The role of human xanthine oxidoreductase (HXOR), anti-HXOR antibodies, and microorganisms in synovial fluid of patients with joint inflammation

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The role of human xanthine oxidoreductase (HXOR), anti-HXOR antibodies, and microorganisms in synovial fluid of patients with joint inflammation

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Abstract This work is to investigate the levels of human xanthine oxidoreductase (HXOR), its antibodies, and microorganisms in synovial fluid of patients with untreated rheumatoid joint diseases. Synovial fluids were collected from sixty-four patients with rheumatoid joint diseases. Sixty-four age-matched individuals were included as control. Xanthine oxidoreductase (XOR) proteins level and anti-XOR antibodies were determined in the blood and synovial fluid, using human XOR as antigen, by enzyme-linked immunosorbent (ELISA) assay. Synovial fluids were cultured for bacteria and fungi. The titers of XOR protein in the synovial fluid of patients with rheumatoid arthritis were 90.43 ± 23.37 µg/ml (mean ± SD, n = 29) and up to 62.42 ± 8.74 µg/ml (mean ± SD, n = 35) in other joint inflammation. Anti-HXOR antibodies titers in patients were 167.72 ± 23.64 µg/ml, n = 64, which was significantly higher in rheumatoid arthritis patients. The results indicated that anti-HXOR antibodies in synovial fluids have a protective role as high concentrations against XOR were detected in inflammatory arthritis. These antibodies play a role in eliminating XOR from synovial fluids. However, immune complex formation could activate complement and participate in propagating the inflammatory cycle. Synovial aspirate ordinary microbial cultures were negative for any bacteria or fungi, but that does not exclude organisms of special culture requirements.

Keywords Synovial fluid · Human xanthine oxidoreductase (HXOR) · ELISA · Rheumatoid joint diseases · Free radical

Introduction

In recent years, free radicals reactions and other reactive intermediates produced in normal metabolic processes have been implicated in the pathogenic mechanism of a wide range of diseases, including inflammatory diseases [1] as acute coronary syndrome [2] and others cardiovascular diseases [3]. Similarly, pathogenic organisms such as parvovirus B19, rubella, hepatitis B and C, herpes [4–10], Epstein–Barr virus [11], and retroviruses could also serve as infectious causes of rheumatoid arthritis–like disease. Synovial human T lymphotropic virus type 1 (HTLV-1) infection is associated with chronic arthritis [4].
Rheumatic joint disease is a chronic syndrome of ambiguous etiology and is characterized by non-specific inflammation of the peripheral joints with joint swelling, morning stiffness, destruction of articular tissues, and joint deformities. One particular type of tissue injury from free radicals is reoxygenation injury following reperfusion of ischemic tissue [12–17]. Rheumatoid synovitis is usually accompanied by increased synovial effusions. Damage to bone and cartilage of synovial tissue is mediated by metabolic free radical sources such as xanthine oxidoreductase (XOR) and others. Xanthine oxidoreductase (XOR) is a complex metalloflavoprotein, which plays a key role in catalyzing the oxidation of a wide range of substrates (purine, pterins, dinucleotides, and aldehydes) [18]. The enzyme is a homodimer of approximately 300KDa [19]. XOR exists into 2 different but interconvertible forms, xanthine dehydrogenase (XDH, EC 1.1.1.204) and xanthine oxidase (XO, EC 1.1.3.22) [19, 20]; the former is the dominant form. XOR produces reactive oxygen species (ROS), superoxide (O\(_2^−\)), or hydrogen peroxide (H\(_2\)O\(_2\)), respectively [21]. 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 [19, 22–25] including inflammatory joint and rheumatic diseases [15–17]. Xanthine oxidase is the first-registered biological source of oxygen free radicals and plays the leading role in the pathogenesis of tissue damage by the production of superoxide anion, hydrogen peroxide, and hydroxyl radical [26]. There is substantial evidence that XOR participates in the pathophysiology of many diseases [27].

Rheumatoid arthritis (RA) is the most common chronic inflammatory arthritis that affects about 1% of adults [28]. There are no specific laboratory tests specific for RA; diagnosis depends on a constellation of signs and symptoms that can be supported by serology and radiographs [29–31]. Some RA patients show evidence of autoimmunity long before the appearance of clinical arthritis [32].

The presence of human anti-XOR antibodies was initially reported by Oster et al. [33]. Bruder et al. [34] confirmed the existence of such antibodies and showed them to be IgG. The amount of anti-XOR antibody in normal healthy humans represents 1–8% of total IgG. Levels of these antibodies were shown to be elevated in patients suffering from myocardial infarction [35]. Ng et al. [36] found by ELISA technique that the IgM anti-XOR antibodies in healthy humans varied from 0.32 to 1.8% of total IgM, whereas IgG anti-XOR level represented only 0.02–0.4% of total IgG. Ng and Lewis [37] reported the presence of circulating XOR-anti-XOR immune complexes (XORICs) in healthy humans. The complex containing antibodies of IgG and IgM classes, representing <20% of total anti-XOR antibodies. The levels of these complexes correlate with the anti-XOR antibody titers [38].

Rheumatoid arthritis (RA) is characterized by the appearance of autoantibodies of types IgM, IgG, and IgA known as rheumatoid factors (RF) that react essentially against autologous IgG [39]. Immune complexes activate complement. Complement activation induces inflammatory reaction [40], which contributes to the constitution of a vascular formation leading to cartilage destruction and bone erosion [41–43]. XOR present in the synovium could be liberated by synovium destruction and could play a role in post-ischemic reperfusion of rheumatoid synovium [17] contributing to the characteristics signs and symptoms of radical attack present in synovial fluid. XOR concentration is significantly raised in patients suffering from RA, up to 60 times in synovium [16] compared with healthy humans or patients with other non-rheumatic diseases.

The detection of xanthine oxidoreductase in human synovium is therefore of importance as a first step in the investigation of possible mechanisms of radical generation peculiar to a reperfusion phase of synovial injury. The aim of this work is to investigate the presence of human xanthine oxidoreductase, its antibodies in synovial fluid, and the possible microbial etiology of the inflammation in untreated patients suffering from rheumatoid arthritis (rheumatoid factor +ve) and other joint inflammatory diseases (rheumatoid factor –ve).

Materials and methods

Subjects

Sixty-four patient with rheumatoid joint disease and 64 age- and sex-matched controls were included in the study. All patients fulfilled the revised American College of Rheumatology (ACR) criteria for rheumatoid arthritis [23]. Consent for all procedures was obtained from each individual. The study was approved by the hospital ethics committee and was in accordance with the ethical standards. Preliminary evaluation consisting of a brief medical history, smoking, alcohol habits, and physical examinations was performed. Patients with any history of liver diseases, diabetes mellitus, respiratory disorders, and cardiovascular diseases were excluded. None had been treated with corticosteroids or disease-modifying antirheumatic drugs prior the study.

A pooled high-titer anti-XOR and immune complexes (XORIC) human sera from volunteer donors were served to build standard curves.

Rheumatoid factor detection

Rheumatoid factor is detected by latex agglutination test using appropriate plates from Behring (Germany) according to the manufacturer’s recommendations. Fifty microliters of latex
coated with human IgG was added to different dilutions from each serum sample. Negative and positive sera were used as controls. After 2 min, a clear agglutination is observed in the positive, indicating the presence of RF. Sera with titer less than 20 UI/ml were considered negative according to the manufacturer’s recommendation.

Milk and reagents

Human breast milk was kindly donated by mothers who had that in excess at the special care unit of the maternity hospitals; human milk was freshly collected and stored at −20°C until use.

Unless otherwise stated, all other reagents were purchased from sigma (Poole, UK).

CRP latex agglutination was purchased from Genzyme diagnostics (Kent, UK).

Purification of human xanthine oxidoreductase (XOR)

Human XOR was purified according to the previously described protocol for human milk [22]. The purified enzyme was dialyzed overnight against 3 l of 50 mM sodium/Bicine buffer pH 8.3 and processed as reported by Godber et al. [44]. Enzyme activity tests were then carried out. Purified XOR enzyme was found to have more than 75% in the oxidase form.

Total protein estimation

Protein estimation for XOR enzyme was carried out according to the method of Bradford [45]. The enzyme purity was assessed on protein/flavin ratio (PFR = A280 nm/A450 nm). An enzyme sample with a PFR value 5–5.2 is widely accepted to be pure.

Xanthine oxidase activity assay

Total xanthine oxidase activity of the synovial fluid was determined by measuring the rate of oxidation of xanthine to uric acid spectrophotometrically at 295 nm in a Cary 100 spectrophotometer, using a molar absorption coefficient (ε) of 9.6 mM⁻¹ [46]. Assays were performed at 25.0 ± 0.2°C in air-saturated 50 mM Na/Bicine buffer, pH 8.3, containing 100 μM xanthine. Total (oxidase plus dehydrogenase) activity was determined as above but in the presence of 500 μM NAD⁺. Dehydrogenase content of an enzyme sample was determined from the ratio of oxidase and total activities.

C-reactive protein (CRP) concentration determination

CRP concentration was determined as recommended by the manufacturer; 2.4 μl of patient serum is added to 120 μl buffer solutions (pH 8.5) and mixed with 120 μl suspension of mouse anti-human CRP monoclonal antibody that is bound to latex (2 mg/ml) and incubated for 5 min. CRP binds to the latex-bound antibody and agglutinates. The resulting agglutination was measured spectrophotometrically at 580 nm, negative and positive control samples were included. Values higher than 9.4 mg/l for females and 8.55 mg/l for males were consider as positive.

Single radial immunodiffusion assay (SRID) to determine levels of total IgG and IgM

Commercially available plates of agarose containing anti-human IgG or anti-human IgM (Behring, Germany) were used as described by Benboubetra et al. [38]. Serial dilutions of purified human IgM or IgG were used to generate standard curves. Human synovial fluids or sera were dialyzed against the commercial buffer and run against anti-human IgG or anti-human IgM on the same plates as the standard curves. Plates were placed in a humidified box and stored for 48–72 h at room temperature until the sizes of the precipitate rings were stable. Standard curve was plotted and used to determine total IgG and IgM anti-human XOR contents in synovial fluids.

Enzyme-linked immunosorbent assay (ELISA)

(a) For anti-HXOR antibodies

Specific human anti-XOR antibodies were determined as previously described by Harrison et al. [35] and Benboubetra et al. [38] with slight difference in enzyme substrate. Orthophenylene diamine (OPD) [47] was used instead of 3,3,5,5-tetramethylbenzidine (TMB). In addition to synovial fluids, each plate (Coster, Spain) included serial dilutions (200–6,400 fold in PBS-Tween) of a standard high-titer pooled serum, in duplicate wells (100 μl/well). The absorbance was measured at 492 nm, in each well using a 96-well plate reader (Diagnostics Pasteur LP200).

(b) For XOR immune complexes (XORIC)

To determine (XORIC) polystyrene, 96-well microtiter plates (Coster, Spain) 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, and processed as described by Stevens et al. [17]. XORIC concentrations were calculated from plotted standard curves of absorbance against log concentration of the standard serum for each plate, and the linear part of the curve was used to calculate the titers as a percentage of
the standard. Data for ELISA were expressed as mean ± SD.

Data from patient groups were compared with each other using Student’s t test. SigmaStat Software were used for statistical analyses. Probability values of 0.05 were considered significant.

Microbiologic testing

Synovial fluids were cultured on Blood agar (BA), MacConkey’s agar (MA), Chocolate Agar (CA), and Sabouraud’s dextrose agar (SA) (Oxoid). BA and MA plates were incubated aerobically at 37°C, CA plates were incubated under microaerophilic condition (3% CO2), and SA plates were incubated at room temperature (25°C) for 72 h. Synovial fluids were centrifuged at 500 rpm for 5 min, and smears from the deposit were Gram and Acid fast stained. The slides were examined by light microscopy with oil immersion lens.

Results

Latex agglutination used in determining IgM-RF factor showed that of the 64 patients, 29 were RA+ (seropositive) and 35 were RA- (seronegative). The Clinical and biochemical characters of the participated subjects are shown in Table 1.

The mean age and BMI for both rheumatoid arthritis and other joint inflammation and control subjects were comparable. ESR (after 1 h) and CRP values were significantly elevated in patients with rheumatoid arthritis (RA+) compared with the values in patient with other joint inflammation (RA−) and to those of the control values.

The total IgG and IgM titers in the synovial fluid of patient with rheumatoid arthritis (RA+) and patients with other joint inflammation (RA−) as determined by single radial immunodiffusion assay (SRIDA) are shown in Table 2.

Total IgG and IgM (mg/ml) levels were significantly higher in patient with rheumatoid arthritis in comparison with the levels in patient with other joint inflammation, respectively.

Total IgG and IgM titers in blood circulation of patient with rheumatoid arthritis (RA+), other joint inflammation (RA−), and in healthy volunteers using SRIDA are shown in Table 3.

Both IgG and IgM were significantly higher in rheumatoid arthritis patients than in the healthy controls. Similarly, the levels were higher in serum of patient with other joint inflammation than controls. IgG levels were more elevated in serum of patient with rheumatoid arthritis compared with other joint inflammation (P < 0.001). On the other hand, total IgM was more elevated in serum of patients with other joint inflammation compared with rheumatoid arthritis.

Human XOR protein, its activity, and anti-human XOR (IgG and IgM) in patient’s synovial fluid were measured; the results are shown in Table 4.

Human xanthine oxidase protein (HXO) concentrations were significantly elevated in patient with rheumatoid arthritis compared with the concentrations in patient with other joint inflammation (P < 0.01).

Human xanthine oxidase (HXO) activity was also significantly elevated in patient with rheumatoid arthritis (100.00 ± 47.24 nmol min−1 mg−1) compared with the activity in patients with other joint inflammation (81.42 ± 33.22 nmol min−1 mg−1) (P < 0.01).

Both IgG and IgM anti-HXOR levels are remarkably high, representing, in the case of anti-HXOR IgM antibodies, 6.6% of the total IgM immunoglobulin in patient with rheumatoid arthritis and 4% in patient with other joint inflammation. However, anti-HXOR IgG was higher in patients with other joint inflammation compared with patients with rheumatoid arthritis.

Levels of free and immune complexes IgG and IgM anti-HXOR antibodies determined by ELISA are shown in Table 5.

Both the free and immune complexes of anti-HXOR (IgG and IgM) were lower in patients with rheumatoid arthritis compared with the levels in patients with other joint inflammation.

Direct examination as well as aerobic and microaerophilic cultures for bacteria and fungi did not reveal the presence of microorganisms.

Discussion

The elevated levels of ESR (erythrocyte sedimentation rate) and CRP (C-reactive protein) reported in this study

<table>
<thead>
<tr>
<th>Type of disease</th>
<th>No. of subjects</th>
<th>Age (years)</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>BMI</th>
<th>ESR (mm)</th>
<th>CRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA+</td>
<td>29</td>
<td>39.1 ± 8.9</td>
<td>159.8 ± 5.8</td>
<td>69.6 ± 2.3</td>
<td>27.3 ± 9.2</td>
<td>66.6 ± 45.2</td>
<td>+</td>
</tr>
<tr>
<td>RA−</td>
<td>35</td>
<td>41.04 ± 9.1</td>
<td>161.6 ± 5.6</td>
<td>69.1 ± 6.0</td>
<td>26.9 ± 9.7</td>
<td>40.3 ± 30.4</td>
<td>+</td>
</tr>
<tr>
<td>Controls</td>
<td>64</td>
<td>42.04 ± 7.7</td>
<td>162.3 ± 4.7</td>
<td>68.7 ± 5.8</td>
<td>25.8 ± 2.3</td>
<td>12.8 ± 4.01</td>
<td>−</td>
</tr>
</tbody>
</table>
indicate the presence of inflammation in both patient groups. Patients positive for rheumatoid factor were labeled as rheumatoid arthritis (RA+), while seronegative patients were labeled as other joint inflammation (RA-).

The level of human xanthine oxidase protein and its activity is significantly high in the synovial fluid of patient with rheumatoid arthritis (90.43 µg/ml, 100.00 nmol min⁻¹ mg⁻¹) (Table 4). The normal level in healthy individual as reported by others [48, 49] is 0.16–0.38 mU/g. This may be due to upregulation of the enzyme by characteristically high levels of cytokines and hypoxic nature of rheumatoid synovium. Xanthine oxidase is the first-registered biological source of oxygen free radicals and plays the leading role in the pathogenesis of tissue damage by the production of superoxide anion, hydrogen peroxide, and hydroxyl radical. The roles of XOR in cytokine induced bone erosion promoting vasculitis have been well documented by others [15, 16].

The slightly higher levels of total IgG and IgM titers in patients sera determined by SRIDA in RA+ patients compared with the levels in other joint inflammation and to the levels in the serum of control healthy are in agreement with [35, 37, 50]. This is expected because of the autoimmune nature of the disease. On the other hand, the

### Table 2: Total IgG and IgM titers in synovial fluid of patients with rheumatoid arthritis (RA+) and other joint inflammation (RA-), using SRIDA

<table>
<thead>
<tr>
<th>Patients</th>
<th>Number of patients</th>
<th>Total IgG (mg/ml)</th>
<th>Total IgM (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA+</td>
<td>29</td>
<td>13.71 ± 4.41⁺</td>
<td>0.97 ± 0.34⁺</td>
</tr>
<tr>
<td>Other joint inflammation</td>
<td>35</td>
<td>9.77 ± 2.71</td>
<td>0.55 ± 0.20</td>
</tr>
</tbody>
</table>

RA rheumatoid arthritis latex agglutination positive, SRIDA single radial immunodiffusion assay

⁺ P < 0.001 significantly higher with RA+ group

### Table 3: Total IgG and IgM titers in sera of patients with rheumatoid arthritis (RA+), other joint inflammation (RA-), and healthy volunteers, using SRIDA

<table>
<thead>
<tr>
<th>Patients</th>
<th>Number of patients</th>
<th>Total IgG (mg/ml)</th>
<th>Total IgM (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA+</td>
<td>29</td>
<td>24.52 ± 9.53⁺</td>
<td>2.23 ± 1.25⁺</td>
</tr>
<tr>
<td>Other joint inflammation</td>
<td>35</td>
<td>19.37 ± 5.6⁺</td>
<td>3.42 ± 0.60⁺</td>
</tr>
<tr>
<td>Pools from healthy individuals</td>
<td>60</td>
<td>13.5 ± 4.41</td>
<td>1.5 ± 0.52</td>
</tr>
</tbody>
</table>

RA rheumatoid arthritis latex agglutination positive, SRIDA single radial immunodiffusion assay

⁺ P < 0.0001 significantly differ from the control subjects

⁺⁺ P < 0.01 significantly differ from the other joint inflammation, Results are means ± S.D in milligrams per deciliter

### Table 4: Human xanthine oxidase protein, (HXOR) activity and anti-HXOR (IgG and IgM) in patient synovial fluids

<table>
<thead>
<tr>
<th>Type of disease</th>
<th>No. of patients</th>
<th>HXOR protein (µg/ml)</th>
<th>HXOR activity mmol min⁻¹ mg⁻¹</th>
<th>Anti-HXOR (IgG) µg/ml</th>
<th>Anti-HXOR (IgM) µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA+</td>
<td>29</td>
<td>90.43 ± 23.37</td>
<td>100.00 ± 47.24</td>
<td>36.37 ± 22.35</td>
<td>131.35 ± 30.39</td>
</tr>
<tr>
<td>Other joint inflammation</td>
<td>35</td>
<td>62.42 ± 8.74</td>
<td>81.42 ± 33.22</td>
<td>46.43 ± 23.62</td>
<td>105.6 ± 31.36</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

### Table 5: IgG and IgM anti-HXOR antibodies, free (HXORAb) and immune complexes (HXORIC) determined by ELISA (µg ml⁻¹) in patient synovial fluid

<table>
<thead>
<tr>
<th>HXORAb</th>
<th>HXORIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
<td>RA+</td>
<td>29</td>
</tr>
<tr>
<td>Others joint inflammation</td>
<td>35</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
results showed that synovial fluids of patients suffering from rheumatoid arthritis and other joint inflammation contained lower levels of total IgG and IgM compared with the levels of these immunoglobulins in patients serum. This disagrees with the finding of others [35, 37, 50]. This may indicate that some of those immunoglobulins were bound to the tissue or are bound in immune complexes, which need to be detected by other techniques. In fact, the pathology of rheumatoid arthritis is attributed to an Arthus-like reaction in which immune complexes mediate the crucial role.

The high-titer anti-HXOR in RA patients may be due to elevated serum and synovial fluid XOR level. In most cases, more than 50% of synovial XOR is present as oxidase form [51]. The detection of relatively high concentration of XOR enzyme in its oxidative form in rheumatoid arthritis patient synovial fluids compared with the concentration of the enzyme in other joint inflammation indicates that the enzyme XOR is liberated with lysosomal enzymes from the synovium and could be involved in cartilage destruction and bone erosion suggesting a positive correlation between the level of the enzyme and the severity of the disease.

The anti-HXOR (IgM) titers, in the synovial fluid samples, were found to be significantly higher in other joint inflammation than in RA+ patients. This finding that is also reported by others [50] could be explained by the fact that IgM anti-XOR is more efficient in immune complex formation and consequently damage in RA+ patients due to activation of the complement system.

The levels of IgM-containing XORICs were considerably higher than those of IgG, reflecting the relative levels of the free specific antibodies. In the case of both IgM and IgG, a high proportion of specific antibodies were in the complex form (90.34 and 77.06, respectively). It is worth noting that, despite the relatively high levels of circulating (complexes) XOR, corresponding enzymic activity is seldom detectable, which most probably reflects the low specific activity of the human enzyme. This was also reported by Abadeh et al. [52] and Yamamoto et al. [53].

Synovial aspirate were never positive for any bacteria or fungi. This was also reported by other [54]. A blood culture may be more revealing if an association between RA disorder and infection with a microorganism exists. Some have reported improvement after treatment of RA patient with antibiotics [55] suggesting a microbial role. It may be useful to look for unconventional organisms such as Chlamydia and Mycoplasma as suggested by other [4, 56].

Rheumatoid arthritis may have complex pathology. In addition to the obvious effect of the rheumatoid factor, Proteus mirabilis was also incriminated. Newkirk et al. [57] have found that IgM and IgA anti-<em>Proteus mirabilis</em> antibodies were significantly higher in patients with rheumatoid arthritis RA+ compared with RA-arthritis, spondyloarthopathy, and undifferentiated arthritis. Similarly, Tani et al. [58] have found that patients with rheumatoid arthritis showed elevated levels of antibodies to <em>Proteus mirabilis</em> compared with ankylosing spondylitis and controls. They suggested an amino acid homology between an outer membrane hemolysin protein of <em>Proteus mirabilis</em> and susceptibility sequence in HLA-DR4 and DR5 subtypes. Also, Senior et al. [59] found that 33% of patients with rheumatoid arthritis had asymptomatic insignificant bacteriuria compared with 4% of age- and gender-matched controls. Similarly, patients had significantly higher levels of IgA, IgM, and IgG antibodies to <em>Proteus mirabilis</em> in blood and urine than controls. It is suggested that this may be the trigger for the rheumatoid arthritis condition.

**Conclusions**

These findings suggest that XOR may play an important role as a source of ROS in RA and in other joint inflammation, which participate in self-maintenance of the diseases. Antibodies against XOR 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. However, immune complex formation could activate the complement system and participate in self-maintenance of the diseases.

**References**


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