Deoxyribonuclease activity of polyclonal IgGs and blood serum in patients with early arthritis

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Abstract

Deoxyribonuclease (DNAse) properties of serum and autoantibodies have been demonstrated in patients with autoimmune and infectious diseases, but conclusive data about DNAse activity of serum and antibodies in patients with early arthritis are lacking. We performed a cross-sectional study to evaluate levels of serum and IgG DNAse activity in patients with early arthritis and investigating patients with early rheumatoid arthritis (ERA) (n = 53) and acute reactive arthritis (AReA) (n = 48). In addition the EA patients, 39 healthy controls matched for sex and age were recruited. Levels of DNAse activity of serum and IgG were assessed by the rivanol clot method and confirmed by agarose gel electrophoresis. Levels of DNAse serum activity were significantly higher in sera of EA patients than in the control subjects (p < 0.0001). Levels of DNAse IgG activity were significantly higher in EA patients than in the control subjects (p < 0.0001). Patients with ERA displayed significantly higher levels of serum and IgG DNAse activity than patients with AReA (p < 0.0001). Regarding the differentiation of ERA from AReA, serum DNAase activity detection showed a sensitivity, specificity, positive likelihood ratio, and negative likelihood ratio of 80.0%, 92.2%, 10.1, and 0.2 respectively, IgG DNAse activity detection showed a sensitivity, specificity, positive likelihood ratio, and negative likelihood ratio of 80.0%, 92.2%, 10.1, and 0.2 respectively. Detection of IgG DNAse activity can be employed for the differential diagnosis of EA.

Keywords:
Deoxyribonuclease, IgG, blood serum, arthritis.

Introduction

Differential diagnosis of early arthritis is a topical problem of modern rheumatology. Most often, difficulties arise in the verification of early rheumatoid arthritis (ERA) and acute reactive arthritis (AReA). A standard method for laboratory diagnosis and diagnostic criteria for RA is a positive IgM rheumatoid factor (IgM RF), but the sensitivity is equal to 50–90% and a specificity of 80–93% [1]. IgM RF detected in the serum for many rheumatic diseases, chronic infections, lung diseases, malignant neoplasms, primary biliary cirrhosis, and in old age [1, 2]. Determination of antibodies to cyclic citrullinated peptide (Anti-CCP) is more useful for the diagnosis of RA than the definition of IgM RF. However, at high specificity (93–99%), the method has sufficiently low values of sensitivity (41–80%) [1], and in the case of ERA sensitivity of 67.3% [3]. Besides anti-CCP were detected in patients with infectious diseases [4]. Search
for new laboratory markers of early rheumatoid arthritis high analytical characteristics is continued.

DNAs serum activity has been implicated in the pathophysiology of autoimmune diseases since the 1950s. The importance of DNase1 has grown up since the description that apoptotic cells can be the source of self-antigens in systemic lupus erythematosus (SLE). Many articles have focused in disturbed apoptosis and in the defects of the apoptotic cell debris as the origin of nucleosomes against which the immune response can be induced. The enzyme DNase1 plays a role in the clearance of apoptotic debris, and is therefore of capital interest in this process [5]. DNase serum activity is much higher in primary biliary cirrhosis (PBC) than in Graves’ disease (GD) and multiple sclerosis (MS) or in healthy subjects [6].

Achieving a vibrant now a new branch of immunology – abzymology (“abzymes”, from the English: antibody + enzyme, catalytic antibodies) made by the new information in the functioning of the immune system, contribute to the solution of various biotechnological problem [7, 8]. Detection of the catalytic activity of immunoglobulins in rheumatic diseases creates the basis for further scientific research on the abzymes as biomarkers of these diseases, as well as the development of diagnostic criteria [7, 8]. In rheumatoid arthritis (RA) was studied pathogenetic role and clinical significance of DNA hydrolyzed antibodies [9], but did not assess the potential use of the results of its determination to the differential diagnosis of RA from other arthritis. Till now in ERA patients catalytic activity of serum and antibodies has not been studied. Recently the diagnosis of autoimmune diseases are invited to assessment of different types of enzymatic activity of serum [10]. Evaluation of serum catalytic activity and study of its diagnostic importance in early arthritis has not yet been performed.

The aim of the study is to analyze the DNase activity of polyclonal IgG and serum in patients with early arthritis (early rheumatoid arthritis (ERA) and acute reactive arthritis (AREA), as well as the possibility of using the results of determination of these types of activity for the differential diagnostics of early arthritis.

Materials and Methods

All reagents used in the work were purchased from Sigma, if not otherwise indicated. Recombinant protein A-Sepharose matrix was purchased from Pierce (USA).

Patients and methods

53 patients with ERA, 47 patients with AREA, associated with Chlamydia trachomatis urogenital infections and 39 healthy persons (control group) were enrolled in the study. The study was performed in accordance with ethical principles based on the Helsinki declaration. The blood sampling protocol conformed to local hospital human ethics committee guidelines. Informed consent was obtained from all patients.

Complete physical examinations had been performed in all patients with EA and controls by one investigator (M. V. Volkava). The diagnosis of RA was established in accordance with 2010 Rheumatoid Arthritis Classification Criteria [11]. The diagnosis of ReA was made in accordance with preliminary international criteria [12].

There were no differences (p > 0.05) in mean age and male / female ratio in the group of examined patients, in comparison with control group (Table 1).

<table>
<thead>
<tr>
<th>Characteristics of patients</th>
<th>ERA</th>
<th>AREA</th>
<th>Controls (healthy donors)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>22 (41.51%)</td>
<td>28 (58.33%)</td>
<td>25 (64.1%)</td>
</tr>
<tr>
<td>Female</td>
<td>31 (58.49%)</td>
<td>19 (41.67%)</td>
<td>14 (35.9%)</td>
</tr>
<tr>
<td>Mean age ± SD</td>
<td>51.1 ± 3.45</td>
<td>39.90 ± 8.26</td>
<td>36.1 ± 1.1</td>
</tr>
<tr>
<td>Average disease duration (months) ± SD</td>
<td>4.29 ± 2.39</td>
<td>3.48 ± 2.44</td>
<td></td>
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</tbody>
</table>

All participants completed questionnaires, which included questions on demographics such as age, sex, educational attainment, occupation, exercise, family history of arthritis, and co-morbidities. We also collected clinical data, such as, disease duration, age at disease onset, manifesting symptoms, initial joint symptoms, previous site of joint and enthesis involvement, and history of extra-articular manifestations. We performed complete joint and enthesis examinations and physical measurements on all patients at study entry. Joint involvement was defined either if a joint showed swelling, tenderness, or a limited range of motion during physical examination or if...
the patient reported having arthralgia/arthritis in a joint during clinical data collection. Enthesitis was defined as either the presence of enthesal tenderness during physical examination or the history of pain at the above enthesal areas. The peripheral joints (wrist, feet) radiographs were obtained. Radiographs in this group of patients were scored using the modified Steinbrocker method. Disease activities in patients with ERA were evaluated using a Disease Activity Score 28 (DAS28).

In patients with ERA 28 patients (58%) were seropositive for RF IgM. Radiographic 1st stage was observed in 14 patients (29%), 2nd stage – 25 patients (52%), 3rd stage – 9 patients (19%). All patients were determined by first functional class. The average value of DAS28 was 5.53 ± 1.69 (95% CI 5.0–6.02). The average Richie index value was 14.81 ± 12.30 (95% CI 11.24–18.39).

In ReA patients, polyarthritis was detected in 34 persons (28.3%), oligoarthritis – in 45 (37.5%), enthesopathies – in 86 patients (71.7%), sacroilitis – in 39 patients (32.5%), spondylitis – in 22 (18.3%). Radiographic 1st stage was detected in 50 patients (41.7%), 2nd stage – in 66 (55.0%), 3rd stage – in 4 (3.3%). *Chlamydia trachomatis* infection was diagnosed in all patients. Verification of urogenital chlamydiosis was made by PCR combined with indirect immunofluorescence testing or ELISA (Vector Best, Novosibirsk, Russia).

All patients who participated in the study on outpatient taking non-steroidal anti-inflammatory drugs. A survey on the catalytic activity performed before the first treatment with antibiotics, basic drugs and glucocorticoids.

The laboratory tests included: complete blood cell count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF), anti-cyclic citrullinated peptide antibody (anti-CCP), T- and B-cell count, phagocytosis index, total IgG concentration.

Electrophoretically and immunochemically homogeneous IgGs were obtained from patients and control sera by the multi-step purification method. Briefly, blood (10 ml) was incubated for 3–5 hrs at 4°C to achieve complete coagulation, serum was carefully separated and centrifuged for 15 minutes at 500 g. It was then mixed with 0.75% rivanol (ethacridine lactate) solution in ratio 1 : 2. The mixture was incubated at 4°C for 2 hrs. The pellet was discarded and rivanol was removed by adsorption on charcoal column. Afterwards the sample was loaded onto a 2-ml recombinant protein A-Sepharose column equilibrated in buffer A (0.1 M phosphate buffered saline (PBS), pH 7.4). The column was washed with 15 ml of buffer A. Non-specifically adsorbed proteins were then washed away with buffer A, containing 1% Triton X-100 and 0.3 M NaCl. Then IgGs of 1, 2 and 4 subclasses were eluted with 0.1 M glycine-HCl (pH 2.8). Ig-containing fractions were collected, immediately neutralized with 1 M Tris-HCl buffer pH 9.0 and intensively dialyzed against 0.9% NaCl. Ab preparations were kept in plastic tubes at –20°C until use. Subsequent sterility control tests of purified IgG samples did not reveal any traces of bacterial or fungal contamination.

Analysis of Abs for homogeneity was conducted in SDS-PAGE under reducing and non-reducing conditions in 3–16% gradient, or 12.5% gel containing 0.1% SDS with Coomassie Brilliant blue R250 stain.

The basic method for DNAse activity measurement relied upon the capacity of rivanol to form a clot with DNA, reversely proportional to nucleic acid depolymerization on DNAse action. The reaction mixture (0.4 ml) contained 0.2 ml calf thymus DNA (300 µg/ml), 0.1 ml of IgG samples (1 mg/ml) and 0.1 ml 0.02 M Tris-HCl (pH 7.4) with 0.01 M MgCl₂ and 0.02% sodium azide, and was incubated for 20 hrs at 37°C. DNAse activity levels were registered after addition of 20 µl 0.75% rivanol solution into the reaction mixture to make clot. Abzyme activity was measured semiquantitatively using arbitrary units (AU) that correspond to a 6-grade visual scale (from 0 to 5 points). Zero level of activity was attributed to a negative DNAse reaction (full clot) whereas 5 AU denoted complete degradation of the DNA-rivanol clot, resulting from DNA depolymerization. DNAse abzyme activity was primarily assessed per 1 mg of IgG (“specific activity”). Then it was recalculated for total IgG concentration in 1 ml of serum.

For direct confirmation of DNA hydrolysis by abzyme IgGs in specified conditions the products of the reaction were analyzed in 1.5% agarose gel electrophoresis with ethydium bromide stain.

**Statistical analysis**

Data distributions were assessed by Shapiro-Wilk’s test. In cases of normal data distribution the results were presented as mean (M) ± standard deviation (SD). Data that did not fit a normal distribution were expressed as median (Me), range (Min-Max) and interquartile range (25th and 75th percentiles). 95% confidence intervals (CI) were calculated for means and medians. Statistical data differences for means and medians were assessed by the Student’s t-test and Mann-Whitney’s U-test, respectively. The significance level was established at 0.05.
Statistical correlations were calculated by the Spearman method. For diagnostic performance analysis, receiver operator curve (ROC) analysis was performed and analytical characteristics of corresponding tests were calculated [13].

**Results**

IgGs fractions were purified from sera of patients and healthy donors with chromatography on Protein A-Sepharose using a special protocol to remove nonspecifically bound proteins. Strong non-covalent protein complexes usually dissociate under treatment with Triton X-100. Homogeneity of abzyme samples was confirmed in SDS-PAGE under non-reducing conditions in 12.5% gel with Coomassie brilliant blue stain (Figure).

Results of DNAse serum and abzyme activity assessment in patients with early arthritis and healthy donors are shown in Tables 2–3.

It has been estimated that levels of DNAse IgG activity in patients with EA were significantly higher than in healthy donors (p < 0.0001). At the same time DNAse IgG activity in patients with ERA exceeded the levels of patients with AReA and (p < 0.0001).

Differences between the levels of serum DNase activity in patients with ERA and AReA compared with a group of healthy subjects were statistically highly significant (p < 0.0001). In patients with ERA value of serum DNase activity exceeded (p < 0.0001) those in patients with OReA.

In patients with ERA IgG DNAse activity is significantly correlated with the number of swollen joints, DAS28 and total T-lymphocytes count (r = 0.66, r = 0.85, r = 0.76, respectively, p < 0.05). In patients with AReA IgG DNAse activity is significantly correlated with total B-lymphocytes count (r = 0.57, p < 0.05) and the number of CD4+ T helper count (r = 0.57, p < 0.05).

Given the statistically highly significant differences between the values of the specific activity of DNase IgG, and serum DNase activity in patients with ERA and AReA we studied the possibility of determining the specific test DNase activity of IgG and serum DNase activity in the differential diagnosis of these diseases. Analytical characteristics of new assays for ERA and AReA differentiation are shown in Table 4.
We performed a comparative analysis of the characteristics of analytical tests developed by the reference methods and differential diagnosis of ERA and AReA based on the definition of the RF IgM and anti-CCP on our own cohort of patients with early arthritis. The results of calculation are summarized in Table 5.

### Table 4. Analytical characteristics of DNase-based tests for early rheumatoid arthritis and acute reactive arthritis

<table>
<thead>
<tr>
<th>Analytical characteristics</th>
<th>DNase IgG activity per 1 mg of IgG</th>
<th>DNAse serum activity</th>
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<tbody>
<tr>
<td></td>
<td>ERA vs AReA</td>
<td>ERA vs AReA</td>
</tr>
<tr>
<td>Test sensitivity</td>
<td>86.05 (95% CI 72.10–94.70)</td>
<td>93.50 (95% CI 82.10–98.60)</td>
</tr>
<tr>
<td>Test specificity</td>
<td>83.33 (95% CI 68.60–93.00)</td>
<td>95.00% (95% CI 83.00–99.20)</td>
</tr>
<tr>
<td>Positive likelihood ratio (PLR)</td>
<td>5.16 (95% CI 4.30–6.20)</td>
<td>18.70 (95% CI 16.80–20.80)</td>
</tr>
<tr>
<td>Negative likelihood ratio (NLR)</td>
<td>0.17 (95% CI 0.06–0.50)</td>
<td>0.069 (95% CI 0.01–0.40)</td>
</tr>
</tbody>
</table>

### Table 5. Analytical characteristics of differential diagnosis tests for ERA and AReA based on the definition of RF IgM and anti-CCP (on our own cohort of patients)

<table>
<thead>
<tr>
<th>Analytical characteristics</th>
<th>RF IgM</th>
<th>Anti-CCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test sensitivity</td>
<td>45.16% (95% CI 27.30–64.00)</td>
<td>73.53% (95% CI 55.60–87.10)</td>
</tr>
<tr>
<td>Test specificity</td>
<td>87.80% (95% CI 73.80–95.90)</td>
<td>93.75% (95% CI 79.20–99.10)</td>
</tr>
<tr>
<td>Positive likelihood ratio (PLR)</td>
<td>3.70 (95% CI 2.50–5.50)</td>
<td>11.76 (95% CI 9.40–14.70)</td>
</tr>
<tr>
<td>Negative likelihood ratio (NLR)</td>
<td>0.62 (95% CI 0.30–1.50)</td>
<td>0.28 (95% CI 0.07–1.2)</td>
</tr>
</tbody>
</table>

### Discussion

Currently, the number of investigations that study associations of catalytic IgG activities and disease characteristics, including activity, severity, remission length, and future prognosis, are greatly increasing.

This study demonstrates significantly elevated levels of DNase serum and abzyme activity in patients with early arthritis compared with healthy subjects for the first time. According to the literature [14, 15], the highest levels of DNase activity of polyclonal antibodies observed in autoimmune diseases. In our study, levels of DNase activity ERA polyclonal IgG were higher (p < 0.0001), than in AReA, which confirms the notion of RA as a progressive systemic autoimmune process in which there are marked immunopathological changes. DNase abzymes can play an important role in the pathogenesis of various autoimmune disorders [16, 17]. DNA hydrolyzing Abs have demonstrated high cytotoxic potential, caused nuclear DNA fragmentation, and induced apoptosis [18, 19]. Thus, catalytic Abs can aggravate destructive effects on host tissues via direct cytotoxic action. It has been also speculated that DNase abzymes may eliminate DNA-containing cytolysis products in early stages of disease, but may later exert proinflammatory activity. The involvement of DNase abzymes in apoptosis regulation needs special attention as, for instance; deregulation of apoptosis is one of the leading factors of epidermis hyperproliferation in psoriasis [20]. Several data indicate that abzymes can play a role as natural regulators of apoptosis and other cytotoxic mechanisms in systemic autoimmune diseases and neoplasms [18].

Taking into account the data on the identification anti-nuclear antibodies, antibodies to double-stranded DNA, perinuclear antibodies in various diseases, can not be excluded that the abzymes with DNase activity, appearing in the early stages of the disease, have an adaptive value, destroying the excess of nucleic acids in cytolysis. With the progression of disease DNase antibodies may
have a direct damaging effects on tissues, realizing their cytotoxicity [14]. Abzymes possessing DNase activity also contribute to the regulation of apoptosis.

Positive correlations of DNase abzymes activity with T-cell count, number of swollen joints and DAS28 provide further hints of a putative role of abzymes in autoimmune reactions in early arthritis.

The enzyme DNase degrades DNA during early apoptosis. Impaired DNase activity might increase susceptibility to autoimmune diseases. DNase1 activity is significantly lower in patients with SLE [21]. Nevertheless, it was not find relationships with any other of the epidemiological, clinical, immunological or therapeutical variables considered [21]. Previous findings indicate the potential relevance of DNase serum activity in patients with monoglandular and polyglandular autoimmune and their clinically healthy relatives. The impaired DNase activity might reduce removal of circulating self- or pathogen-derived DNA thereby favoring autoimmune mechanisms by Toll-like receptor 9 co-activation [22]. In other way mitochondrial DNA that escapes from autophagy cell-autonomously leads to Toll-like receptor 9-mediated inflammatory responses in cardiomyocytes and is capable of inducing myocarditis and dilated cardiomyopathy [23]. There are a few studies of DNase serum activity in patients with RA, and lack of these studies in patients with early arthritis. It has been shown that the association of DNASE2-1066 GG homozygosity with RA was limited to rheumatoid factor-positive disease, but was not influenced by the presence of anti-cyclic citrullinated peptide or antinuclear antibodies [24].

The design of new serum- and abzyme-based laboratory tests with high discriminating power might be a novel promising approach fulfilling the requirements for a diagnostic marker in systemic rheumatic diseases. Development of diagnostic test and differential diagnostic test based on an assessment of IgG catalytic activity is considered by many authors current and future direction of modern abzymology [25, 26]. Resulting the determination of specific DNase activity of IgG and serum DNase activity, we have developed tests and differential diagnosis of ERA from AReA, as well as assess their diagnostic utility by calculating the analytical parameters. Here, for the first time we present a test design for the discrimination of ERA from AReA, by means of DNase serum and IgG assessment, and calculated its analytical performance.

According to the Immunologic Laboratory Testing Guidelines, laboratory tests are recommended for clinical practice as “very useful”, when the majority of reported data reveal positive likelihood ratios > 5 or negative likelihood ratios < 0.2. The test for differential diagnosis of ERA and AReA on the results of the determination of specific IgG DNase activity meets the criteria for useful diagnostic tests, and test the differential diagnosis of ERA and AReA the evaluation of serum DNase activity meets the criteria of the most useful diagnostic tests. Reference methods for differential diagnosis of ERA and AReA inferior to the analytical characteristics developed tests based on the evaluation of specific DNase activity of IgG and serum DNase activity.

Conclusion

In patients with early rheumatoid arthritis and acute reactive arthritis levels of DNase activity of polyclonal IgG, and serum DNase activity significant (p < 0.001) higher than control values in healthy individuals. Patients with early rheumatoid arthritis have higher levels of IgG and serum DNase activity compared with patients with acute reactive arthritis.

A test of differential diagnosis of early rheumatoid arthritis and acute reactive arthritis, based on a DNase activity of IgG in their operating characteristics meet the criteria of useful diagnostic tests in rheumatology, and the test based on the assessment of serum DNase activity is most useful in rheumatological practice.

References

5. Martínez VF, Balada E, Ordi-Ros J, Vilardell-Tar


Deoxyribonuclease activity of polyclonal IgGs and blood serum in patients with early arthritis

Asmenų, sergančių ankstyvu artritu polikloninių imunoglobulinų G ir kraujo serumo deoksiribonukleazės aktyvumas

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Santrauka
Serumo deoksiribonukleazės (DRN) savybės ir autoantikūnių tiriami asmenims, sergantiems autoimuninėmis ir infekcinių ligomis, bet galutinių duomenų apie serumo DRN aktyvumą ir autoantikūnius asmenims, sergantiems ankstyvu artritu yra nepakankamai. Mes atlikome skerspjūvio tyrimą ir ištyrėme serumo ir Ig G DRN aktyvumą asmenims, sergantiems ankstyvu artritu bei nustatėme asmenis su ankstyva reumatodinio artrito forma (ARA) (n = 53) ir ūminiu reaktyviniu artritu (ūReA) (n = 48). Taip pat ištyrėme 39 sveikus tos pačios lyties ir amžiaus kontrolinės grupės asmenis. Serumo ir Ig G DRN aktyvumas nustatytas rivanolio krešulio metodu ir paviršinio agrozės gelio testu elektroforezės metodu. Serumo DRN aktyvumas buvo reikšmingai didesnis ARA sergantiems asmenims serume palyginti su kontroline grupe (p < 0,0001). Ig G DRN aktyvumas buvo reikšmingai didesnis serume ARA sergantiems asmenims palyginti su kontrolinės grupės (p < 0,0001). Asmenims, sergantiems ARA nustatytas reikšmingai didesnis serumo ir Ig G DRN aktyvumas palyginti su asmenų, sergančių ūReA (p < 0,0001). Tiryti ARA ir ūReA asmenis, serumo DRN aktyvumo nustatymas yra 80 proc. jautrus, 92,2 proc. specifinis, nustatyta 10,1 proc. teigiama tikimybė ir 0,2 proc. neigiama tikimybė, Ig G DRN aktyvumo nustatymas yra 80 proc. jautrus, 92,2 proc. specifinis, nustatyta 10,1 proc. teigiama tikimybė ir 0,2 proc. neigiama tikimybė. Ig G DRN aktyvumo nustatymas gali būti taikomas ankstyvo artrito diagnostikoje.

Raktažodžiai:
deoksirobomukleazė, imunoglobulinas G, serumas, artritas.