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Inflammation Parameters, Xanthine Oxidase and Anti-Xanthine Oxidase Antibodies in Synovial Fluid of Patients Suffering from Arthritis

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ABSTRACT

Objective: Rheumatoid arthritis (RA) is an autoimmune disease where sera and synovial fluid (SF) of suffering patients contain immune complexes formed from autoantibodies to several proteins. SF from humans with joint diseases was examined for the presence of some inflammatory parameters and autoantibodies.

Materials and Methods: Antibodies in their free and complex forms were assayed by indirect ELISA. The immunoprecipitation technique was used to evaluate total IgG and IgM and complement.

Results: The results showed that most RA SF was anti-ASLO negative, but they were CRP positive. Levels of complement components (C3 and C4) were highest in the group of mono-/oligo-arthritis and lowest in RA. The results showed that xanthine oxidase (XO) presence and activity were important in SF of RA patients. Moreover, free and complex anti-XO antibodies were detected in all SF with different titers throughout the groups of patients where IgG was lower than IgM.

Conclusion: The studied parameters of inflammation and auto-antibodies especially against XO could serve as an evaluation of the severity of joint inflammation and in RA pathogenesis understanding.

Keywords: Autoantibodies, joint inflammation, streptolysin, Rheumatoid arthritis, xanthine oxidase, complement

INTRODUCTION

Rheumatoid arthritis (RA) is the most severe and destructive of all joint diseases affecting approximately 1% of the worldwide adult population and more often women than men. In the Western world more than 50% of RA patients are older than 65 years at diagnosis (1). The main pathological changes that occur in RA are of an autoimmune nature, chronic synovitis, cartilage destruction and bone erosion (2,3). It is also the most common systemic autoimmune disease and is characterized by the presence of various autoantibodies in serum and synovial fluid (SF) (4,5). This distinguishes RA from other joint diseases (6) where autoimmune phenomena are rarely observed. The immunological process is a crucial factor in the pathophysiology of RA with the presence of immune cells like lymphocytes, phagocytes and plasma cells in the pannus (7,8), whereas SF encloses immunoglobulins, complement, and anti-γ-globulins (9,10). Serologically, RA is known by the existence of a variety of autoantibodies like anti-citrullinated protein, anti-carbamylated protein, peptidylarginine deiminase (PAD4) etc., which possess great roles in RA pathogenesis and diagnosis (6). Synovitis is thought to be caused by the action of interleukin-1, tumor necrosis factor-alpha, prostaglandin E2, matrix metalloproteinases and nitric oxide (11). In addition, xanthine oxidase generates reactive oxygen species (ROS), which lead to chronic damage in joints of RA and osteoarthritis patients (12,13). We have pre-

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viously described the presence of active xanthine oxidase and anti-xanthine oxidase antibodies in synovial fluids of patients suffering from different joint inflammations (10,14). The present study aims to evaluate inflammatory parameters in the SF of patients suffering from RA and other joint illness. For this reason, free and complexed anti-xanthine oxidase (XO) antibodies, Anti-Streptolysin antibodies (ASLO), C-reactive protein (CRP), complement factors (C3, C4), and total immunoglobulins (IgG and IgM) were examined. In addition to their diagnostic use, the presence of autoantibodies should give deeper understanding of the mechanism of joint inflammations.

MATERIALS AND METHODS

SF samples were collected from adult volunteers during regular medical examinations in Dr. Zenoun, Dr. Benouar, Dr. Lebdani and Dr. Ait Majbar's clinics. Patients and volunteers have signed an informed consent following a detailed explanation of the undertaken procedures. Samples (n=137) were divided into five groups as shown in Table 1: rheumatoid arthritis group (RAG), mono/oligo arthritis group (MOA), a group suffering from gonarthrosis of the knee (GKG), hemarthrosis and mechanical inflammations group (HMI) and with other types of joint inflammations (OJI).Approval was obtained from the ethics local committee (NrECA/003/22). Collected samples were centrifuged at 3000 x g (Hettich Universal centrifuge) for 15 min, then distributed to small aliquots and kept at -20°C until use. To reduce viscosity, an aliquot of each SF was incubated with Hyaluronidase enzyme (Sigma, Germany) at 20 µg/ml of SF, for 3 h at 37°C then centrifuged (15) with modifications, and the supernatant was used in various estimations, in parallel to non-treated SF.

Inclusion and exclusion criteria of RA samples were the same as previously explained by Arrar et al. (10). Human sera, pooled as a standard serum, were obtained from the Centre of blood of Setif. Latex positive sera were collected from Central Laboratory of the University Hospital of Setif, Dr Latri and Dr Messai laboratories (Setif).

Xanthine Oxidase Preparation and Activity

Xanthine oxidase (XO) was purified from human milk, kindly provided by volunteers, according to Baghiani et al. (16) and Abadeh et al. (17). It was supplemented with 2.5 mM dithioerythritol, 1 mM EDTA and 1.25 mM sodium salicylate. It was centrifuged at 3000 × g for 20 min and the cream was collected. An equal volume of 0.2 M K_2HPO_4, containing 2.5 mM dithioerythritol, 1 mM EDTA and 1.25 mM sodium salicylate was added to the cream, followed by slow addition of cold (-20°C) butanol to give 15% (v/v) and then 15% (w/v) solid $(NH_4)_2SO_4$ with constant stirring. The resultant slurry was stirred for 1 h and then centrifuged at 13,000 × g for 20 min. The aqueous lower phase was separated and 20% (w/v) solid $(NH_4)_2SO_4$ was added, with stirring. The suspension was allowed to stand at 4°C for 2 h, after which the separated top phase was collected and centrifuged at 10,000 × g for 30 min. The top phase was again collected and suspended in a small volume of buffer A (0.2 M Na_2HPO_4, containing 2.5 mM dithioerythritol, 1 mM EDTA and 1.25 mM sodium salicylate, pH 6.0) and dialyzed against the same buffer overnight. The precipitate was removed by centrifugation at 40,000 g for 1 h. The supernatant was applied to a column (1 cm/8 cm) of heparin-agarose (Sigma, type 1) equilibrated with heparin buffer. The column was washed with 0.1 M NaCl in buffer A and xanthine oxidase was then eluted by using buffer A, containing, additionally, 5% (w/v) $(NH_4)_2SO_4$. Protein-containing fractions were combined and dialyzed overnight against 50 mM sodium bicine, pH 8.3 (3L) buffer aliquoted (0.5 ml) and stored at -20°C.The purity of the enzyme was based on protein/flavin ratio (PFR = A_{280}/A_{450}), and SDS-PAGE using vertical slab gels. XO activity was determined by measuring the generation of uric acid at 295 nm (16).

Anti-XO Antibodies Preparation

Antibodies against XO enzyme were obtained by immunizing New Zealand white rabbits (18). After obtaining IgG by precipitation with 18% sodium sulphate the purity and the immune reactivity were tested by SDS-PAGE and indirect ELISA, respectively (10).

Indirect ELISA Protocol

To determine titers of rabbit immune-serum, normal or latex positive human sera or synovial liquids against XO, 5 µg/ml of human xanthine oxidase (HXO) in $NaHCO_3/Na_2CO_3$ solution (50 mM, pH 9.6) were coated on well microtitration plates (100 µl).

Table 1. Repartition of sample among different groups of patients

	Group of patients	Number	Age
Synovial fluids	Rheumatoid arthritis (RAG)	26	52±15
	Mono-/oligo-arthritis (MOA)	22	61±19
	Gonarthrosis of the knee (GKG)	24	63±4
	Hemarthrosis/mechanical inflammations (HMI)	30	39±5
	Other joint inflammations (OJI)	35	49±11
Sera	Healthy donors	65	38±13
	Latex positive patients	69	42±12

After overloading of the free sites by casein, series of serum or SF dilutions were added and incubated for 1 h 30 min. at 37°C, then goat anti-rabbit IgG or anti-human IgG/IgM antibodies labeled with horse radish peroxidase diluted in PBS-Tween were added and incubated for 1 h at 37°C. The revelation of the interaction was done by the substrate solution containing orthophenylene diamine (Sigma, Germany) and H_2O_2 (0.07%, v/v) in sodium citrate (0.1 M) Na_2HPO_4 (0.2 M), pH6. After an incubation during 10 to 15 min in the dark, the reaction was stopped with 50 µl/well of H_2SO_4 (1N) and the absorbance measured at 492 nm with a microplate reader (Diagnostics Pasteur LP200), (10).

For XO-anti-XO immune-complex estimation, rabbit anti-HXO serum (diluted 40 times in $NaHCO_3/Na_2CO_3$, 50mM, pH 9.6) was coated on the microplates then serum or SF was added; after that the same steps as above were followed.

The same steps of ELISA for the titration of antibody, in their free and complex forms, were followed to calibrate standard human serum according to Arrar et al. (10).

Detection of Xanthine Oxidase

For the detection of XO enzyme in SF, the microplates were coated with diluted SF (100 times in $NaHCO_3/Na_2CO_3$, pH 9.6, 50 mM), and then incubated with a series of dilutions of rabbit anti-XO for 90 min at 37°C. The immune reaction was detected using peroxidase-labeled anti-rabbit IgG.

Determination of Rheumatoid Factor

The detection of RF was conducted using a hemagglutination method according to the manufacturer (Spinreact, Spain). The presence of visible agglutination of erythrocytes indicates a RF concentration equal or greater than 8 IU/ml.

Determination of C-reactive Protein Level

The samples were tested for the detection of CRP by an agglutination test (RapiTex® CRP), by dispensing 40 µl of undiluted patient sample and 40 µlof each CRP positive and negative control serum, each onto a different field of a test plate. Absorption solution (40 µl)was dispensed alongside each sample or control sera. A volume of 40 µl of RapiTex-CRP was added to the absorption solution. After thoroughly mixing each set of drops with stirring rods for 2 min., agglutination was checked for 2 min. The sensitivity of the test is approximately 6mg/ml for a sample volume of 40µl when the test is read after 2 min.

Determination of Anti-streptolysin and Complement Components

To detect ASLO antibodies, an agglutination test was used according to the manufacturer's instructions (RapiTex®ASLO, 2003). Samples with an ASLO content of ≥200 IU/ml ± 20%, exhibit clear agglutination. Samples with an ASLO content of <200 IU/ml show no agglutination. Also, complement factors (C3c, C4) were assayed following the protocol assigned by the manufacture (Turbiquant®). The results are directly given as g/l. The measurement range valid for the TurbiTime System when using 1:21 diluted samples is C3c (0.3- 4.5 g/l) and C4 (0.045- 1.17 g/l).

Determination of Total Immunoglobulins G and M

Turbiquant®Immunoglobuline test was used to determine IgG and IgM antibodies in SF and sera. Samples were diluted 1/21 with isotonic saline solution. The vial reagent was placedin the reagent receptacle of the TurbiTime System. Volumes of 20 µl (IgG) and 200 µl (IgM) of the diluted samples were placed in a cuvette. The quantification, given as g/l, is automatically performed after adding the reagent. The measurement range valid for the TurbiTime System when using 1:21 diluted samples is IgG (0.85- 145 g/l) and IgM (0.17- 33.5 g/l).

Statistical Analysis

All results were calculated as three or more determinations and expressed as mean±standard deviation (SD). Statistical analyses were carried out by using SigmaStat. Comparison of groups was carried out with Student's *t* test. P values less than 0.05 were considered statistically significant.

RESULTS

Total IgG and IgM Immunoglobulins in SF

The results obtained show that total IgG and IgM are present in the SF in most cases. IgG are the most represented with the highest concentrations in patients with mono or oligo-arthritis (between 8.41 and 18.60 mg/l) and less in patients with RA, while almost equal in the other samples. For IgM-type antibodies, concentrations are much lower than IgG and the highest percentage was recorded in patients OJI (2.21 mg/l), and the lowest percentage in the GKG samples (Figure 1).

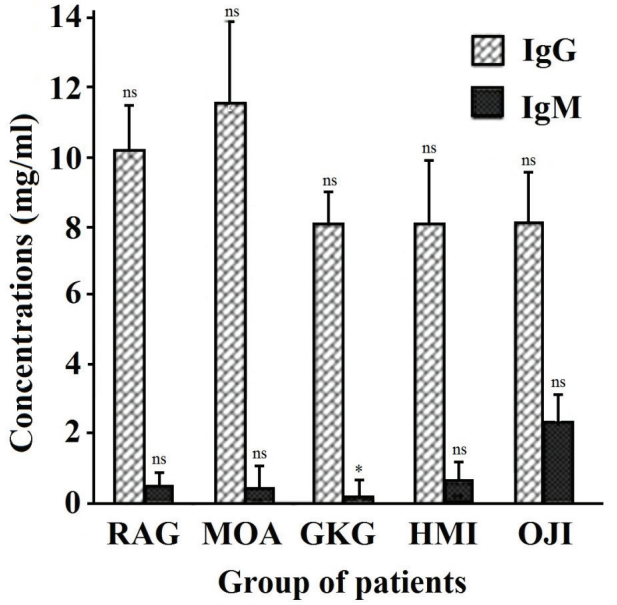

Figure 1. Total IgG and IgM in synovial fluid, using Turbiquant technique.

RAG: Rheumatoid arthritis group, MOA: mono/oligo-arthritis, GKG: gonarthrosis of the knee group, HMI: hemarthrosis/mechanical inflammations, OJI: Other joint inflammations. ns: non-significant, *p<0.05, compared to normal serum.

Inflammation Parameters

Only SF of patients with Rheumatoid arthritis (92.3%) and HMI (50%) were positive for IgM-RF by Latex agglutination test, except a sample of a 20-year-old girl and another of a 57-year-old woman with RA, who were negative (<8 IU/ml; Figure 2). ELISA revealed that SF of all patients was positive except for the group OJI, with the highest percentage in SF from the RAG group.

Results of the detection of CRP are plotted in Figure 2. Samples of subject RAG were mostly positive CRP. This confirms that the incidence was of inflammatory origin. While in the category of mono-/oligo-arthritis (MOA), only 27.27% of the samples were CRP-positive, as well as in the case of HMI (23.33%) and of GKG (18.18%). In the category of OJI, only samples with gout and rheumatoid popliteal cyst were CRP positive.

The test of ASLO antibodies is classified among the rheumatologic markers (19). The results revealed that only two SF from the mono-/oligo-arthritis group were positive (a 44-year- old man with mono-arthritis, and a 68-year-old woman with oligo-arthritis). Also, in GKG only two SF were positive (a 60-year-old woman and an 80-year-old man). In the group with hemarthrosis and mechanical inflammations only one sample was positive (a 40-year-old man with post traumatic injury) (Figure 2).

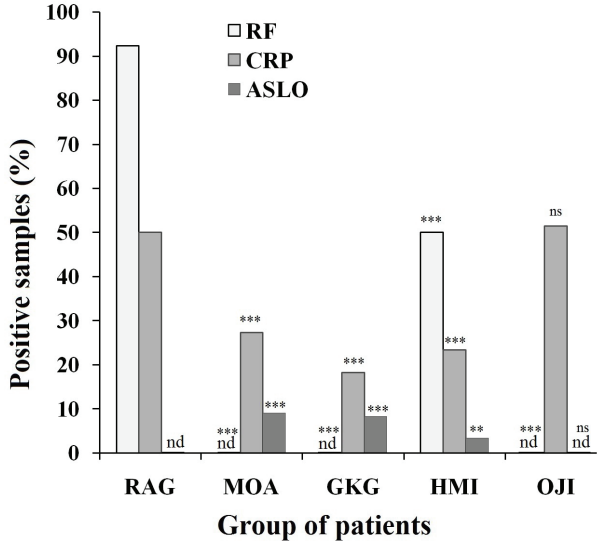

Figure 2. Detection of inflammation markers.

RF: rheumatoid factor, CRP: c-reactive protein and ASLO: anti-streptolysin antibodies. RAG: Rheumatoid arthritis group, MOA: mono/oligo-arthritis, GKG: gonarthrosis of the knee group, HMI: hemarthrosis/mechanical inflammations, OJI: Other joint inflammations. nd: not detected, ns: non-significant, **p<0.01, ***p<0.001, compared to the most severe disease (RAG).

The levels of C3 and C4 can serve as important factors linked to infectious and immune complex diseases (20). For this reason, the concentrations of these two components were evaluated in SF. Results presented in Figure 3 showed that in the category of RA patients (RAG), the concentration of C_3 factor was less than 30 mg/dl in most samples and reached 70.9 mg/dl in a sample where detected C_4 factor was absent, and 59.4 mg/dl in the sample that contained the greatest concentration of C_4 (24.8 mg/dl). In SF from the rest of the samples, C_4 concentrations ranged between 4.5 and 8.17 mg/dl. On the whole, C_3 and C_4 concentrations were much lower than their concentration in the serum, which ranged between 70 to 180 g/l for C_3 and between 10 to 80 mg/dl for C_4 (21). As for the category with MOA, values of C_3 factor ranged between 33.1 mg/dl to concentrations greater than 450 mg/dl, at an average of 148.86 mg/dl, where most values were beyond the concentration of this factor in normal serum. While the concentration of C_4 factor was weakly compared with C_3 factor, concentrations ranged from traces in one of the samples to 25.6 mg/dl, and at a mean of 15.67 mg/dl.

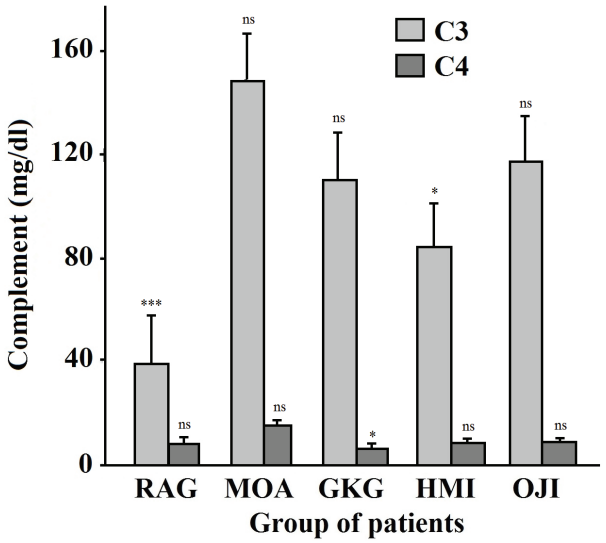

Figure 3. Estimation of C3 and C4 concentrations in SF.

RAG: Rheumatoid arthritis group, MOA: mono/oligo-arthritis, GKG: gonarthrosis of the knee group, HMI: hemarthrosis/mechanical inflammations, OJI: Other joint inflammations. ns: non-significant, *p<0.05, ***p<0.001, compared to normal serum.

The concentration of C_3 factor in the group with GKG was 110.581 mg/dl. However, most of values were above the natural level of this factor in normal serum and some of them were less than the normal concentration (less than 30 mg/dl). The concentration of C_4 factor in four samples was less than 4.5 mg/dl, while in the remaining samples it ranged between 5.06 and 13.2 mg/dl at a rate of 7.037 mg/dl. The highest concentration of C_3 that exceeds 450 mg/dl was observed in a sample of a patient suffering from gout, where the concentration of C_4 was found to be equal to 10.5 mg/dl.

Presence and Activity of Xanthine Oxidase

The presence of XO in SF was detected using indirect ELISA. All studied synovial fluids were XO-positive. The interactions varied

between 320 and 5120 titers. The highest recorded titers were in the RA groups (RAG, MOA).

The presence of XO in SF was detected by using spectrophotometric technique (16). XO activity in SF was estimated by adding 50 µl of each SF into reaction medium, and reading was performed within 10 min due to the viscosity of the liquid and the slow reaction (Figure 4). XO activity in samples of the RA group ranged between 0 and 5 mIU/ml. For the group with mono-/oligo-arthritis, enzymatic activity is concentrated around 1.43 mIU/ml. Gonarthrosis samples showed no enzymatic activity in 75% of samples. In most samples, we obtained different activities of XO enzyme; the highest rate was recorded in the two samples which have gout disease, and joint chondrocalcinosis. A lower rate was registered in the two cases of hemarthrosis and sprained knee. The activity was completely absent in ankylosing spondylitis, hygroma and rheumatoid popliteal cyst samples.

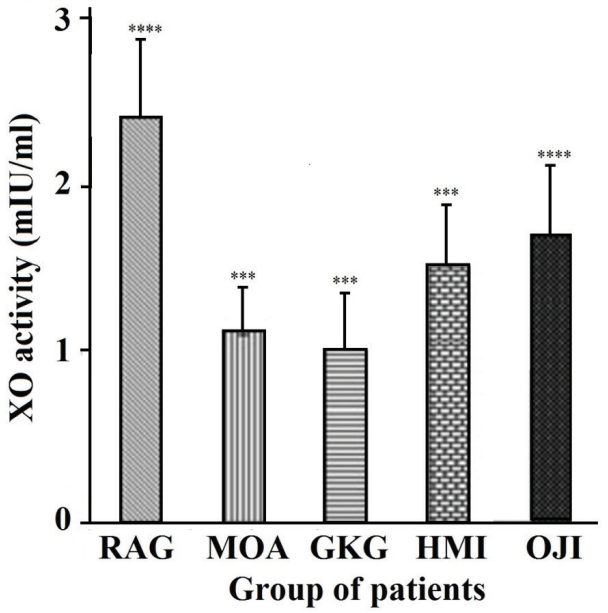

Figure 4. Total xhantine oxidase (XO) activities in synovial fluid.

RAG: Rheumatoid arthritis group, MOA: mono/oligo-arthritis, GKG: gonarthrosis of the knee group, HMI: hemarthrosis/mechanical inflammations, OJI: Other joint inflammations. ***p<0.001, ****p<0.0001 compared to standard serum.

The results of enzymatic activity indicate that in more than 96% of SF, XO exist as oxidase form; the XO activity was completely inhibited with allopurinol as a specific inhibitor for XO.

Unexpectedly, we noticed a significant decrease in the activity after treatment of the samples with hyaluronidase. This decrease is probably due to the impact of the active center of the enzyme by centrifugation and the length of the time of incubation during the treatment of the sampleswith hyaluronidase.

Anti-xanthine Oxidase Antibodies

The results showed that in the RAG, the treatment with hyaluronidase enhanced anti-XO IgM percentage both in free and complex forms, while there was no change in IgG level. IgG antibodies were lower than IgM in the two forms (Figure 5B).

Most of the anti-XO antibodies in the SF of patients in the MOA category were present as IgM class, but with levels lower than those in the RA group (Figure 5A). After the treatment of the SF with hyaluronidase, the levels of IgM antibodies slightly increased while those of IgG type decreased (Figure 5B).

Unlike SF in the RA group, the largest proportion of anti-XO antibodies within GKG was IgG, either in free or immune complexed form (Figure 5A). After treatment of the SF with hyaluronidase enzyme, no change in proportion of free antibodies was detected, but that of immune complexes declined (Figure 5B).

In the HMI group, most of the anti-XO antibodies were IgM in the two forms (Figure 5A). Treatment of the SF with hyaluronidase enzyme ledto a slight increase in the proportion of IgMan-

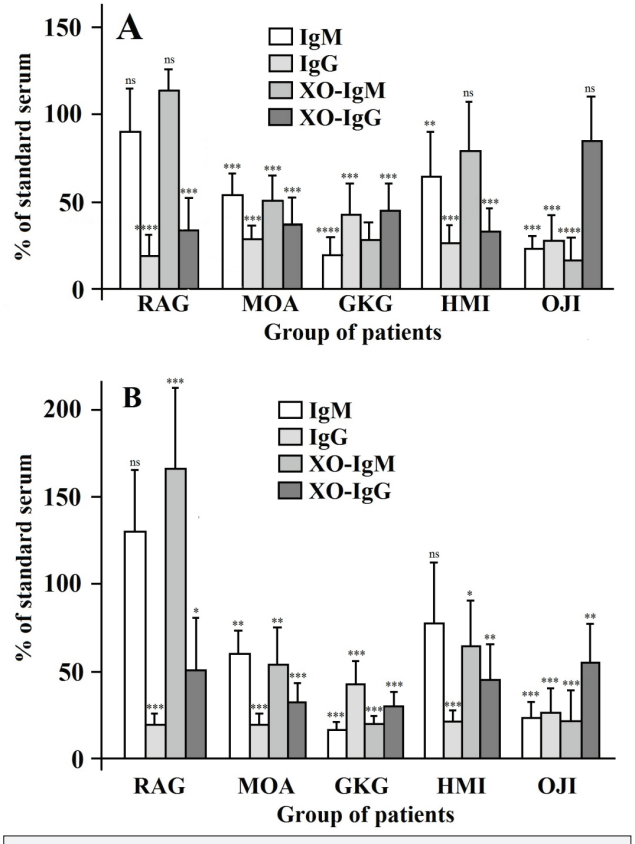

Figure 5. Free and complex anti-XO IgG and IgM represented as % of standard serum±SD in SF of patients. A and B: SF without and with hyaluronidase, respectively.

RAG: Rheumatoid arthritis group, MOA: mono/oligo-arthritis, GKG: gonarthrosis of the knee group, HMI: hemarthrosis/mechanical inflammations, OJI: Other joint inflammations. ns: non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to standard serum.

ti-XOR antibodies and IgG-XO immune complexes and a slight decrease in the proportion of IgM-XO immune complexes (Figure 5B).

The OJI group includes patients with joint pain with inflammatory origin. The levels of antibodies were close in all types of anti-XO antibodies, except for immune-complexes containing IgG that exceed four times the value of the rest of other antibodies (Figure 5A). Treatment of SF with hyaluronidase did not affect the level of antibodies, ether in free or complexes form containing IgM, while it led to a significant decrease (about 30%) in the proportion of immune complexes containing IgG (Figure 5B).

DISCUSSION

The highest levels of total IgG and IgM titers were registered in the SF of patients with arthritis (RAG and MOA) and other joint inflammations (OJI) compared to the levels in the control serum, and are in agreement with previous studies (10, 22). These high levels are due to the auto-immune nature of the diseases.

The presence of CRP found in this study indicates the presence of inflammation in the SF of patients. CRP levels increase in response to tissue damaging processes (up to 1000 fold), (23) and in inflammatory arthropathies like RA (24). In RA serum there is a good correlation between CRP levels and disease severity.

Rheumatoid factor is an important biomarker in RA. However, a positive test for RF is a diagnostic and prognostic indicator of RA (25, 26). The serum levels of RF do not reflect disease activity since they are found in patients with other autoimmune and non-autoimmune diseases, as well as in healthy subjects (27). In the current study, the presence of RF was greater in RA patients with high activity. Using ELISA, all SF were positive due to the high sensitivity of this technique.

The most widely used oropharyngeal culture in clinical practice includes the detection of anti-streptococcal antibodies such as ASLO and anti-deoxyribonuclease B. Of these two tests, ASLO was the first developed and is the most commonly used test (28, 29). In the present study no correlation of ASLO and the severity of the rheumatic inflammation was found. This finding agrees with the results previously obtained by Geerts et al. (19), who concluded that little or no scientific evidence was found for the use of ASLO in patients with pharyngitis, post-streptococcal glomerulonephritis and in adults with rheumatoid arthritis.

Complement activation participates in the pathogenesis of several autoimmune and inflammatory diseases including RA (30). The evidence of complement activation in the SF of RA patients is probably due to the presence of immune complexes. Levels of complement components are generally depressed in the SF of patients with RA, reflecting their consumption. On the other hand, elevated levels of cleavage products have been observed in SF. TNFα-inhibitor effectiveness in arthritis is exerted by the reduction of complement activation. Complement could be an attractive therapeutic target both in RA and in PsA (31).

The presence of active XO is significantly higher in the SF of patients with RAG compared to the other groups. This result agrees with the finding of Blake et al. (32) who showed that XO concentration is significantly raised up to 60 times in the synovium of patients with RA. This could be due to the up-regulation of the enzyme by high levels of cytokines, hypoxic nature and the symptoms of radical attack present in rheumatoid synovium (33). The roles of XOR in cytokine induced bone erosion promoting vasculitis were well documented by Miesel and Zuber (34). As we have previously found (14), more than 50% of XO is in its oxidase form. This finding indicates that XOR is liberated from the pannus (33) and correlates well with the severity of RA.

Anti-XO antibodies of IgM class were found to be higher in RA, followed by mechanical origin infections, hemarthrosis, and mono-/oligo-arthritis, then by other joint inflammation and gonarthrosis. A greater proportion of immune complexes IgM-XOR were recorded in the RA group, followed by mechanical infections and hemarthrosis; the lowest levels were recorded in patients with other joint inflammations. The highest percentage of immune complexes IgG-XOR was recorded in mechanical infections and the hemarthrosis category, and was close to the rest of the groups. These results confirm our previous study (10, 35) where the presence of these antibodies was well discussed. Al Muhtaseb et al. (35) indicated that anti-XO antibodies in SF possess a protective role in inflammatory arthritis, where they play a role in eliminating XOR from SF. However, immune complexes could activate complement and participate in propagating the inflammatory cycle. Treatment of SF with hyaluronidase gives variable effects either in free or complexed antibodies (IgG and IgM). Similar effects were observed by Brouwers et al. (36) in the case of cytokines. In contrast, these authors found that the treatment with this enzyme resulted in a lower coefficient of variation for IgG measurements.

IgM anti-XOR antibodies titers and IgM-containing XORICs in the SF of RAG patients were significantly higher than in joint pathologies. It is noteworthy that XOR activity was detectable; even it could be in part affected by immune complex formation (36).

CONCLUSION

The current study suggests that XOR may play a negative role in arthritis by generating ROS which causes self-maintenance of the disease. Furthermore, anti-XO antibodies in sera and SF may participate in eliminating the enzyme and stop its negative action. However, XORIC formation could activate complement which has a crucial role in the self-maintenance of the illness.

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Ethics Committee Approval: This study was approved by the ethics local committee (NrECA/003/22).

Informed Consent: Written consent was obtained from the participants.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- L.A.; Data Acquisition- N.H.; Data Analysis/Interpretation- N.H., L.A.; Drafting Manuscript- N.H.; Critical Revision of Manuscript- L.A.; Final Approval and Accountability- L.A., N.H.

Conflict of Interest: Authors declared no conflict of interest.

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