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ORIGINAL ARTICLE

Detection of 16α-Hydroxyestrone-histone 1 Adduct as High-Affinity Antigen for Rheumatoid Arthritis Autoantibodies

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Abstract

Increased concentrations of 16α-hydroxyestrone (16α-OHE_1) have been observed in rheumatoid arthritis (RA), but the underlying mechanism of this remains elusive. Here we aimed to identify the role played by 16α-OHE_1 in RA. In 40 RA patients, the specificities of antibodies from the sera of these patients were checked by direct binding, inhibition ELISA, and quantitative precipitation titration. Competition ELISA was also used for the estimation of 16α-OHE_1 in the serum of different RA patients. RA IgG from a patient's sera showed strong recognition to 16α-OHE_1-H_1 (histone 1) adduct in comparison to control subjects ($p < 0.001$), as the formation of this adduct brings out various biochemical changes that might generate neo-epitopes, which have been well-recognized by these antibodies. The affinity of RA antibodies for 16α-OHE_1-H_1 (1.10×10^{-7} M) was high, as detected by the Langmuir plot. Comparing RA patients to the controls, no significant differences were detected in the level of 16α-OHE_1 or 2-hydroxyestrone/16α-OHE_1 ratio. 16α-OHE_1-H_1 might have an antigenic role and function as a high-affinity antigen for RA autoantibodies and, therefore, could be used as a biomarker for this disease.

Keywords 16α-Hydroxyestrone · Histone · Rheumatoid arthritis · Antibodies · ELISA

Introduction

Estrogen has anti-inflammatory or pro-inflammatory roles depending on various factors, including intracellular metabolism of estrogen, leading to the formation of biologically active metabolites that have anti- or pro-inflammatory functions (Straub 2007). Experimental evidence shows that estrogen could function as an enhancer of the humoral immunological responses and play an important role in the pathophysiology of rheumatic disease (Cutolo et al. 2012). Estrogens have their biological effect through their peripheral metabolites rather than their serum level. In rheumatoid arthritis (RA) synovial tissues, these estrogen metabolites cause stimulation of cell proliferation and cytokine production (Cutolo et al. 2012). These RA synovial cells mainly produce the cell proliferative 16α-hydroxyestrone (16α-OHE_1), which is the downstream estrogen metabolite that does not inhibit tumor necrosis factor (Schmidt et al. 2009). Therefore, a high concentration of 16α-hydroxylated estrogens is an unfavorable sign in synovial inflammation and synovial tissue hyperplasia (Cutolo et al. 2012; Schmidt et al. 2009).

Compared to males, being female is associated with having a worse disease activity score in RA (Kuiper et al. 2001; Tengstrand et al. 2004). Women with RA under premenopausal condition showed an increased level of estrogen with anticardiolipin autoantibodies (Seriolo et al. 1999). In the early 1950s, studies reported a lower level of estrogen in the urine of RA patients compared to controls, but the type of estrogen metabolites was not reported (Enzinger 1954). Recently, Weidler et al. (2004) reported increased renal excretion of 16α-OHE_1 in relation to 2-hydroxyestrone in RA patients. 16α-Hydroxyestrogen is an active metabolite that is mitogenic and has a proliferative effect, whereas the 2-hydroxy metabolite seems to have low biological activity due to its conversion into *O*-methylated estrogens. A similar study also reported an increase in the concentration of 4- and 16α-hydroxylated estrogen in the synovial fluid (SF) of RA

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patients when compared with controls (Castagnetta et al. 2003). The molar ratio of estrogen to androgen was also reported to be increased in these patients. These data show that the serum level of estrogen in RA patients is similar to healthy controls, but the level of estrogen in relation to androgen was higher in inflamed synovial tissue and synovial fluid. Each of these factors somehow contributes to the pro-inflammatory state in RA.

One of the earlier studies of the 1980s reported a higher level of 16α-OHE_1 in the urine of systemic lupus erythematosus (SLE) patients (Lahita et al. 1979, 1981). Recently, these studies were confirmed in RA patients, where low levels of 2-hydroxyestrogens were detected (Weidler et al. 2004). The 2-hydroxylated estrogens were found to be ten times lower in patients with RA and SLE than in healthy controls, whereas the ratio of 16α-OHE_1/2-hydroxyestrogens was 20 times higher in these patients (Weidler et al. 2004). Moreover, a significantly higher 16α-OHE_1/4-hydroxyestradiol (4-OHE_2) ratio was detected in RA patients (Castagnetta et al. 2003). SLE patients have more peripheral hydroxylation of estrogen, which is known to elevate B cell differentiation and stimulate T cells (Lahita 1996). Also, data from our laboratory demonstrate that similar estrogen metabolites (catecholestrogens) appear to play an important role in the production of RA autoantibodies (Khan and Assiri 2011). Histone 1 (H_1) is a component of chromatin found in almost every eukaryotic cell. Antibodies against H_1 have been described in patients with RA (Pauls et al. 1989). It is interesting to note that 16α-OHE_1 is capable of making an adduct with H_1 via a stabilized Schiff base and subsequent rearrangement (Yu and Fishman 1985). This stable adduct is formed between 16α-OHE_1 and H_1, and both are somehow related to RA. Therefore, it is possible that the 16α-OHE_1-H_1 adduct contributes to the production of RA antibodies.

To test this hypothesis, here we aimed to evaluate 16α-OHE_1-H_1 adduct as a possible biomarker for RA and to probe the possible antigenic role of the16α-OHE_1-H_1 adduct in the production of RA autoantibodies. Furthermore, we also investigate whether induced (ant-16α-OHE_1-H_1) antibodies can be used as a probe for the estimation of 16α-OHE_1 concentration in the serum of RA patients. The role of 16α-OHE_1-H_1 adduct in RA etiopathogenesis has been discussed and evaluated.

Materials and Methods

Patients and Controls

Blood samples were taken from 40 patients (age: 61 ± 9 years; 4 male and 36 female) with rheumatoid arthritis who fulfilled the American College of Rheumatology (ACR) classification criteria for RA (Arnett et al. 1988). Thirty normal individuals (age: 60 ± 4 years; 3 males and 27 females), who serve as controls, were without any symptoms of RA. None of the controls had any history of illness (viral or another disease) during the study, which was confirmed by a doctor. During the recruitment, all patients had taken nonsteroidal anti-inflammatory drugs, various disease-modifying antirheumatic drugs or both. The clinical and laboratory data concerning these subjects are given in Table 1. All serum samples were heated at 56 °C for 30 min to inactivate complement protein and stored at − 20 °C with 0.2% sodium azide. Serum samples were centrifuged at 1500×*g* for 10 min before being treated with heat to inactivate complement protein. The study was approved by the Institutional Ethics Committee of the Centre, and informed consent was obtained from each subject.

16α-OHE_1-H_1 Adduct Formation

16α-OHE_1-H_1 adduct was formed as described by Yu and Fishman (1985) with slight modifications. 16α-Hydroxyestrone (1–10 mM) was incubated with 1 mg of histone (histone 1) in 0.1 M potassium phosphate pH 6.0. One micromol of sodium cyanoborohydride was added, and the reaction mixture was incubated at 37 °C for 48 h with gentle shaking. 16α-$OHE_1$was dissolved in ethanol, such that the final concentration of ethanol in the reaction mixture was 0.1%. Excess of 16α-OHE_1 and H_1 was removed by dialyzing the reaction mixture against phosphate buffer saline (PBS) pH 7.4.

Antibodies Against 16α-OHE_1-H_1

Antibodies against 16α-OHE_1-H_1, along with suitable controls, were produced in experimental animals (female rabbits, random bred, New Zealand White, $n = 5$) as described by Khan and Qureshi (2015). Briefly, antigens (50 µg) were emulsified with an equal volume of complete Freund's adjuvant (Sigma-Aldrich, USA) and injected intramuscularly in female rabbits. Subsequent injections were given in incomplete Freund's adjuvant. Each animal received a total of 400 µg of antigen (weekly) in the course of eight injections. Blood was collected from a marginal vein of the ear. Pre-immune serum was collected before immunization and served as a negative control. The sera were collected and stored at − 20 °C with 0.1% sodium azide as preservatives.

ELISA

Direct binding ELISA was performed for antibody screening in RA/immunized sera, as described previously (Khan et al. 2008). Competition ELISA was performed to evaluate the specific binding of circulating RA/immunized antibodies to 16α-OHE_1-H_1 adduct (Khan et al. 2008). Briefly, 100 µl of

Table 1 Clinical data and estimation of 16α-OHE_1 by anti-16α-OHE_1-H_1 antibodies in different RA patients

	RA patients ($n=40$)	Control ($n=30$)
Age, years	61 ± 9	60 ± 4
Men/women	4/36	3/27
Disease duration (years)	9.1 ± 3.2	–
CRP level, mg/dl	8.4 ± 2.8[a]	2.3 ± 0.8
ESR	34 ± 4.7[a]	20 ± 2.5
SF nuclear cells	18,920 ± 4188	–
SF protein (g/l)	48.7 ± 3.5	–
WBC count, $\times 10^3$/l	11.2 ± 2.9[a]	4.5 ± 1.2
Rheumatoid factor positive (%)	70 (28/40)	–
Anti-CCP positive (%)	65 (26/40)	–
Medication		
Methotrexate dose mg/week (mean) ($n=31$)	12.37	–
Use of NSAIDs (%)	57.5 (23/40)	–
Infliximab (3 mg/kg) in combination with methotrexate (n)	30	–
Adalimumab 40 mg/every other week (n)	10	–
16α-Hydroxyestrone estimation in serum ($n=5$) by[b]		
Anti-16α-OHE_1-H_1 antibodies	13.1 ± 1.2 pg/ml[c]	12.9 ± 4.2 pg/ml[d]
Human 16α-hydroxyestrone ELISA antibodies	13.3 ± 2.2 pg/ml	–
2-/16α-OHE_1	5.8 ± 1.3	5.9 ± 2.1

ESR erythrocytes sedimentation rate, *CRP* C reactive protein, *SF* synovial fluid, *WBC* white blood cell

[a]RA vs normal subjects $p<0.05$ (Student's t test). *2-/16α-OHE_1* 2-hydroxyestrone/16α-hydroxyestrone ratio

[b]The amount of 16α-OHE_1 level was measured by ELISA and the values are presented in mean ± SD

[c]Correlation coefficient $r=0.96$ ($p<0.001$). 2-/16α-OHE_1 ratio was measure by commercially available kit

[d]$n=8$

16α-OHE_1-H_1 adduct (2.5 µg/ml) was coated onto microtiter plates, incubated for 2 h at room temperature and overnight at 4 °C. The plates were washed with Tris buffer saline-Tween 20 (TBS-T) and blocked with 150 µl of 1.5% bovine serum albumin (BSA). Immune complexes were prepared by mixing 100 µl of 1:100 dilution of RA/immunized sera with increasing amounts (0–20 µg/ml) of the various antigens, and incubated at 37 °C for 2 h and overnight at 4 °C. One hundred microlitres of immune complex was added to each well, followed by anti-human IgG-alkaline phosphatase conjugate. The reaction was developed with p-nitrophenyl phosphate as substrate and absorbance were recorded at 410 nm on a microplate reader (Labsystem Multiskan EX, Finland). For the estimation of estrogen metabolites, we used the Human 16α-hydroxyestrone ELISA Kit (Glory Science Co. Lt, USA) for 16α-OHE_1 and the Estramet 2-hydroxyestrogen/16α-OHE_1 ELISA Kit (CD Diagnostics, NY, USA) for the 2-/16α-OHE_1 ratio.

Purification of Anti-16α-OHE_1-H_1 Antibodies

Immunoglobulin G (IgG) was isolated from RA sera (or immunized animal) on a Protein A Agarose column (Goding 1978). The homogeneity of the isolated IgG was checked on 7.5% PAGE. IgG concentration was determined by considering 1.40 $OD_{280}=1.0$ mg/ml.

Formation and Quantitation of Immune Complexes

Formation and quantitation of immune complexes have been done as described earlier (Khan et al. 2008). Briefly, 100 µg of IgG was incubated with varying amounts (0–40 µg) of the various antigens in an assay volume of 500 µl. Normal IgG served as control. The mixture was incubated for 2 h at room temperature and overnight at 4 °C. The immune complex**es** were pelleted, washed twice with PBS, and dissolved in 250 µl 1N NaCl. Free protein and complex bound protein were measured by colorimetric methods (Bradford 1976). The binding data were analyzed for antibody affinity (Langumir 1918).

Statistical Analysis

The statistical significance of differences, relative to control values, were determined using the Student's t test (IBM-SPSS, Statistic 22, New York, USA). A value of $p<0.05$ was considered statistically significant. The values are presented as means ± SD, wherever indicated.

Results

Formation of 16α-OHE_1-H_1 Adduct

The electrophoretic patterns of the 16α-OHE_1-H_1 adduct, as well as 16α-OHE_1 and H_1 separately, are shown in Fig. 1. Incubation of 16α-OHE_1 and H_1 results in the formation of a 16α-OHE_1-H_1 adduct, as evident from its reduced gel migration (Fig. 1a, lane 1) compared to either 16α-OHE_1 or H_1 (Fig. 1a, lane 2 and 3). Formation of the 16α-OHE_1-H_1 adduct is causing reduced migration in the gel due to the higher molecular weight of the complex (Fig. 1a). 16α-OHE_1-H_1 adduct showed higher absorbance ($OD_{280}=0.48$) and 35.9% UV hyperchromicity at 280 nm, indicating the formation of a higher molecular adduct relative to H_1 ($OD_{280}=0.36$) (Fig. 1b). The adduct formation between 16α-OHE_1 and H_1 occurs by stabilized Schiff base and subsequent rearrangement (Yu and Fishman 1985).

Anti-16α-OHE_1-H_1 Antibodies in the Sera of RA Patients

Sera were collected from 105 RA patients, and a total of 40 RA sera that showed high binding were selected for the study. Sera from 40 RA patients and 30 healthy subjects were checked for binding to the 16α-OHE_1-H_1, H_1, and 16α-OHE_1 by ELISA. In the direct binding ELISA, nearly all of the sera showed a higher recognition to 16α-OHE_1-H_1 compared to either 16α-OHE_1 ($p<0.001$) or H_1 ($p<0.05$) (Fig. 2). No appreciable binding was detected using the sera of healthy subjects with either of the antigens. Binding specificities were also checked using 16α-OHE_1 and H_1 separately, and it was found that their binding is far less compared to 16α-OHE_1-H_1. 16α-OHE_1 does not show any binding with either of RA autoantibodies or normal controls. Specific recognition of circulating autoantibodies in RA with 16α-OHE_1-H_1, 16α-OHE_1, and H_1 was also evaluated by competitive ELISA. 16α-OHE_1-H_1 showed maximum inhibition of $58\pm9.1\%$ (range 44.5–73.3%) in the sera from 40 RA patients, while H_1 showed lower inhibition ranging 15.8–69.5% (average $45.3\pm3.8\%$). 16α-OHE_1 showed no appreciable inhibition with RA antibodies (average $11.3\pm3.8\%$) (Fig. 3a).

Affinity chromatography on a Protein A-Agarose column was used to purify RA IgG. The purity of eluted immunoglobulin was checked by SDS–PAGE under non-reducing conditions, in which a single homogenous band was detected. In competition, ELISA, RA IgG showed an inhibition ranging 53.1–85.8% (average: $65.8\pm9.3\%$) for 16α-OHE_1-H_1, while for H_1, this was 18.3–71.5% (average: $49.8\pm3.5\%$). The inhibition of RA IgG was also checked with 16α-OHE_1, wherein IgG inhibition ranged 6.3–33.8% (average: $13.5\pm5.1\%$) (Fig. 3b). The antigen–antibody affinity was also characterized by quantitative precipitation titration curve. For this preparation, a varying amount of 16α-OHE_1-H_1, 16α-OHE_1, and H_1 were incubated with 100 µg of RA IgG ($n=6$). Normal human IgG ($n=3$) was used as an RA antibody-negative control. A maximum of 25 µg of 16α-OHE_1-H_1 adduct was bound to 76 µg of RA IgG. Although with H_1, a maximum of 31 µg of H_1 was bound by 63 µg of RA IgG. Similarly, 32 µg of 16α-OHE_1 was bound to 60 µg of RA IgG. A Langmuir plot was used to evaluate the affinity constant (Fig. 4). The affinity constants of RA IgG for the 16α-OHE_1-H_1, H_1 and 16α-OHE_1 were found to be 1.23×10^{-7}, 1.31×10^{-6}, and 1.23×10^{-6}, respectively. The binding specificity was also checked according to the medication taken during the study. We have taken about 30 patients' sera and divided them into four different groups, where we categorized them into those

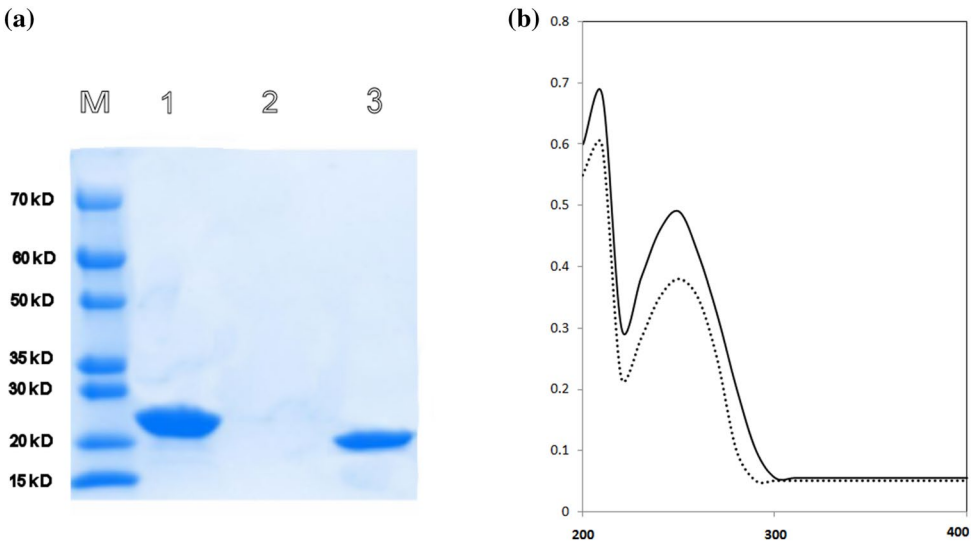

Fig. 1 **a** SDS–polyacrylamide gel electrophoresis of 16α-OHE_1-H_1 adduct and controls. Lanes: M—molecular weight markers (15–70 kD); 1—16α-OHE_1-H_1 adduct; 2—16α-OHE_1; 3—Histone 1 (H_1). **b** UV absorption spectra of Histone 1 (dashed line) and 16α-OHE_1-H_1 adduct (continuous line)

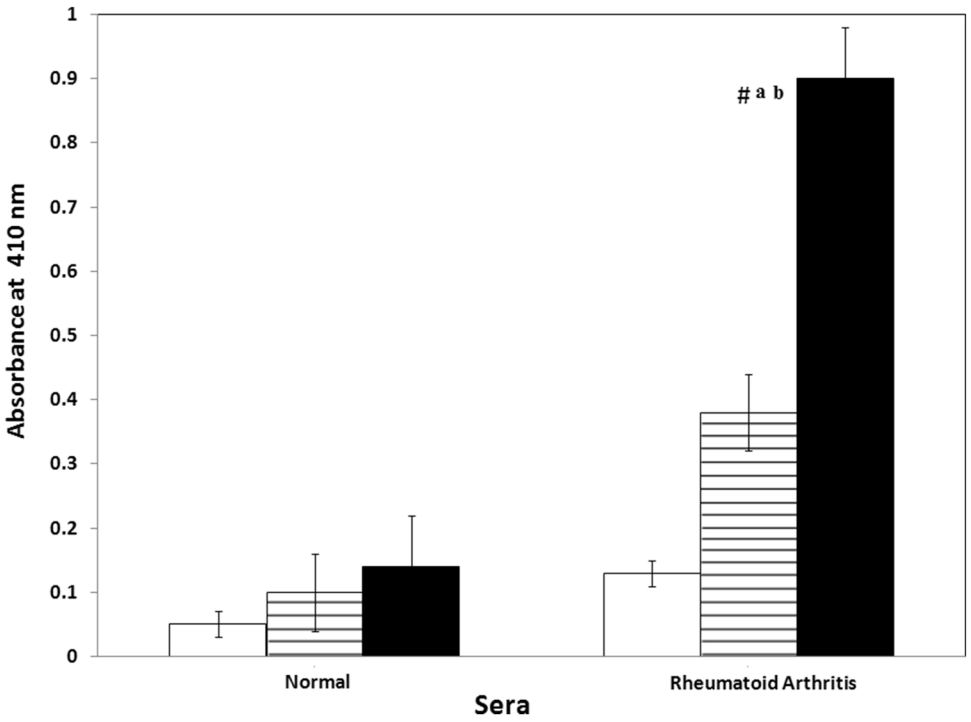

Fig. 2 Direct binding ELISA of control and RA patients. Direct binding enzyme-linked immunosorbent assay of control ($n=30$) and RA antibodies ($n=40$) to 16α-OHE_1-H_1 (black bars), H_1 (stripes bar) and 16α-OHE_1 (white bar). Microtitre plates were coated with 100 µl of respective antigen (2.5 µg/ml). The reaction was developed with p-nitrophenyl phosphate as the substrate and the absorbance was recorded at 410 nm as describe in "Materials and Methods". Each histogram represents the mean±SD. $^{\#}p<0.001$, significantly higher binding than normal sera; $^{a}p<0.05$, significantly higher binding than H_1 in RA; $^{b}p<0.001$, significantly higher binding than 16α-OHE_1 in RA

patients who had taken infliximab + methotrexate, adalimumab, NSAIDS or methotrexate. By this approach, we found that the binding of 16α-OHE_1-H_1 with those patients' sera who had taken infliximab + methotrexate was 35.3%, which is the lowest among all other groups. The bindings of the other groups' sera with 16α-OHE_1-H_1 were found to be adalimumab = 45.8%, NSAIDS = 63.8% and methotrexate = 50.8% (Fig. 5).

Anti-16α-OHE_1-H_1 Antibodies and Their Characterization

The immunogenicity of 16α-OHE_1-H_1 along with suitable controls was also checked by inducing antibodies in experimental animal (female rabbits) against them. Direct binding and competition ELISA was used to confirm the antigenic specificities of these induced antibodies. 16α-OHE_1-H_1 adduct was found to be highly immunogenic. Antiserum showed high titer antibodies (≥ 1:25,600) in experimental animals. In contrast, pre-immune sera served as a negative control and did not show any appreciable binding to 16α-OHE_1-H_1. After immunization with either 16α-OHE_1 or H_1, the antiserum showed lower binding with these antigens. The titer shown by these antigens (16α-OHE_1 and H_1) was low in comparison to 16α-OHE_1-H_1. The antigenic specificity was further ascertained by competition ELISA. A maximum of 73.4% inhibition in antibody activity was observed at a 20 µg/ml, and 50% inhibition was achieved at only 5.8 µg/ml (Fig. 6a). While for 16α-OHE_1, a maximum of 65.3% antibody inhibition was obtained at an immunogen concentration of 20 µg/ml, 50% inhibition was achieved at 13.5 µg/ml of immunogen (Fig. 6a). In case of H_1, a maximum of 71.3% inhibition in antibody activity was observed at an H_1 concentration of 20 µg/ml, and 50% inhibition was achieved at 11.3 µg/ml of immunogen.

IgG was purified from immune and pre-immune rabbit's sera by affinity chromatography on a Protein A Agarose column. Direct binding ELISA showed substantial binding of induced antibodies (anti-16α-OHE_1-H_1 antibodies) with 16α-OHE_1-H_1. Pre-immune IgG served as a negative control, showing negligible binding. The specificity of the induced IgG was also evaluated by competition ELISA using different types of antigens. In the competition ELISA, the anti-16α-OHE_1-H_1 antibodies showed strong binding for 16α-OHE_1-H_1, causing 93.1% inhibition in antibody binding at 20 µg/ml of the immunogen concentration (Fig. 6b). Fifty percent inhibition was achieved only at 3.1 µg/ml of the immunogen. For 16α-OHE_1, a maximum of 83.4% inhibition in antibody binding was observed with immunogen as the inhibitor. The concentration required for 50% inhibition was achieved at 9.3 µg/ml. For H_1, a maximum of 90.3% inhibition in antibody was achieved with the immunogen, and 50% inhibition was observed at 6.8 µg/ml (Fig. 6b). Immuno-cross-reactivity of anti-16α-OHE_1-H_1 antibodies was also checked by competition inhibition assay using 16α-OHE_1, H_1, chromatin, 4-OHE_1, 2-OHE_1, 16α-OHE_1-DNA, and 16α-OHE_1-chromatin as the inhibitor. The anti-16α-OHE_1-H_1 antibodies showed a very low degree of binding towards all of the other antigens, except 16α-OHE_1. However, anti-16α-OHE_1 antibodies showed a high degree of

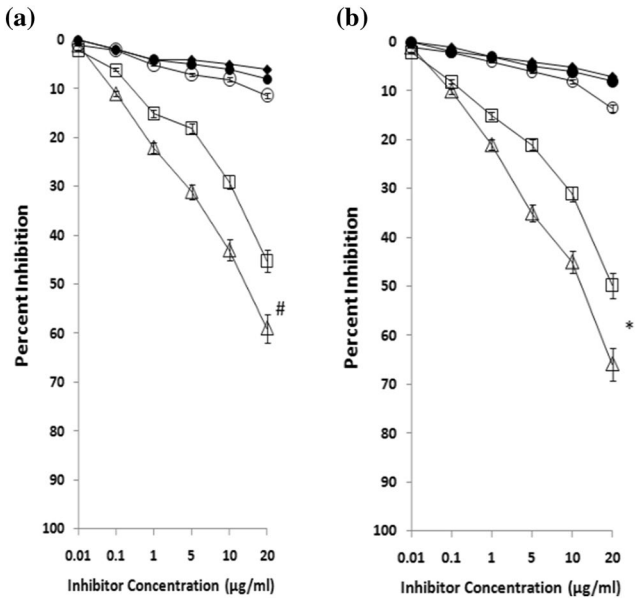

Fig. 3 Inhibition ELISA of control and RA patients. **a** Inhibition ELISA of RA sera (unfilled triangle, unfilled circle, unfilled square) and Control sera (filled circle, filled triangle) pre-incubated with 16α-OHE_1-H_1, 16α-OHE_1, H_1 on binding to 16α-OHE_1-H_1 coated plates. **b** Inhibition of RA IgG pre-incubated with 16α-OHE_1-H_1 (unfilled triangle), 16α-OHE_1 (unfilled circle), H_1 (unfilled square) on binding to 16α-OHE_1-H_1 coated plates. (filled triangle, filled circle) Represent the inhibition of Normal anti-16α-OHE_1-H_1 and H_1 IgG binding to 16α-OHE_1-H_1 and H_1. Microtire plates were coated with 100 µl of antigen (2.5 µg/ml). Immune complexes were prepared by mixing 100 µl of 1:100 dilution of serum antibodies from RA patients and control individuals with increasing amount (0–20 µg/ml) of antigens at 37 °C. *Note* inhibition values for Control sera and IgG with 16α-OHE_1 were negligible and not shown. $^{\#}p < 0.001$, significantly higher inhibition than 16α-OHE_1 in RA sera; $^{*}p < 0.001$, $p < 0.05$, significantly higher inhibition than 16α-OHE_1, H_1 in RA IgG

binding towards 16α-OHE_1-H_1, followed by 4-OHE_1 in addition to its own antigen (16α-OHE_1) (Table 2). Anti-H_1 antibodies showed less cross-reactivity with 16α-OHE_1-H_1 inaddition to the binding shown towards its own immunogen (H_1).

Serum levels of 16α-OHE_1 in RA patients ($n = 5$) were measured using anti-16α-OHE_1-H_1 antibodies, which was further confirmed using a commercially available kit (Human 16α-hydroxyestrone ELISA Kit, Glory Science Co. Lt, USA). The mean value of 16α-OHE_1, as estimated by anti-16α-OHE_1-H_1 antibodies, was 13.1 pg/ml, which is comparable to the value obtained using the Human 16α-hydroxyestrone ELISA Kit (13.3 pg/ml) (Table 1). Also, comparing RA patients to healthy controls, we have found no significant difference in the level of 16α-OHE_1 or 2-hydroxyestrone/16α-OHE_1 ratio ($n = 8$). Our results suggest that anti-16α-OHE_1-H_1 antibodies could be efficiently used as an immune-chemical probe to estimate 16α-OHE_1 concentrations in the serum of various RA patients.

Discussion

Sex hormones function as modulators of autoimmune disease onset or perpetuation, including RA (Cutolo et al. 2003). Estrogen can function as a stimulator for humoral immunity. Compared to a control group, the serum level of estrogen was found to be normal in RA patients, while SF level was significantly higher in both male and female RA patients (Castagnetta et al. 2003). This increase in the concentration of estrogen in RA SF was of a particular type, which is characterized by the presence of hydroxylated forms, particularly 16α-OHE_1 and 4-OHE_2 (Castagnetta et al. 2003), which are known for their mitogenic stimulating role. The other conversion product of estrogen is 2-hydroxyestrogen has been shown to function as a growth inhibitor by promoting the effect of estradiol (Schneider et al. 1984). If we compare the urinary concentration of these metabolites, urinary 2-hydroxyestrone is found to be 10 times lower in RA patients than in healthy controls, whereas 16α-OHE_1/2-hydroxyestrogen ratios are 20 times higher in these patients (Weidler et al. 2004). Therefore, the relative loss of 2-hydroxyestrogen in relation to 16α-hydroxylated estrogen might be an important aspect of mediating the proliferative state of synovial cells in RA. Synovial cells produce an increased concentration of 16α-OHE_1 in RA patients (Schmidt et al. 2009). Despite the normal concentration of 16α-OHE_1 in the serum and urine of RA patients, there is an increased 16α-OHE_1 found in the inflamed synovial tissues, where active immune cells are plentiful. Therefore, 16α-OHE_1 could form an H_1 adduct and might cause the production of autoantibodies. As a consequence, these autoantibodies might trigger inflammation in the joints of these RA patients. Given all these points, this study demonstrates the specificity and binding affinities of RA autoantibodies against 16α-OHE_1-H_1. A higher molecular weight adduct is formed between 16α-OHE_1 and H_1. This adduct showed about 35.9% UV hyperchromicity at 280 nm, and formation has taken place by stabilized Schiff base and subsequent rearrangement (Yu and Fishman 1985).

Autoantibodies are common features of rheumatic disease. However, rheumatoid factor has been considered a hallmark of autoantibodies found in RA (Zutshi et al. 1969) and was found to have modest specificity (~70%) for this disease. Today, several newly characterized autoantibodies have been used as diagnostic indicators for RA, including antikeratin, anticitrullinated peptides, anti-RA3, anti-Sa, and anti-p68 autoantibodies, that are shown to have > 90% specificity for RA (Steiner and Smolen 2002). Autoantibodies against cartilaginous collagen have been found in the sera of some RA patients (Morgan et al. 1987). Cross-reactive natural autoantibodies are also present in

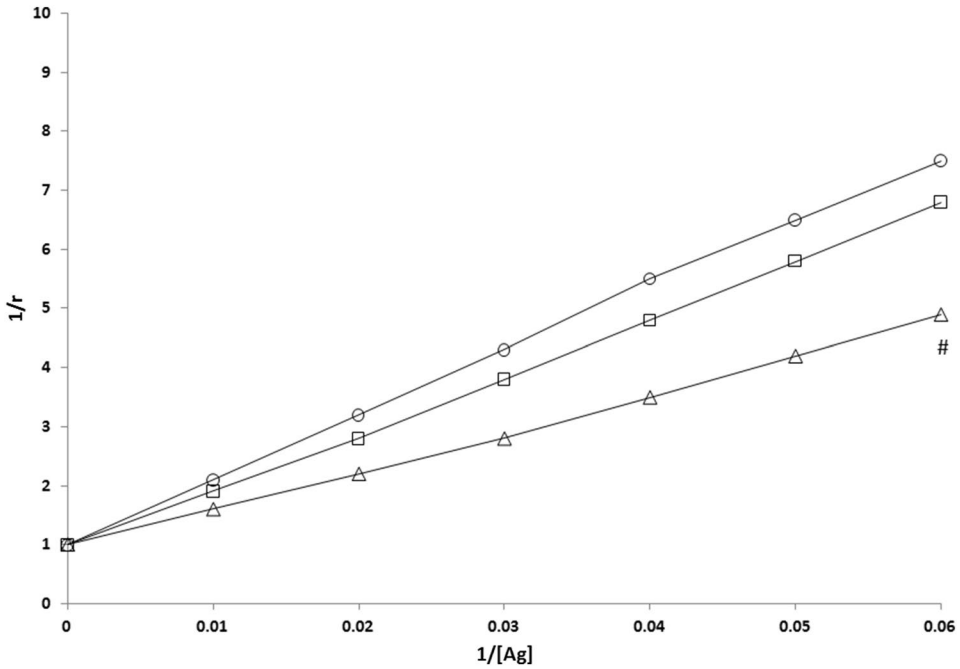

Fig. 4 Determination of apparent association constant by Langmuir plot. Antigens were 16α-OHE_1-H_1 (unfilled triangle), H_1 (unfilled square) and 16α-OHE_1 (unfilled circle). Immune complexes were prepared by incubating 100 µg of IgG with varying amount of different antigens (0–100 µg) in an assay volume of 500 µl for 2 h at room temperature and overnight at 4 °C. The binding data were analyzed for antibody affinity as described in "Materials and Methods". #Significantly higher binding than H_1 ($p < 0.05$) and 16α-OHE_1 ($p < 0.001$)

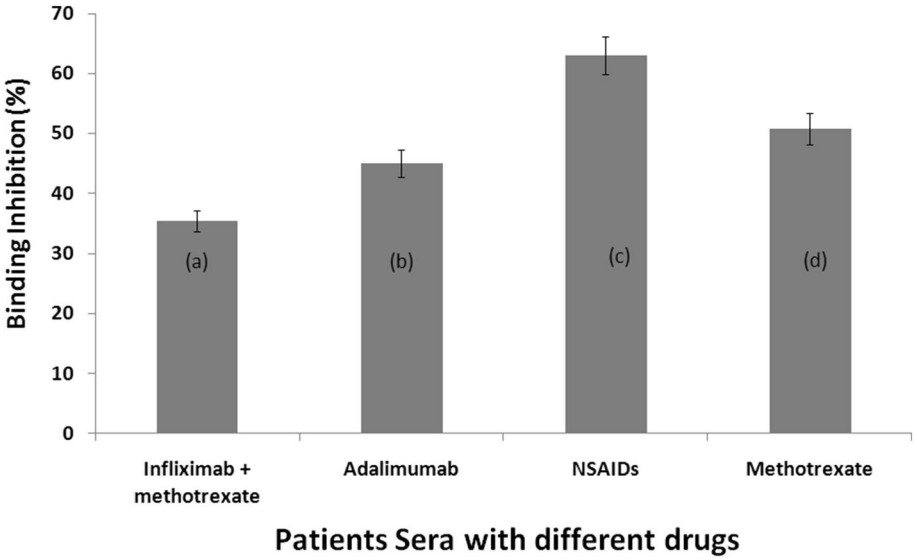

Fig. 5 Effect of treatment on efficacy of RA patients IgG in competition ELISA. IgG from patients who have taken **a** Infliximab + methotrexate, **b** Adalimumab, **c** NSAIDS and **d** Methotrexate were compared with IgG from three patients that showed maximum inhibition among other RA patients and which were used as reference for 100% binding inhibition (actual inhibition was 80–85%). Microtitre plates were coated with 100 µl of 16α-OHE_1-H_1 (2.5 µg/ml)

the sera of RA patients (George and Shoenfeld 1996). Sera from some RA patients were also tested for the presence of antibodies against histones, and it was found that nearly 24% of the antinuclear antibodies positive sera reacted to histone-dependent nuclear antigen (Aitcheson et al. 1980). Thus, 50% of rheumatoid factor possess cross-reactivity with nuclear components and histones are involved in a significant number of these reactions. The binding of antibodies from sera of 40 RA and 30 normal control subjects to 16α-OHE_1-H_1 was studied by direct binding and inhibition ELISA. The 16α-OHE_1-H_1 adduct showed preferentially higher binding with RA sera compared with controls ($p < 0.001$). These data demonstrate that 16α-OHE_1-H_1 can function as an effective inhibitor, showing a substantial difference in the recognition of 16α-OHE_1-H_1 over H_1 or 16α-OHE_1. The present study is in agreement with the earlier reports demonstrating increased recognition of estrogen modified DNA by RA autoantibodies (Khan and Assiri 2011). Formation of adduct exposes unique epitopes on the molecule that might cause an increase in the binding of these autoantibodies to 16α-OHE_1-H_1.

To further confirm the recognition of 16α-OHE_1-H_1 by antibodies in RA, we further evaluated the specificity of autoantibodies by quantitative precipitation titration.

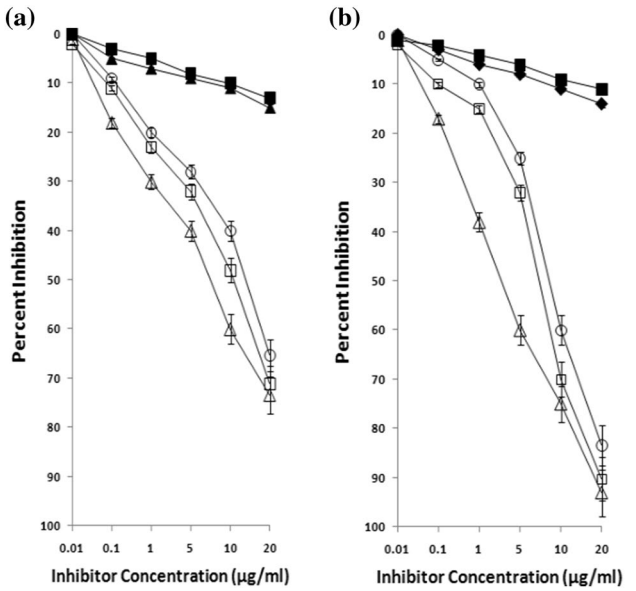

Fig. 6 Inhibition ELISA of immunized antigens. **a** Inhibition ELISA of immune sera (unfilled triangle, unfilled square, unfilled circle) and pre-immune sera (filled square, filled trianlge) pre-incubated with 16α-OHE_1-H_1, H_1, 16α-OHE_1, on binding to antigen coated plate. **b** Inhibition ELISA of immune IgG pre-incubated with 16α-OHE_1-H_1 (unfilled triangle), 16α-OHE_1 (unfilled circle), H_1 (unfilled square), on the binding to antigen coated plates. (filled square, filled triangle) represent the inhibition of pre-immune anti-16α-OHE_1-H_1 and H_1 IgG binding to 16α-OHE_1-H_1 and H_1. The experiments were carried out by incubating ELISA plate with 100 µl of respective antigens (2.5 µg/ml) as described in "Materials and Methods"

The affinity constant of the order of 10^{-7} indicates better recognition of 16α-OHE_1-H_1 over 16α-OHE_1 or H_1. The higher recognition of 16α-OHE_1-H_1 demonstrates the possible participation of 16α-OHE_1-H_1 in RA pathogenesis. As mentioned above, RA synovial cells produce an increased concentration of 16α-OHE_1 that is capable of forming an H_1 adduct in inflamed synovial tissues and triggering the production of autoantibodies. As a result, these autoantibodies might be involved in the pathogenesis of RA. Therefore, 16α-OHE_1-H_1 presents unique antigenic determinants for the recognition of RA autoantibodies. We also tested the effect of medication on the binding of RA IgG to 16α-OHE_1-H_1 during the study. We categorized our RA patients into four groups based on which medication they had been taking during the study. 16α-OHE_1-H_1 binding in the different groups showed that sera from the patients who have taken infliximab + methotrexate interfered with the binding between 16α-OHE_1-H_1 and IgG in these patients. This might be due to the presence of existing IgG from the medication of infliximab + methotrexate that might interfere in the binding of IgG with 16α-OHE_1-H_1. Binding might also be hindered by normal existing IgG. The other remaining groups showed higher binding with 16α-OHE_1-H_1, demonstrating reduced inhibition by IgG with 16α-OHE_1-H_1. The induced antibodies (anti-16α-OHE_1-H_1 antibodies) demonstrate immune cross-reactivity with 16α-OHE_1 in addition to binding to its own immunogen. This is similar to the anti-16α-OHE_1 antibodies data, in which these antibodies showed high recognition to 16α-OHE_1-H_1, indicating that the epitopes on 16α-OHE_1-H_1 were efficiently bounded by anti-16α-OHE_1 antibodies.

Here we have attempted to describe the possible role of 16α-OHE_1-H_1 in the generation of autoantibodies in RA patients. In conclusion, our study indicates the possible antigenic role of 16α-OHE_1-H_1 adducts in RA patients, which might be used as a marker for this disease. However, further studies are needed to confirm the utilization of these adducts as a biomarker for RA.

Table 2 Relative affinity of induced antibodies towards different inhibitors

Inhibitors	Maximum % inhibition at 20 µg/mL	Concentration for 50% inhibition (µg/mL)	Percent relative affinity
Anti-16α-OHE_1-H_1 antibodies			
16α-OHE_1-H_1	93.1	3.1	100
16α-OHE_1	73.8	4.8	64.5
H_1	23.1	–[a]	–
Chromatin (human)	9.8	–	–
16α-OHE_1-chromatin	10.1	–	–
16α-OHE_1-DNA (human)	4.5	–	–
4-OHE_1	5.7	–	–
2-OHE_1	8.9	–	–
Anti-16α-OHE_1 antibodies			
16α-OHE_1	83.4	9.3	100
16α-OHE_1-H_1	75.8	10.8	86.1
H_1	9.1	–	–
Chromatin (human)	7.8	–	–
16α-OHE_1-chromatin	9.1	–	–
16α-OHE_1-DNA (human)	5.6	–	–
4-OHE_1	20.1	–	–
2-OHE_1	8.9	–	–
Anti-H_1 antibodies			
H_1	90.3	7.8	100
16α-OHE_1-H_1	28.3	–	–
16α-OHE_1	3.8	–	–
Chromatin (human)	19.8	–	–
16α-OHE_1-chromatin	18.1	–	–
16α-OHE_1-DNA (human)	8.1	–	–
4-OHE_1	2.9	–	–
2-OHE_1	3.2	–	–

The experiments were carried out by incubating ELISA plate with 100 µl of different antigens (16α-OHE_1-H_1, 16α-OHE_1, H_1) at 2.5 µg/ml. After washing, unoccupied sites were blocked with 1.5% BSA in TBS. Immune complexes (formed by incubating varying amount of inhibitors with constant amount of IgG) were added to the well, followed by anti-immunoglobulin alkaline phosphatase conjugate. The reaction was developed with *p*-nitrophenyl phosphate as the substrate and absorbance was recorded at 410 nm

[a]50% inhibition was not obtained. 4-OHE_1: 4-hydroxyestrone, 2-OHE_1: 2-hydroxyestrone

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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