

# Anti-xanthine oxidase antibodies in sera and synovial fluid of patients with rheumatoid arthritis and other joint inflammations

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## ABSTRACT

**الأهداف:** دراسة مستويات مضادات مؤكسدة الكزانثين (XOR) في السائل الزلالي، وكذلك في المصل للمرضى الذين يعانون من مشاكل رثوانية، وذلك من أجل تقييم العلاقة المحتملة بين عيارات الأجسام المضادة وشدة المرض.

**الطريقة:** تم الحصول على الأمصال والسائل الزلالي خلال الفترة ما بين 2001م وحتى 2007م، من متبرعين متطوعين بمستشفى سطيف الجامعي - الجزائر، بعد أخذ موافقة المرضى. تم تحديد مستويات عامل المناعة البشري (IgG) ومستويات عامل المقاومة البشري (IgM) للأجسام المضادة لـ (XOR) باستعمال (XOR) البقري كمستضد مع طريقة (ELISA).

**النتائج:** تتفق معايير مصل عامل المقاومة البشري (IgG anti-bovine milk XOR) لدى ثلاثين من الأشخاص السليمين ( $2.74 \pm 2.31 \mu\text{g/mL}$ ) مع تلك التي وردت في الأدبيات. تبين وجود ارتفاع ملحوظ في معايرة عامل المناعة البشري (IgG) ومستويات عامل المقاومة البشري (IgM) المضادات لـ (bovine milk XOR) بشكل ملحوظ في المصل من المرضى المصابين بالتهاب المفصلي الرثواني (RA) وعينات الأشخاص الموجبين للآنتيكس. تبين وجود ارتفاع ملحوظ في معايرة الأجسام المضادة للسائل الزلالي (IgM) لدى المرضى المصابين بالتهاب المفاصل الرثواني (XOR)، مقارنة مع المرضى المصابين بالتهابات مفصلية أخرى.

**خاتمة:** تمت ملاحظة التركيزات العالية للأجسام المضادة مقابل (XOR) لدى المرضى المصابين بالتهاب المفاصل الرثواني (RA). قد تلعب هذه الأجسام المضادة دوراً رئيسياً في التهاب المفصلي الرثواني (RA) عن طريق تثبيط أكسدة كلا من (الكزانثين) وأنشطة أكسدة (NADH) لـ (XOR). وقد تلعب أيضاً دوراً رئيسياً في تحديد (XOR) من مصل والسائل الزلالي (دور إيجابي). ولكن لسوء الحظ، تصفية المركب المناعي يمكن أيضاً تكوين تنشيط المتممة والمشاركة في المحافظة الذاتية للتهاب.

**Objectives:** To study anti-bovine milk xanthine oxidoreductase (XOR) antibody levels in synovial fluid as well as in serum of patients suffering from

rheumatoid affections to assess a possible correlation between antibody titres and severity of disease.

**Methods:** Sera and synovial fluids were collected from volunteer donors at Setif University Hospital, Setif, Algeria from 2001-2007 with the consent of patients. Human IgG and IgM levels of free and bound anti-bovine milk XOR antibodies were determined using bovine XOR as antigen, with enzyme-linked immunosorbent assay (ELISA).

**Results:** Serum IgG anti-(bovine milk XOR) titres in 30 healthy normal subjects ( $2.74 \pm 2.31 \mu\text{g/mL}$ ) are in agreement with that reported in the literature. Immunoglobulin G and IgM anti-(bovine milk XOR) antibody titres were found to be significantly higher in serum from patients with rheumatoid arthritis (RA), and latex positives subjects. Synovial IgM antibody titres to bovine XOR were found to be significantly higher in rheumatoid arthritis patients compared to patients with other joint inflammations.

**Conclusion:** In rheumatoid arthritis patients, high concentrations of antibodies against XOR were noticed. These antibodies may play a major role in RA by inhibiting both xanthine and NADH oxidase activities of XOR. They may also play a key role in eliminating XOR from serum and synovial fluid (positive role) but unfortunately, immune complex formation could also activate complement and participate in self maintenance of inflammation.

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Xanthine oxidoreductase (XOR) is a complex metalloflavoprotein, which catalyses the oxidation of a wide range of substrates (purines, dinucleotides, pterins and aldehydes).<sup>1</sup> The enzyme is a homodimer of approximately 300 kDa.<sup>2</sup> Xanthine oxidoreductase exists into 2 distinct, however, interconvertible forms; xanthine dehydrogenase (XDH, EC 1.1.1.204) and xanthine oxidase (XO, EC 1.1.3.22).<sup>2,3</sup> Xanthine oxidoreductase produces reactive oxygen species (ROS), superoxide ( $O_2^-$ ), or hydrogen peroxide ( $H_2O_2$ ).<sup>4</sup> The capacity to generate such ROS has led to a great deal of interest in XOR as a pathogenic factor in many instances of ischemia-reperfusion injury in various organs<sup>5-8</sup> including rheumatic diseases.<sup>9-11</sup> The presence of human anti-XOR antibodies were initially reported by Oster et al<sup>12</sup> and Bruder et al<sup>13</sup> confirmed the existence of such antibodies, and showed them to be of IgG type, in normal healthy subjects representing 1-8% of total IgG. Levels of these antibodies were shown to be elevated in patients suffering from a myocardial infarction.<sup>14</sup> Using ELISA, Ng et al<sup>15</sup> found that the IgM anti-XOR antibodies in healthy subjects varied from 0.32-1.8% total IgM, whereas the IgG anti-XOR level represented only 0.02-0.4% of total IgG. Ng and Lewis<sup>16</sup> reported the presence, in healthy subjects, of circulating XOR-anti-XOR immune complexes (XORICs), containing antibodies of IgG and IgM classes, representing <20% of total anti-XOR antibodies. Levels of these complexes are in good correlation with anti-XOR antibody titres.<sup>17</sup> Rheumatoid arthritis (RA) is characterized by the appearance of auto-antibodies (IgM, IgA, and IgG) known as rheumatoid factors (RF) that react essentially against autologous IgG.<sup>18</sup> Immune complexes of IgG-RF-complement precipitation<sup>19</sup> induce an inflammatory reaction, which contributes to the constitution of a vascular formation known as "pannus" leading to cartilage destruction and bone erosion.<sup>20-22</sup>

In rheumatoid arthritis, lysosomal enzymes such as hydrolases, collagenases, mucopolysaccharidases and elastase,<sup>18</sup> as well as intra-articular immune complexes<sup>21</sup> and ROS<sup>22</sup> are massively released. Xanthine oxidoreductase present in the synovium<sup>11</sup> could also be liberated by synovium destruction and could play a role in post-ischemic reperfusion of rheumatoid synovium contributing to the characteristic signs of radical attack present in synovial fluid. Xanthine oxidoreductase concentration is significantly raised in patients suffering from RA, up to 50 times in serum<sup>9</sup> and up to 60 times in synovium<sup>10</sup> compared to healthy subjects or patients with other non-rheumatic diseases. The presence of high XOR concentration among RA patients is likely to be at the origin of specific anti-XOR antibody genesis. The aim of this study is to investigate the presence of free and bound anti-XOR antibodies in serum and synovial fluid from patients suffering from rheumatoid affections.

**Methods.** This study was carried out in the Laboratory of Immunology at Setif University, Setif Algeria from 2001-2007. Blood samples were collected from adults volunteers after consent of patients at the Setif University Hospital, Setif, Algeria from 40 subjects with inflammatory joints (latex positive [LP]) and 36 subjects suffering from RA according to the American College of Rheumatology (ACR) criteria,<sup>23</sup> among which 11 were LP (RA<sup>+</sup>) and 24 negative (RA<sup>-</sup>). Approval was obtained from the ethics local committee prior to the commencement of the study. Synovial fluid samples were collected from adults suffering from RA or only with joint inflammations without rheumatoid factor. Serum A1 is a pooled high titre anti-XOR and immune complexes (XORIC) human serum serving to build standard curves. Bovine milk was freshly collected from a dairy (Ain Sfiha, Setif, Algeria). Bovine xanthine oxidoreductase (BXOR) was purified according to the protocol previously described.<sup>17,24,25</sup> The obtained purified enzyme was dialysed overnight against 3 L of 50 mM sodium bicine buffer pH 8.3, in which enzyme activity tests were carried out. The enzyme concentration was estimated using the extinction coefficient of the FAD ( $\epsilon_{450nm} = 36000 M^{-1} cm^{-1}$ ).<sup>1</sup> The purity of enzyme was estimated using the following criteria, protein/flavin ratio (PFR=A280nm/A450nm), UV visible spectrum and polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate (SDS-PAGE) pattern. The latter was performed in 10% acrylamide according to Laemmli.<sup>26</sup> To determine levels of total IgG and IgM, available plates of agarose containing anti-human IgG or anti-human IgM (kits from Behring Germany) were used. The method of single radial immunodiffusion assay (SRIDA) was the same as reported previously.<sup>17</sup> Serial dilutions of purified human IgM or human IgG were used to generate standard curves. Human sera or synovial fluids were dialyzed against the commercial buffer and run against anti-human IgG or anti-human IgM on the same plate as the standard curves. Plates were placed in a humidified box and stored for 48-72 hours at room temperature until the sizes of the precipitate rings were stable. Standard curve was plotted and used to determine total IgG and IgM contents in sera and synovial fluids. Rheumatoid factor is tested by latex agglutination test using appropriate plates from Behring (Germany). Fifty  $\mu$ L from each individual serum, at different dilutions were loaded then supplemented with 50  $\mu$ L of latex coated with human IgG. Positive and negative sera were used as controls. After 2 minutes a clear agglutination is observed in positive indicating the presence of RF. Sera with titre <20 UI/mL were considered negative according to the manufacturer recommendations. Specific human serum anti-XOR antibodies were determined as previously described<sup>14,17</sup> with a slight difference in enzyme substrate where

orthophenylene diamine (OPD)<sup>27</sup> was used instead of 3,3',5,5' tetramethylbenzidine (TMB). In addition to samples of sera or synovial fluids, each plate included serial dilutions (200-6400 fold in PBS-Tween) of a standard high-titre pooled serum, A1, in duplicate wells (100 µL per well). To determine XOR immune complexes microtitre plates were coated (100 µL/well) with diluted (1 in 40) rabbit anti-human XOR serum in sodium hydrogen carbonate, pH 9.6, then incubated overnight at 4°C. All following steps were similar to those reported previously.<sup>17</sup> A standard curve of absorbance against log concentration of A1 was plotted for each plate, and the linear part of the curve was used to calculate titres as a percentage of the standard. Omission of antigen or of the serum always led to minimal levels of background absorbance.

Data was expressed as mean ± SD. Data from groups of patients were compared to normals using students t test. Statistical analyses were carried out by using the SigmaStat Software. Probability values of 0.05 were considered significant.

**Results.** Agglutination and ELISA used in determining IgM-RF in all samples showed that ELISA was more sensitive especially on the RA<sup>-</sup> samples, which were shown to be negative by the former technique and positive with the latter. For the other samples,

LP and RA<sup>+</sup> a good correlation was observed between the 2 methods. The IgG and IgM titres, determined by SRIDA, of 40 LP sera and 36 sera from RA (RA<sup>+</sup> and RA<sup>-</sup>) patients were normal or slightly elevated as represented in Table 1. Using standard high-titre A1 pooled serum (total IgM: 3.00±0.2 mg/mL<sup>-1</sup>, total IgG: 15.0±1.5 mg/mL<sup>-1</sup>, specific IgM anti-BXOR 50.4±3.3 µg/mL<sup>-1</sup>, specific IgG anti-BXOR 7.5±1.4 µg/mL<sup>-1</sup>),<sup>17</sup> specific IgG and IgM anti-BXOR were determined in 30 healthy human sera. The IgG titres, determined by ELISA (Table 2), were comprised between 0.31 and 9.59 µg/mL<sup>-1</sup> with an average of 2.74±2.31 µg/mL<sup>-1</sup>. Figure 1 shows the distribution of IgG anti-XOR among healthy sera where titres varied from 0.31-4.04 µg/mL<sup>-1</sup> in 76.7% (23 samples) of cases. The IgM anti-BXOR titres in the same samples varied from 5.98 -91.27 µg/mL<sup>-1</sup> with an average of 30.14±39.68 µg/mL<sup>-1</sup>. The distribution is presented by Figure 2. Specific IgG anti-BXO titres in sera of LP subjects were found to be significantly (*p*=0.001) higher than normals (Table 2). Distribution of titres among patients is represented in Figure 1 with an average of 9.28±5.55 µg/mL<sup>-1</sup>. Equally, IgM anti-BXO were significantly higher (*p*<0.001) compared to normals, with an average of 161.71±187.03 µg/mL<sup>-1</sup> (Figure 2). Sera from 36 RA patients show a significant increase of IgG anti-(bovine XOR) antibody titres with a

**Table 1** - The IgG and IgM titres in latex positive and rheumatoid arthritis patient sera determined by ELISA.

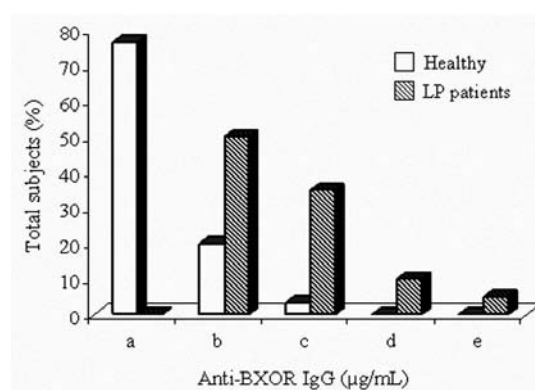
Patient sera Ig titres	Latex positive sera (n= 40)	Rheumatoid arthritis sera (n=36)		Normal subjects
		RA <sup>+</sup> (n=12)	RA <sup>-</sup> (n=24)	
IgG (mg/mL)	22.7 ± 6.00	24.52 ± 9.53	19.37 ± 5.6	15.59 ± 15.2
IgM (mg/mL)	2.49 ± 1.039	2.23 ± 1.25	3.42 ± 1.25	1.81 ± 0.52

Ig - immunoglobulins, RA - rheumatoid arthritis

**Table 2** - Levels of IgG and IgM class anti-bovine xanthine oxidoreductase (BXOR) antibodies (µg.mL<sup>-1</sup>) in healthy human, latex positive (LP), and rheumatoid arthritis (RA) sera.

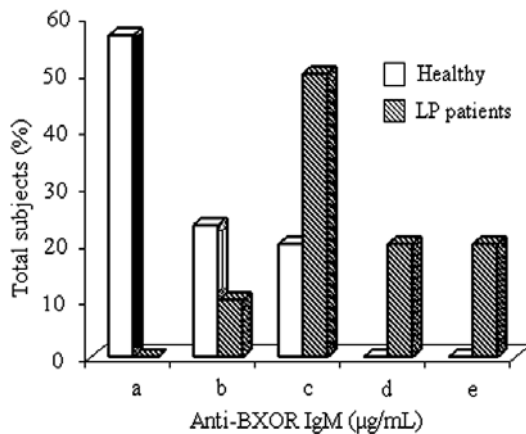
Patients	IgG	IgM	P-value
Healthy	2.74 ± 2.31	30.14 ± 39.68	
LP	9.28 ± 5.55	161.71 ± 187.03	0.001
RA <sup>+</sup>	5.34 ± 2.88	133.43 ± 101.98	0.0009
RA <sup>-</sup>	5.55 ± 2.06	60.76 ± 38.39	0.008

Serum was collected from healthy donors and patients then stored at -20°C until assay for antibodies by ELISA. Values are means ± SD.



**Figure 1** - Distribution of anti-bovine xanthine oxidoreductase (BXOR) IgG levels in serum samples of latex positive (LP) subjects compared to healthy human. a: <4.04, b: 4.05-8.07, c: 8.08-13.45, d: 13.46-18.83, e: >18.8 µg/mL.

mean of  $5.34 \pm 2.88 \mu\text{g/mL}^{-1}$  among RA<sup>+</sup> and  $5.55 \pm 2.06 \mu\text{g/mL}^{-1}$  among RA<sup>-</sup> subjects, compared to normals (Table 2). Equally, the IgM anti-(bovine XOR) titres were significantly higher ( $p < 0.001$ ). The means were  $133.43 \pm 101.98 \mu\text{g/mL}^{-1}$  for RA<sup>+</sup> and  $60.76 \pm 38.39 \mu\text{g/mL}^{-1}$  for RA<sup>-</sup>. These titres represent  $8.70 \pm 6.55\%$  and  $4.00 \pm 1.16\%$  of total IgM in RA<sup>+</sup> and RA<sup>-</sup> patients. The increase in IgM anti-XOR was reported in patients who suffered from myocardial infarction.<sup>14</sup> Total IgG and IgM in synovial fluids from 38 patients with different joint inflammations are shown in Table 3.



**Figure 2** - Distribution of anti-bovine xanthine oxidoreductase (BXOR) IgM levels in serum samples of latex positive (LP) patients compared to healthy human. a:  $< 33.57$ , b:  $33.57-43.16$ , c:  $43.17-143.86$ , d:  $143.87-239.77$ , e:  $> 239.77 \mu\text{g/mL}$ .

**Table 3** - Total IgG and IgM in synovial fluids of patients with rheumatoid arthritis and other joint inflammations, assayed by SRIDA.

Patients	Total IgG (mg/mL)	Total IgM (mg/mL)
RA (n=12)	$11.33 \pm 4.41$	$0.97 \pm 0.74$
Hydarthrosis (n=12)	$6.83 \pm 2.71$	$0.125 \pm 0.20$
Hemarthrosis and other inflammations (n=14)	$12.71 \pm 7.16$	$0.98 \pm 0.70$

RA - rheumatoid arthritis, SRIDA - single radial immunodiffusion assay

Tested by SRIDA, synovial fluids (SF) of patients suffering from RA contain total IgG ( $7.0-18.0 \text{ mg/mL}^{-1}$ ) and IgM ( $0.30-2.45 \text{ mg/mL}^{-1}$ ) levels similar to the normal concentrations of these immunoglobulines in serum. The SF from patients with hemarthrosis and other inflammations have nearly the same titres of IgG ( $5.98-28.02 \text{ mg/mL}^{-1}$ ) and IgM ( $0.0-1.92 \text{ mg/mL}^{-1}$ ) as RA cases. The SF from patients with hydarthrosis contains low titres of IgG ( $2.97-11.03 \text{ mg/mL}^{-1}$ ) and very low concentrations of IgM ( $0-0.45 \text{ mg/mL}^{-1}$ ). Only RA synovial fluids were IgM-RF positive on latex agglutination test, but when assayed by ELISA all SF were positive. Antibodies to XOR, either in free form or as immune complexes, were determined in synovial fluids, using ELISA, in all 38 samples and mean of each group are represented in Table 4. It is worth mentioning here that in 4 cases, beside the lack of IgM complexes, one of them is also IgG complex free.

**Discussion.** Using a very sensitive ELISA method, the obtained IgG anti-(Bovine XOR) titres in 30 healthy normal subjects ( $0.31$  and  $9.59 \mu\text{g/mL}^{-1}$  with an average of  $2.74 \pm 2.31 \mu\text{g/mL}^{-1}$ ) were in correlation with what reported by Harrison et al<sup>14</sup> who showed that healthy subjects contained  $39.9 \pm 29.6\%$  of standard A1 high titre pooled serum. Furthermore, Ng et al<sup>15</sup> showed a higher titre with an average of  $14.7 \mu\text{g/mL}^{-1}$  among the 220 subject sera studied. Bruder et al<sup>13</sup> reported that IgG anti-XOR titre in healthy subjects represent 1-8% total IgG, most of these antibodies were found to be against the human enzyme. The present study shows that IgG anti-(bovine XOR) titres were distributed between  $0.13$  and  $9.59 \mu\text{g/mL}^{-1}$ , around 77% of them were less than  $4.04 \mu\text{g/mL}^{-1}$  (Figure 1). The IgM anti-(bovine XOR) titres, on the same 30 samples, were found to be significantly higher ( $5.98-91.27 \mu\text{g/mL}^{-1}$  with an average of  $30.14 \pm 39.68 \mu\text{g/mL}^{-1}$ ) compared to average titres of  $20.11 \pm 14.92 \mu\text{g/mL}^{-1}$  and  $11.8 \mu\text{g/mL}^{-1}$  reported by Harrison et al<sup>14</sup> and Ng et al.<sup>15</sup> The increase in anti-(bovine XOR) antibody titres in RA patients could well be a result of elevated serum XOR in these patients. The interference of RF can not be ruled out, in

**Table 4** - IgG and IgM anti-(bovine XOR) free antibodies (XORAb) and immune complexes (XORIC) determined by ELISA ( $\mu\text{g mL}^{-1}$ ).

Patients	XORAb		XORIC	
	IgG	IgM	IgG	IgM
RA (n=12)	$3.18 \pm 0.41$	$37.05 \pm 20.89$	$8.11 \pm 4.08$	$44.19 \pm 36.67$
Hydarthrosis (n=12)	$2.43 \pm 0.62$	$20.73 \pm 5.85$	$8.48 \pm 1.49$	$37.34 \pm 15.29$
Hemarthrosis and other inflammations (n=14)	$3.31 \pm 1.05$	$38.14 \pm 14.19$	$9.70 \pm 1.51$	$66.01 \pm 21.33$

XOR - xanthine oxidoreductase, RA - rheumatoid arthritis

fact Abadeh et al<sup>28</sup> showed that the removal of serum RF led to a disappearance between RA and normals sera.

We are trying to find out if there is any correlation between these antibodies and RF titre in RA patients. High levels of anti-XOR antibodies in human sera especially in sera of patients with RA raise the question on their origin. They could be a result of an exogenous stimulation by cow's milk XOR<sup>12,13</sup> or raised against endogenous enzyme released following blood vessel injury.<sup>14,17</sup> The question arises on the origin of these antibodies in the SF. They could be, as in the normal serum, auto-antibodies against endogenous enzyme found with high levels in RA pathology,<sup>8,9</sup> however, it is conceivable that they could result from ingestion of cow's milk or its products.<sup>8,12,16</sup> The presence of high titres of anti-XOR antibodies in the synovial fluid of patients suffering from RA and other joint inflammations is of great interest. Like in the serum, these antibodies are present in both forms (free and immune complexes). They could be involved in the inhibition of xanthine oxidase together with nitrotyrosine formed in swelling joints<sup>29</sup> (positive role), at the same time they could also activate complement and participate in self-maintenance of inflammation (negative role).

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