) plays a significant role in the process of inflammation-caused tissue damage. ADA is an enzyme which regulates cellular and extracellular concentration of adenosine and deoxyadenosine along with 5'-nucleotidase (5'-NT) and adenosine kinase. The increase of ADA in serum of RA patients is related to its release from damaged cells⁴. This enzyme has the metabolic significance due to its role in the catabolism of purine derivatives of adenosine and deoxyadenosine, as well as in proliferative activity of immunocompetent cells⁵. ADA is a ligand of CD26 protein (ADA binding protein-ADAbp) which, in interaction with CD26, has a stimulating effect on T cell receptor (TCR), i.e. acts as a mediator in T cell activation⁶. Activation of T-cell response initiates a sequence of reactions which result in increased inflammation, thickening of synovial membrane and deterioration of cartilage and bones.

The central role of 5'- nucleotidase is the production of extracellular nucleotides, adenosine being the most important one. Ecto-5'-NT is believed to be the marker of human B-lymphocyte maturation, having in mind that it is increased during normal development, and reduced in immunodeficiency conditions⁷. Even though circulating monocytes show the low activity of ecto-5'-NT, differentiation of monocytes results in increased activity of this enzyme. Additionally, increased values of this enzyme in synovial fluid are significant in terms of diagnostics⁸.

Clinical presentation of RA is characterized by numerous symptoms and signs which refer to joints, periarticular structures and internal organs. The basic features of RA are a symmetrical pain, swelling, morning stiffness and specific radiological changes. Clearly defined parameters of clinical

presentation and complex composite indices, obtained by the combination of subjective and objective parameters, are used for the presentation of the disease activity.

In the era of biological targeted therapies, MTX is still the gold standard in the treatment of RA⁹. After absorption, 10% of this drug is converted in 7-hydroxy MTX in the liver, and afterwards, both are excreted through kidneys¹⁰⁻¹². Several pharmacological mechanisms of MTX effects are explained, including anti-inflammatory effect mediated by stimulating adenosine receptors^{13, 14}. MTX causes reduction of ADA level in three ways: firstly, MTX can directly inhibit ADA; secondly, MTX inhibits ADA indirectly through aminoimidazole carboxamide ribose-5-phosphate (AICAR) and its metabolites; and thirdly, ADA can be inhibited indirectly to compensate the adenosine reduction. The reduction of ADA causes an increase in adenosine level and consequently anti-inflammatory effect¹⁴.

Biomarkers which refer to synthesis and degradation of cartilage and bones, inflammation and autoimmune processes which might have clinical significance in the assessment of the diseases' presentation have been the subject of interest in the past ten years. Purine metabolism enzyme testing has become more frequent, having in mind that understanding pathophysiological role these enzymes have in the progression of RA may be useful, not only in diagnostics but also in the process of monitoring the clinical course of the disease and therapy effects.

The aim of the study was to define 5'-nucleotidase and adenosine deaminase activity in the serum of patients with rheumatoid arthritis treated with methotrexate and patients with rheumatoid arthritis who were not treated with methotrexate, as well as to determine the correlation between the enzymes' activities and the disease activity.

Methods

The study included 160 patients suffering from RA; 60 of them were not treated with methotrexate (average age 56.8; 68.3% female patients) and 100 patients were treated with methotrexate (average age 59.8 years; 88% female patients), as well as 60 healthy controls (average age 58.8; 66.6% female patients). All included patients were treated at the Clinic for Rheumatology of the Institute for Treatment and Rehabilitation "Niška Banja", and the study was approved by the local Ethics Committee.

Diagnosis and classification of RA were performed based on revised American College of Rheumatology (ACR) classification criteria from 1987¹⁵. Patients suffering from chronic inflammatory diseases (connective tissue diseases,

other systemic diseases), chronic respiratory, cardiac and kidney insufficiency, severe acute diseases and other diseases which might modify inflammatory response were not included in the study. General methodological approach implied a prospective analysis of medical history, as well as clinical and laboratory indicators.

Clinical assessment

Clinical assessment of patients implied physical examination with special emphasis on locomotor system. The number of painful and swelling joints was recorded. Subjective assessment of general health was defined by means of visual analogue scale (VAS) – 100 mm.

Disease activity score-sedimentation rate (DAS28 SE), a combined index which included palpation sensitivity and swelling of 28 joints: shoulders, elbows, metacarpophalangeal (MCP) joints, proximointerphalangeal (PIP) joints and knees, sedimentation rate and patients' assessment of general health, was used for determining disease activity. DAS28 SE score was a number on a scale from 0 to 10 which showed current disease activity. Disease activity level was considered low in case $DAS\ 28 \leq 3.2$, medium in case $3.2 < DAS\ 28 \leq 5.1$ or high if $DAS\ 28 > 5.1$. In case of $DAS\ 28 < 2.6$ the disease was considered to be in remission¹⁶.

Laboratory assessment

Patients were taken a blood sample from cubital vein early in the morning before they had breakfast (no food intake for 12 hours). Blood samples for laboratory analysis were kept in heparinized blood collection tubes. Peripheral blood was taken by means of venipuncture and the serum was separated by centrifugation at room temperature at 3500 cycle/min for 15 minutes. The samples were used for determining: erythrocytes (Er), hemoglobin (Hb), thrombocytes (Tr), leukocytes (Le), erythrocyte sedimentation rate (ESR), 5'-NT and ADA.

The activity of 5'-NT was defined by means of spectrophotometry, using Wood and Williams' method¹⁷. The method was based on defining released inorganic phos-

phorus from adenosine monophosphate (AMP). Aliquot of 0,5 ml of serum was incubated for 30 minutes at 37°C in the presence of barbiturate buffer pH = 7.8 with manganese as an activator and 10 µmol Mn-AMP as a substrate. After adding hydrazine sulfate-tin chloride solution and ammonium molybdate, released inorganic phosphorus was defined by means of spectrophotometry at 618 nm.

Adenosine deaminase assay kit produced by Diazyme was used for determining ADA activity in the serum. ADA determination was based on the enzymatic conversion of adenosine to inosine which was converted to hypoxanthine with the aid of purine nucleoside phosphorylase (PNP). Hypoxanthine was then converted to uric acid and hydrogen peroxide (H₂O₂) with the assistance of xanthine oxidase (XOD). One ADA unit was defined as the quantity of enzyme necessary for releasing 1 µmol/min of inosine from adenosine at 37°C. Adenosine deaminase activity was expressed as unit *per liter*.

Statistical assessment was carried out in Excel 7.0 and SPSS 11.0 in Windows 98 environment. The results were shown in tables. Comparison of mean values of continuous variables of the tested groups was carried out by means of ANOVA test with additional *Post-hoc* analysis (Dunnnett's T3 test). Bivariate correlation analysis [(Spearman's correlation coefficient (r) for nominal and ordinal data and Pearson's correlation coefficient (C) for continuous numerical data] was used for the analysis of interrelation between the disease activity and activities of 5'-NT and ADA. Significance level $p < 0.05$ was accepted as significant.

Results

The study included 160 patients with RA; 60 of them were not treated with MTX (41 female, 19 male) and 100 patients were treated with MTX (80 female, 20 male), as well as 60 healthy controls (40 female, 20 male). The average age of the patients was similar for all groups. The average duration of the disease in patients who were not treated with MTX was 12 months, and 120 months in patients treated with MTX.

Basic characteristics of the tested groups of RA patients and the controls were shown in Table 1.

Table 1

| Demographic and clinical characteristics of patients with rheumatoid arthritis (RA) and controls | | | |
|--|--------------------------------------|------------------------------------|---------------------|
| Characteristics of patients | RA without MTX treatment (n = 60) | RA with MTX treatment (n = 100) | Control (n = 60) |
| Age (years), $\bar{x} \pm SD$ | 56.86 ± 12.96 | 59.81 ± 11.82 | 58.86 ± 18.05 |
| Gender (female), n (%) | 41 (68.3) | 88 (88) | 40 (66.6) |
| Disease duration (months), $\bar{x} \pm SD$ | 12.5 ± 7.2** | 124.4 ± 81.0 | - |
| No. of painful joints, $\bar{x} \pm SD$ | 12.2 ± 7.5 | 12.3 ± 7.6 | - |
| No. of swollen joints, $\bar{x} \pm SD$ | 4.1 ± 3.0 | 3.2 ± 2.0 | - |
| ESR (mm/h), $\bar{x} \pm SD$ | 35.8 ± 27.8 | 29.9 ± 20.1 | 8.6 ± 4.3** |
| VAS general health score (0–100 mm scale), $\bar{x} \pm SD$ | 32.86 ± 19.9 | 40.02 ± 22.7 | 17 ± 16,5** |
| DAS28 score, $\bar{x} \pm SD$ | 5.13 ± 1.3 | 4.66 ± 1.4 | 1.02 ± 1.23** |

\bar{x} – mean; SD – standard deviation; ESR – erythrocyte sedimentation rate; VAS – visual analogue scale; DAS – disease activity score; MTX – methotrexate; ** $p < 0.01$ as compared to other groups.

There was no statistically significant difference in the level of the disease activity assessed by DAS28 score in the above mentioned groups of patients. However, numerical results showed that the value of DAS28 score in patients not treated with MTX was 5.11 which pointed to the high activity of the disease. The average DAS28 score in patients treated with MTX was 4.66 which pointed to moderate activity of the disease. χ^2 -test showed that the controls had a significantly lower DAS28 score ($p < 0.01$).

Values of the 5'-NT and ADA are shown in Table 2.

ADA values were significantly different in all tested groups. *Post-hoc* analysis (Dunnett's T3 test) showed that ADA values in the control group were significantly smaller as compared to those in the RA groups ($p < 0.001$), and that ADA value in the MTX group was significantly smaller as compared to that in the no MTX group ($p < 0.001$). There was no statistically significant difference in 5'-NT values among groups (Table 2).

The correlation between DAS28 score and the serum activities of 5'-NT and ADA by groups is shown in Table 3.

The connection between the activities of the tested enzymes and MTX treatment was analyzed by means of binary logistic regression. The analysis showed that the entered model could explain 47% of the variance of the dependent variable, which pointed to the strong predictive value of the model. The increase of ADA activities was found in the RA group without MTX treatment (OR = 0.98, 95% CI 0.97–0.99).

Having in mind that we recorded significant difference in the diseases duration for the MTX and no MTX groups, we tested the correlation between the disease duration and the serum activity of ADA and 5'-NT and recorded mean negative correlation between ADA activity and the disease duration, i.e. the longer the disease, the less ADA activity. However, if we disregarded the effects of MTX treatment, we could see the stronger negative correlation between the two variables (significance level of 0.001).

Discussion

Previous research indicates that activities of some purine enzymes in rheumatoid arthritis differ from those in healthy controls. However, the question which referred to the way these enzymes affected the RA activity remained unanswered.

We did not record the significant difference in 5'-NT activity in RA patients and the controls. One study showed that the activities of serum 5'-nucleotidase were increased in patients with RA, i.e. increase of serum 5'-NT appeared at 30% to 66% of patients with RA¹⁸. Some researchers pointed that this enzyme was present in synovial fluid at 58% of RA patients and that its concentration was much larger than in the serum. Therefore, testing 5'-NT in synovial fluid could have significant diagnostic value for this type of patients¹⁹. Literature data on role and origin of 5'-NT in RA was quite contradictory. Additionally, they believed that 5'-NT activiti-

Table 2
Values of 5-nucleotidase (5'-NT) and adenosine deaminase (ADA) serum activities in tested groups of patients

| Enzyme | RA without MTX treatment | RA with MTX treatment | Control |
|-------------------------------|--------------------------|-----------------------|-------------------|
| ADA (IJ/L), $\bar{x} \pm SD$ | 19.32 \pm 5.43** | 12.05 \pm 6.57** | 5.09 \pm 1.51** |
| 5'NT (IJ/L), $\bar{x} \pm SD$ | 37.63 \pm 23.71 | 39.25 \pm 15.01 | 39.15 \pm 21.25 |

\bar{x} – mean; SD – standard deviation; ** $p < 0.01$ as compared to other groups;

ANOVA and *Post-hoc* Dunnett's T3 test; RA – rheumatoid arthritis; MTX – methotrexate.

Table 3
Correlation coefficients between disease activity score (DAS28) and enzyme activity in patients with rheumatoid arthritis (RA) and controls

| Groups of patients | 5'-NT | ADA |
|--------------------------|--------|--------|
| RA without MTX treatment | 0.108 | -0.115 |
| RA with MTX treatment | 0.128 | 0.170 |
| Control | -0.033 | 0.105 |

5'-NT – 5-nucleotidase; ADA – adenosine deaminase; MTX – methotrexate.

Even though the groups were of similar age structure, we tested the effect of the age on the activities of the tested enzymes. The results obtained by ANOVA analysis showed no statistically significant difference in ADA and 5'-NT enzymes serum activities in terms of age.

The result obtained by *t*-test of independent samples did not show statistically significant difference in serum activities of ADA and 5'-NT in terms of genders.

es in synovial fluid represented a useful indicator of inflammatory activity in synovial membrane, but that 5'-NT in serum did not have the same significance. The majority of other researchers believed that the importance of this enzyme in RA inflammatory response was negligible, because the increase of 5'-NT in synovial fluid was not statistically significant even though it was verified. The goal of our study was to test the activity of this enzyme in the serum. However, our

further research would most definitely test synovial fluid in patients with RA.

The analysis showed statistically significant higher values of ADA in patients with RA than in healthy controls. Mean value of ADA in the serum of RA patients with MTX therapy was 12.05 ± 6.57 U/L, the value of analyzed enzyme in patients with RA without MTX was 19.32 ± 5.43 , while the recorded value for healthy controls was 5.09 ± 1.51 U/L. The results of most previous studies showed higher values of ADA - for instance, one group of authors²⁰ stated that patients with RA had ADA of 26.67 U/L \pm 8.74 U/L and healthy controls 19.79 ± 5.63 . Surekha et al.²¹ presented high serum activity of ADA in patients with RA (59.79 U/L \pm 21.09 U/L), while the value in healthy controls' was 20.71 ± 5.63 . Aside from the disagreement between the activities of the enzyme, this analysis confirmed the results of the above mentioned analyses^{20, 21} which stated that patient with RA showed higher activity of ADA in the serum. Unlike the results that we obtained, other studies^{22, 23} showed similar results of ADA activity in patients with RA and Mn control group.

The results of previous research, which referred to the interrelation between purine cycle enzymes and clinical presentation of RA, were contradictory, especially those which referred to the interrelation between RA activity and purine enzyme activity. The majority of studies showed a positive correlation between ADA activity in serum and the disease activity. A group of authors²⁴ tested the total serum of activity ADA in RA patients in different disease stages and showed that ADA activity was in significant correlation with DAS28. Some authors stated that ADA activity in the serum could predict RA activities²⁵. Results of several studies^{24, 26} showed that ADA and its isoenzymes could be used as alternative parameters for showing the disease activity. Namely, they recorded clear correlation between DAS28 score in RA and catalytic activity of total ADA and ADA2 isoenzymes. Additionally, they stated that isoenzyme form of ADA 2 was increased along with the disease progression.

Unlike the above-stated results, one study²⁷ showed no positive correlation between ADA activity and parameters of RA activities, especially in patients who had been treated with the nonsteroidal anti-inflammatory drug (NSAIL) and anti-TNF inhibitors. The latest research²⁸ which included 110 RA patients and 55 controls did not record any positive correlation between ADA values and the disease activity. However, the research confirmed increased activity of ADA in the serum of RA patients as compared to that in the control group.

Attempting to continue previous research, we analyzed the correlation between serum ADA and the disease activities and showed that the disease activity measured by DAS28 score was not in correlation with serum ADA activity. Even though our results showed increased serum ADA activity in the RA group without MTX (Table 2), we did not obtain a positive correlation between the disease activity and ADA activity in the serum.

Literature data on role and origin of 5'-NT in RA were also contradictory. Most authors agreed that increased levels of this enzyme were present in the active disease, while lower levels were expected in remission stage. Erer et al.²⁷

showed that the level of 5'-NT was increased with the disease activity increase and stated that synovial 5'-NT was probably isoenzyme of the serum 5'-NT. Our results showed that there was no significant correlation between DAS28 score and 5'-NT activity in the MTX and no MTX groups. Additionally, we did not record the change of 5'-NT activity in terms of age. Contrary to our results, one extensive research showed that the level of 5'-NT dropped with age, as well as that the values of 5'-NT were by 27% lower at male than at female patients²⁹.

It would be very hard to compare the results of our study with previous ones because the enzymes were tested on different enzyme kits. Additionally, tests were carried out on different groups of patients (in terms of gender and age distribution) with different disease duration and therapeutic approach. Stolk et al.²⁹ pointed out that majority of RA patients who participated in the research at medical and research centers had already been treated with NSAIL, while large number of them was treated with some of the disease-modifying antirheumatic drugs (DMARD) and therefore the effects that such treatment had on ADA and 5'-NT activity cannot be excluded.

It should be emphasized that almost all of our patients used NSAIL, while three patients were treated with paracetamol. Patients with RA and controls did not use corticosteroids during the month they were included in the study. The average dose of MTX applied in patients with RA was 14,5 mg a week.

It is well-known that reduction of the local concentration of adenosine, by ADA contributes to joint inflammation in RA. MTX increases the concentration of extracellular adenosine at inflammation area^{30, 31}. Analysis of results confirmed that activity of ADA was significantly lower in RA patients treated with MTX therapy than in patients who were not treated with MTX. These results are in accordance with the mechanism of MTX action on adenosine metabolism, i.e. ADA inhibition. Previously, researchers^{22, 32, 33} also presented a significant difference between the ADA activity in patients with RA treated with MTX and in patients who did not receive MTX in their therapy. On the other hand, we did not find any positive correlations between the activity of 5'-NT and MTX treatment.

The disease duration in patients with no MTX treatment was 12 months, which was significantly less as compared to a group treated with MTX (disease duration approximately 120 months). Our results indicated that the longer the disease, the less serum ADA activity. However, if we disregarded the effects of MTX treatment, we could see the stronger negative correlation between the two variables. The activity of 5'-NT in the tested groups did not depend on the disease duration. A study¹³, which monitored the changes of purine cycle enzyme activities during MTX treatment of RA patients, showed similar results. There were no differences in enzyme activities after 6 weeks of MTX treatment. However, the study recorded a significant drop in the ADA activity and no changes in 5'-NT activity after 48 weeks.

Recent research¹³ did not show mutual dependence between 5'-NT and ADA activities, and gender. We did not find the changes in 5'-NT and ADA activities in terms of

gender. However, Stolk et al.²⁹ showed that the activity of 5'-NT was by 27% lower in male than in female patients.

Our research did not record the significant effect of age on ADA and 5'-NT activities. It should be emphasized that some researches showed the decrease of 5'-NT with age increase^{29, 34}.

Conclusion

We demonstrated that serum adenosine deaminase activity was increased in patients with rheumatoid arthritis, as well as that the application of methotrexate led to its decrease. Serum activity of 5'-nucleotidase was not increased in

patients with rheumatoid arthritis and did not depend on methotrexate treatment. Serum adenosine deaminase and 5'-nucleotidase activities were not good indicators of rheumatoid arthritis activity.

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