Advances in Medical Sciences 64 (2019) 72–78

Contents lists available at ScienceDirect

Advances in Medical Sciences

journal homepage: www.elsevier.com/locate/advms

Original research article

16 α-Hydroxyestrone induced adduct generate high affinity autoantibodies in SLE

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ARTICLE INFO

Keywords:
16 α-Hydroxyestrone
Histone H1
SLE
Autoantibodies
ELISA

ABSTRACT

Purpose: Increased 16 α-hydroxyestrone (16 α-OHE_1) and autoantibodies against histone H1 (H_1) have been well described in systemic lupus erythematosus (SLE), but the combination effects of 16 α-OHE_1 and H_1 remains unclear. Here, we tried to assess the affinity and binding specificity of SLE autoantibodies against the 16 α-OHE_1-H_1 adduct. IgG was induced against this adduct and was also used as immunochemical probe for the estimation of 16 α-OHE_1 in the serum of SLE patients.

Materials and methods: The affinity and binding specificities of SLE autoantibodies against 16 α-OHE_1-H_1 were determined by direct binding and inhibition ELISA as well as quantitative precipitation titration in 60 patients and 30 control subjects.

Results: Purified SLE autoantibodies showed greater recognition to 16 α-OHE_1-H_1 over H_1 ($p < 0.05$) or 16 α-OHE_1 ($p < 0.001$). The relative affinity of SLE autoantibodies for 16 α-OHE_1-H_1, H_1 and 16 α-OHE_1 was 1.41×10^{-7}, 1.31×10^{-6}, and 1.03×10^{-6}, respectively. The concentration of 16 α-OHE_1 in the sera of SLE patients was significantly higher than controls ($p < 0.05$) as estimated by anti-16 α-OHE_1-H_1 antibodies.

Conclusions: High affinity of 16 α-OHE_1-H_1 with SLE autoantibodies might suggest an antigenic role of this adduct in the production of these autoantibodies. The anti-16 α-OHE_1-H_1 antibody is a good immunochemical probe to measure 16 α-OHE_1 in different SLE sera.

1. Introduction

Systemic lupus erythematosus is a chronic disease that depends on various factors including changes in the hormonal concentration during the course of this disease. It is 9-fold more common in women than men [1]. Hormone-like estrogen have both, anti-inflammatory and pro-inflammatory, functions depending on various influencing factors [2]. One of these factors is the production of free radicals through the oxidation of estrogen to generate catecholestrogen metabolites that might damage DNA and other nuclear components [3]. Other compounds of estrogen such as 16 α-hydroxyestrone are active metabolites that play an important role in SLE [4]. Earlier studies have shown an increased concentration of serum 16 α-hydroxyestrone in SLE patients [5]. The other metabolites of estrogen such as 2-hydroxyestrone (2-OHE_1) were ten times lower in SLE patients, and the ratio of 16 α-hydroxyestrone/2-hydroestrone was 20 times higher in these patients [4]. The phenomenon of α-hydroxylation of estrogen was increased in SLE and can increase the production of active estrogen metabolites to accelerate B- and T-cell differentiation and activation [6]. The other mechanism through which estrogen metabolites play an important role in SLE pathogenesis is the process of quinone-semiquinone redox cycling of estrogen. This can generate reactive oxygen species (ROS) that damage DNA. This might alter its structure leading to changes in its antigenicity producing SLE autoantibodies that cross-react with native DNA [7]. An increased level of 16 α-hydroxyestrone was found in the serum and urine of SLE patients. Earlier studies from the 1980s demonstrate higher level of 16 α-hydroxyestrone in the urine of SLE patients compared to healthy controls [5,8]. Recently, one study reported elevated level of 16 α-hydroxyestrone and lower level of 2 hydroxyestrone in SLE patients [4].

Histone H1 (H_1) is one of the five main histones found in chromatin in every eukaryotic cell. Autoantibodies against H_1 were found in the serum of different SLE patients [9]. These antibodies are a heterogeneous population that was directed against epitopes found on the variable region of H_1 molecule [9]. Other studies reported autoantibodies against H_1 in SLE patients. The anti-histone antibodies markers are more prevalent in SLE patients, and there is a strong correlation between the level of antibodies to H_1 and SLE disease activity index [10].

Autoantibodies against H_1 are a highly specific marker for SLE and

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https://doi.org/10.1016/j.advms.2018.11.002
Received 8 April 2018; Accepted 23 November 2018
Available online 05 December 2018

are associated with increased disease activity [11]. The data revealed a strong correlation of anti-H_1 antibodies with SLE disease activity. This was even better than the correlation of anti-dsDNA and other antibodies [11]. In conclusion, anti-H_1 antibodies are a highly specific biomarker for SLE that is even comparable to the anti-DNA antibodies. H_1 is the major auto-antigen for B- and T-cells in SLE that stimulate the production of pro-inflammatory Th1 cytokines that further produces autoantibodies [12]. H_1 can form an adduct with 16 α-hydroxyestrone [13]. Covalent adduct formation was maximal for H_1 and occurs via stabilized Schiff base and subsequent rearrangements [13].

A stable adduct occurs between 16 α-hydroxyestrone and H_1, and both are somehow directly related with SLE. Thus, there is a good opportunity to use this adduct as an immunological biomarker for SLE. To test this important hypothesis, this study was designed to evaluate the 16 α-OHE_1-H_1 adduct as a possible immunological biomarker for SLE and further probe its antigenic role in the production of SLE autoantibodies. Furthermore, induced antibodies against this adduct were also used as immunochemical probe for the estimation of 16 α-OHE_1 concentration in the serum of SLE patients.

2. Materials and methods

2.1. Serum samples

SLE sera were collected from patients who showed high titer antibodies that satisfied the American Rheumatism Association criteria for the diagnosis of SLE disease [14]. We studied 60 SLE patients (54 females and 5 males) with a mean age of 47 ± 4.5 years with a mean disease duration of about 13 ± 4.8 years. The mean disease activity was evaluated by a modified SLE disease activity index; this was 4.8 (range 3–9). Healthy normal individuals (n = 30) free from any disease (26 females and 4 males, mean age 42 ± 8.1 years) served as controls for the study (Table 1). The control group comprises women/men coming to the hospital for routine check up and hospital staff acting as blood donors. They were mostly white/Caucasian. All samples were preheated to deactivate complement protein at 56 °C for 30 min before the start of any experiment. After deactivation, all serum samples were stored at −20 °C.

2.2. 16 α-OHE_1-H_1 adduct formation

The 16 α-OHE_1-H_1 adduct formation occurs as described earlier [13] with some modifications: 16 α-hydroxyestrone (1–10 mM) was incubated with 1 mg of H_1 in 0.1 M potassium phosphate (pH 6.0); then, 1 μmol of sodium cyanoborohydride was added to the reaction mixture and incubated for 48 h at 37 °C with gentle shaking. The 16 α-OHE_1 was dissolved in ethanol prior to the incubation in such a way that the final concentration of the ethanol in the reaction mixture was 0.1%. Dialyzed samples were used for all further experimental procedures.

Table 1
Clinical data and levels of 16 alpha-hydroxyestrone estimated by anti-16 α-OHE_1-H_1 antibodies in the serum of different SLE patients.

S. No.	SLE	Normal Subjects
Number and gender (M/F)	6/54	4/26
Age (years)	47 ± 4.5	42 ± 8.1
Disease duration (years)	13 ± 4.8	-
Disease activity	4.8	-
Treatment		
Prednisolone (n = 16)	9.4 mg/ml	-
Azathioprine (n = 13)	100 mg/ml	-
Methotrexate (n = 11)	7.5 mg/week	-
Cyclophosphamide (n = 10)	100 mg/day	-
Untreated (n = 10)	-	-
[#]16 α-hydroxyestrone estimation in serum by:		
Anti-16 α-OHE_1-H_1 antibodies (n = 20)	37.9 ± 16.8 pg/ml[*]	12.9 ± 5.3 pg/ml
Human 16 α-hydroxyestrone ELISA Kit (n = 20)	37.1 ± 13.3 pg/ml	12.8 ± 7.1 pg/ml

[#] The amount of serum 16 α-hydroxyestrone level was measured by ELISA and the values are presented in pg/ml. Correlation coefficient r = 0.98 ($p < 0.001$).

[*] Significantly higher than control ($p < 0.05$).

2.3. IgG against 16 α-OHE_1-H_1 in experimental animals

IgG against 16 α-OHE_1-H_1 along with suitable controls (16 α-OHE_1 and H_1) were induced in rabbits (female, random bred, New Zealand white, n = 6) as described previously [15]. Pre-immune sera were collected before immunization and served as controls.

2.4. ELISA

A direct binding ELISA was performed for antibody screening in the sera from SLE patients/immunized animals [3]. A competition ELISA was performed to determine the specific binding of SLE/immunized antibodies to 16 α-OHE_1-H_1, H_1, and 16 α-OHE_1 [3]. Briefly, 100 μl of an antigen aliquot (16 α-OHE_1-H_1, H_1 and 16 α-OHE_1, 2.5 μg/ml) was coated onto microtiter plates, incubated for 2 h at room temperature, and then incubated overnight at 4 °C. The plates were washed with TBS -T (50 mM Tris, 150 m NaCl, and 0.05 Tween 20, pH 7.6) and blocked with 150 μl of 1.5% bovine serum albumin (BSA). Immune complexes were prepared by mixing 100 μl of serum diluted at 1:100 ratio with different concentration of antigens (0–20 μg/ml). The reaction mixture was incubated at 37 °C initially for 4 h and 24 h at 4 °C; 100 μl of this mixture was added to the well as an immune complex. Anti-human IgG-alkaline phosphatase conjugate (Sigma-Aldrich) was added to the reaction mixture followed by the addition of p-nitrophenyl phosphate as substrate. The absorbance was read at 410 nm, and results were presented accordingly.

2.5. Purification of IgG

IgG either from SLE patient's sera or immunized animals were purified on Protein A-Agarose with suitable dilution [16]. The purity and homogeneity of purified IgG was checked on 7.5% PAGE and by spectroscopy.

2.6. Immune complex formation and their quantification

Immune complex formation and quantification were done as described earlier [17]. Briefly, 100 μg of patients IgG was incubated with variable amount of different antigens (16 α-OHE_1-H_1, H_1, and 16 α-OHE_1) in a total volume of 500 μl. Normal human IgG serves as the negative control. The immune complexes were incubated initially at 37 °C for 4 h and 24 h at 4 °C. The reaction mixture was pelleted and then washed twice with PBS and finally dissolved in NaCl (250 μl). Bound protein in immune complex and free protein was estimated by colorimetry [18]. The binding affinity was determined by estimating the affinity constant [19].

2.7. Statistical analysis

The significance of difference from control values was determined with the Student's *t*-test (IBM SPSS, Statistics 22). A value of $p < 0.05$ was considered statistically significant. The values are presented as the mean ± SD wherever indicated.

2.8. Ethical approval

All procedures performed in this study involving human participants were in accordance with the 1964 Helsinki declaration and its

Table 2
Characterization of 16 α-OHE_1-H_1 adduct and control.

Parameters	H_1	16 α-OHE_1-H_1 Adduct
Hyperchromacity at 280 nm	-	35.9%
Melting temperature (Tm) °C	54 ± 8.1	64 ± 3.4
Onset of melting (°C)	48 ± 6.1	60 ± 8.2
Circular dichroism (mdeg)		
Wavelength (nm)		
+ 190	0.890	1.328
−199	−34.83	−32.41
−220	−9.23	−11.39
Mobility on SDS-PAGE	More	Less

The experiment was done by incubating 16 α-OHE_1-H_1 and H_1 in 0.1 M potassium phosphate, pH 6.0, containing 1 μ mol of sodium cyanoborohydride and 0.1% ethanol at 37 °C for 48 h.
Hyperchromacity of 16 α-OHE_1-H_1 was calculated by measuring OD of 16 α-OHE_1-H_1 as compared to H_1.
CD spectral analysis was recorded at three wavelengths that showed maximum difference in the ellipticity at these wavelengths.

later amendments. All subjects gave their written informed consent to the study, and the protocol was approved by Institutional Ethics Committee (No.: 345/2016).

3. Results

3.1. Characterization of 16 α-OHE_1-H_1 adduct

Treatment of 16 α-OHE_1 with H_1 results in the formation of high molecular weight adducts that are shown to have less migration on the SDS-PAGE [20]. Formation of the adduct occurs via the Schiff base followed by subsequent rearrangement [13]. The 16 α-OHE_1-H_1 showed 35.9% UV hyperchromacity demonstrating a stark conformational changes in circular dichroism in the adduct in comparison to the controls (Table 2). The thermal stability of the adduct was evaluated and found to have a substantially high melting temperature of about 10 °C in the 16 α-OHE_1-H_1 adduct over H_1 histone. UV absorption and thermal denaturation studies of 16 α-OHE_1-H_1 adducts showed the formation of strong and stable adducts that undergo modifications and alterations in their structure upon adduct formation.

3.2. Detection of anti-16 α-OHE_1-H_1 autoantibodies in SLE sera

Serum samples from 60 SLE patients and 30 normal subjects were tested for binding to 16 α-OHE_1-H_1, H_1, and 16 α-OHE_1 by ELISA. Nearly all sera showed stronger binding to 16 α-OHE_1-H_1 adduct versus either H_1 or 16 α-OHE_1 ($p < 0.05$, $p < 0.001$). Normal sera had no appreciable binding to either antigen (Fig. 1). Competition ELISA was also used to evaluate binding specificities of SLE autoantibodies against these antigens. The specificity of SLE autoantibodies showed the highest binding with 16 α-OHE_1-H_1 adducts showing about 60.4 ± 4.1% (46.8–77.3%) inhibition in the antibody activity. H_1 showed less inhibition in the antibody activity - this was about 43.1 ± 3.9 (22.8–62.9%). The 16 α-OHE_1 showed no appreciable inhibition in SLE autoantibodies (11 ± 5.2%) (Fig. 2).

The SLE autoantibodies from the patient's sera were purified from any contamination by affinity chromatography on Protein A-Agarose column (Sigma, St. Louis, Missouri, USA). The purity of the purified IgG from the patients was checked with SDS-PAGE; there was a single homogenous band. In the competition binding assay, the 16 α-OHE_1-H_1 inhibited the antibody activity to about 72.1 ± 9.8% (52.3%–88.3%), but it was 50.3 ± 4.1% for H_1 (27.5%–70.7%). The inhibition of SLE autoantibodies was also probed with 16 α-OHE_1; this value was 14.1 ± 4.2% (5.8%–23.8%) (Fig. 3). Binding specificities of SLE autoantibodies were also probed according to the medication taken by these SLE patients. We divided them into 5 groups based on the

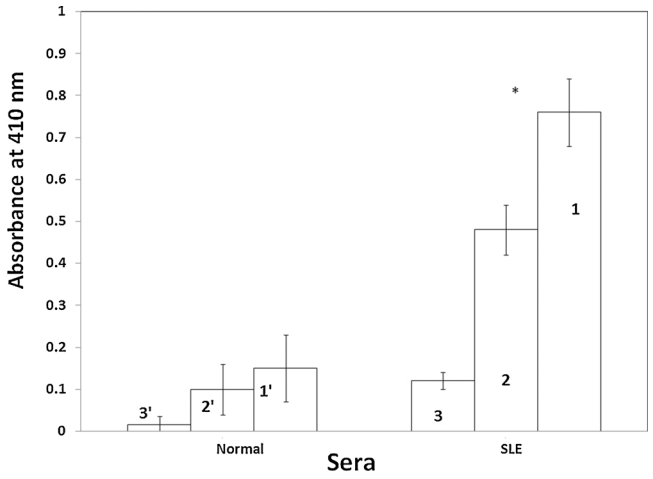

Fig. 1. Direct binding enzyme-linked immunosorbent assay of normal (n = 30) and SLE autoantibodies (n = 60) to 16 α-OHE_1-H_1 (1, 1'), H_1 (2, 2') and 16 α-OHE_1 (3, 3'). Microtitre plates were coated with 2.5 μg/ml of respective antigen and the values are recorded at 410 nm. Significantly higher than control (#P < 0.001).

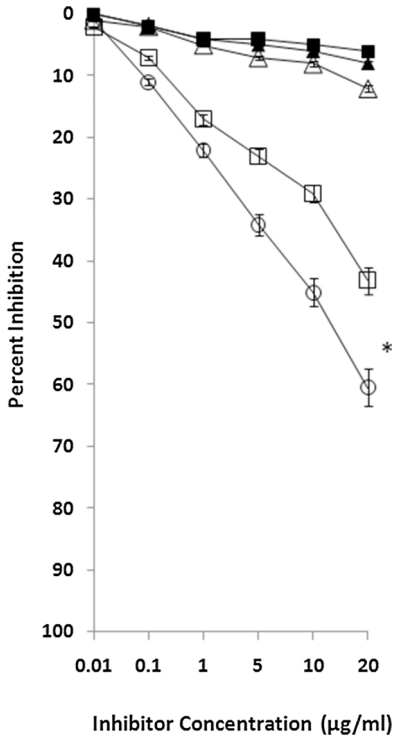

Fig. 2. Inhibition ELISA of anti-(16 α-OHE_1-H_1, H_1, 16 α-OHE_1) SLE (-○-, -□-, -Δ-) and normal (■, ▲) sera with 16 α-OHE_1-H_1, H_1, 16 α-OHE_1. Microtitre plates were coated with respective antigens (2.5 μg/mL). *Note:* Inhibition values for normal sera with 16 α-OHE_1 were negligible and are not shown. *Significantly higher inhibition than H_1 ($p < 0.05$) and 16 α-OHE_1 ($p < 0.001$).

medication they used. Ten were untreated, 16 used prednisolone, 13 used azathioprine, 11 were on methotrexate, and 10 used cyclophasphamide (Fig. 4). The inhibition values were the highest (61.3 ± 6.8%) for those patients who did not use any medication. The inhibition values for patients on medication were the following: 15.3 ± 5.3% for prednisolone patients, 20.3 ± 5.2% for those on azathioprine, 35.9 ± 6.3% for methotrexate patients and 41.5 ± 6.7% for cyclophasphamide patients.

The antibody interaction with different antigens (16 α-OHE_1-H_1, H_1

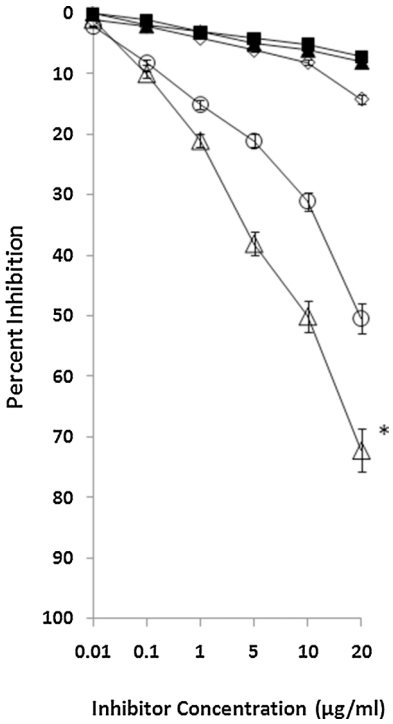

Fig. 3. Inhibition of SLE anti-(16 α-OHE_1-H_1, H_1, 16 α-OHE_1) IgG binding to 16 α-OHE_1-H_1 (-Δ-), H_1 (-○-), 16 α-OHE_1 (-◊-). (▲, ■) Represent the inhibition of Normal anti-16 α-OHE_1-H_1 and H_1 IgG binding to 16 α-OHE_1-H_1 and H_1. Microtitre plates were coated with respective antigens (2.5 µg/ml). Inhibition values for normal IgG with 16 α-OHE_1 were negligible and are not shown. *Significantly higher inhibition than H_1 ($p < 0.05$) and 16 α-OHE_1 ($p < 0.001$).

and 16 α-OHE_1) was also characterized by solid phase precipitin titration. The SLE autoantibody (100 µg) was incubated with varying amount of different antigens (16 α-OHE_1-H_1, H_1 and 16 α-OHE_1) in an assay volume of 500 µl. After waiting for 2 h at 37 °C and overnight at 4 °C, the concentration of free antigens and antigen-antibody complexes was estimated to evaluate free and bound antigen. Normal human IgG served as the negative control. This was also treated with similar conditions. The binding of 16 α-OHE_1-H_1 with their respective IgG was the highest among all other antigens (i.e. H_1, 16 α-OHE_1). A maximum of 21 µg of 16 α-OHE_1-H_1 was bound to about 80 µg of SLE autoantibodies. The H_1 had 38 µg of H_1 bound to 65 µg of SLE IgG. Similarly, 41 µg of 16 α-OHE_1 was bound to 53 µg of SLE IgG. A precipitin titration curve was used to evaluate the affinity of the SLE IgG with their respective antigens (Fig. 5). The affinity constants of SLE autoantibodies for 16 α-OHE_1-H_1, H_1, and 16 α-OHE_1 were 1.41×10^{-7}, 1.31×10^{-6} and 1.03×10^{-6}, respectively.

3.3. Antigenic specificity of anti-16 α-OHE_1-H_1 antibodies and estimation of 16 α-hydroxyestrone

Direct binding ELISA showed that 16 α-OHE_1-H_1 is a potent immunogen inducing high titer antibodies in rabbits. Pre-immune serum was the negative control and had no binding with the immunogen. The H_1 had similar results as 16 α-OHE_1-H_1. The 16 α-OHE_1 titer was low versus 16 α-OHE_1-H_1. In competition ELISA, anti-16 α-OHE_1-H_1 antibodies from the serum showed an inhibition of about 73.4 ± 3.1% in antibody activity at a 20 µg/ml. Inhibition values for H_1 and 16 α-OHE_1 with their respective induced IgG were 71.3 ± 5.3% and 65.3 ± 8.1% at 20 µg/ml of the immunogen concentration (Fig. 6). Induced IgG was purified on Protein A-Agarose from the experimental animal, and their specificity was checked by inhibition binding assays. In competition ELISA, anti-16 α-OHE_1-H_1 antibodies showed strong recognition to 16 α-OHE_1-H_1 inhibiting 93.1 ± 8.3% of antibody activity at 20 µg/ml (Fig. 6). The 50% inhibition was achieved only at 3.1 ± 0.3 µg/ml of the immunogen. The inhibition values for H_1 and 16 α-OHE_1 were 90.3 ± 3.1% and 83.4 ± 9.1%, respectively.

Immuno-cross reactivity of anti-16 α-OHE_1-H_1 antibodies was also checked with other antigens like H_1, 16 α-OHE_1, 16 α-OHE_1-chromatin, 4-hydroxyestrone, and 2-hydroxyestrone as inhibitors. Anti-16 α-OHE_1-H_1 antibodies showed less inhibition with the other antigens except for 16 α-OHE_1 (inhibition = 73.8 ± 3.9%). The anti-16 α-OHE_1 antibodies had similar cross-reactivity with 16 α-OHE_1-H_1 (inhibition = 75.8 ± 8.3%). The anti-H_1 antibodies had no cross-reactivity with either antigen. The anti-16 α-OHE_1-H_1 and anti-16 α-OHE_1 antibodies

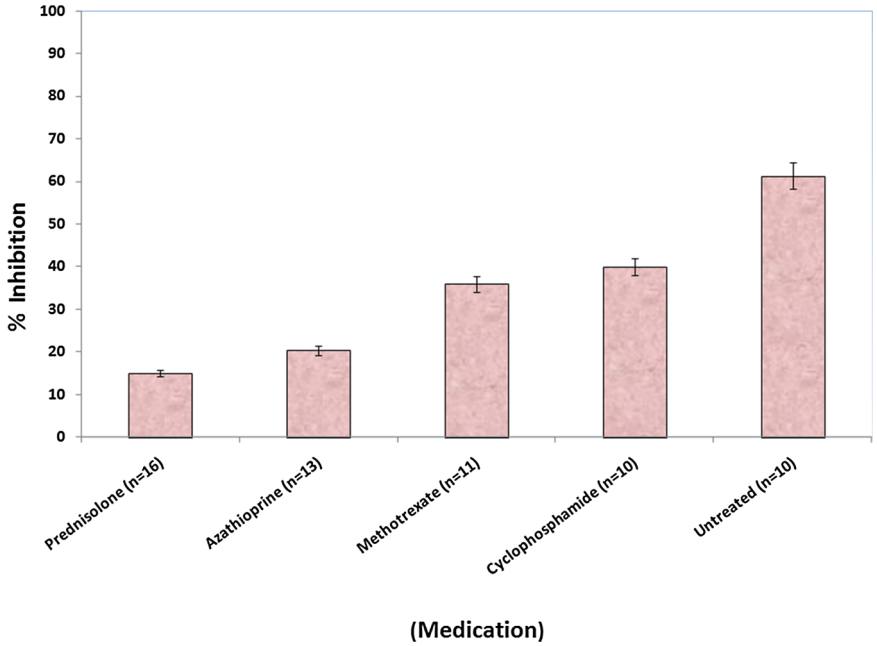

Fig. 4. Detection of autoantibodies according to medication used in different SLE patients by competition ELISA. Microtire plates were coated with respective antigens (2.5 µg/ml) and values are presented in % inhibition as described in 'Material and methods' section.

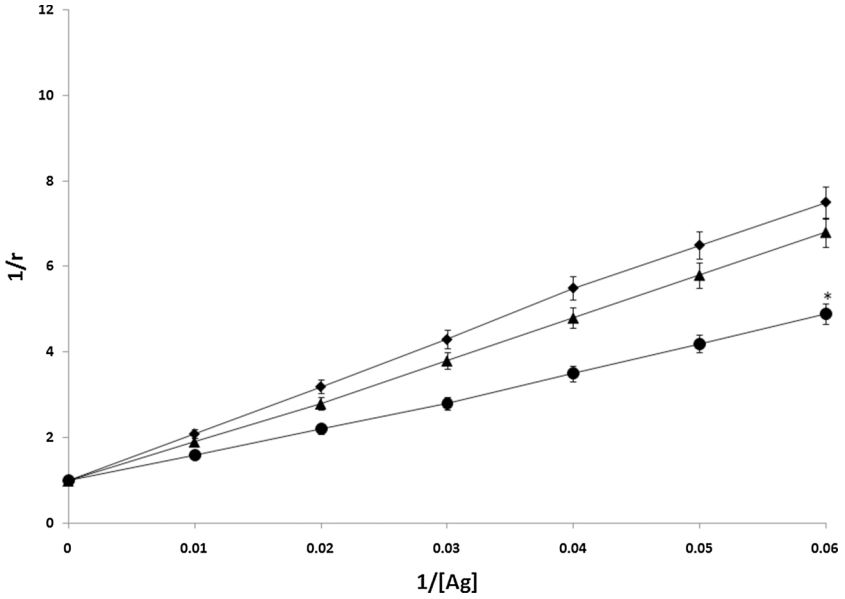

Fig. 5. Determination of apparent association constant by Langmuir plot. Antigens were 16 α-OHE_1-H_1 (-●-), H_1 (-▲-) and 16 α-OHE_1 (-♦-). **Note:* Significantly higher binding than 16 α-OHE_1 ($p < 0.001$).

showed cross-reactivity towards other antigen, and thus the anti-16 α-OHE_1-H_1 antibodies can be used as immunochemical probe for estimating 16 α-hydroxyestrone in the serum of SLE patients. Serum levels of 16 α-OHE_1 in SLE patients (n = 10) were measured with anti-16 α-OHE_1-H_1 antibodies and further confirmed with a commercially available kit (Human 16 α-hydroxyestrone ELISA Kit, Glory Science Co. Lt, USA). The mean level of 16 α-OHE_1 was estimated by anti-16 α-OHE_1-H_1 antibodies in SLE patients - the value was 37.9 ± 16.8 pg/ml, which is comparable to the value obtained by Human 16 α-hydroxyestrone ELISA Kit (i.e. 37.1 ± 13.1 pg/ml). In healthy controls (n = 20), the mean value was found to be 12.9 ± 5.3 pg/ml (Table 1). We have found significantly higher levels of 16 α-hydroxyestrone in the sera of SLE patients in comparison to the normal controls ($p < 0.05$).

4. Discussion

Estrogens and their metabolites seem to play an important role in various autoimmune diseases [4,7,15–17]. Abnormal estrogen metabolism was described in SLE resulting in an abnormally high concentration of 16 α-hydroxyestrone in these patients [4,5]. One of the important properties of these metabolites is that they can directly or indirectly affect various autoimmune processes in this disease. In SLE, the action of estradiol depends on their conversion to either 16 α-hydroxyestrone or 2-hydroxyestrone, which guides them to function as pro- or anti-inflammatory agents [4]. Most studies correlate the urinary concentration of estrogen metabolites with the progression of SLE. The urinary loss of 2 hydroxyestrone was found to be ten times greater in controls than SLE patients. If we compare with 16 α-hydroxyestrone, no such changes were observed in these patients - they were exactly the same as healthy controls. The ratio of 16 α-OHE_1/2-OHE_1 was 20 times higher in SLE patients than controls [4]. Thus, the conversion rate to 16 α-OHE_1 is greatly upregulated, which might contribute to the maintenance of proliferative state in SLE [4].

SLE is characterized by a large group of serious clinical manifestations which produces autoantibodies against cell components. The presence of these autoantibodies is specific for SLE [21] and serves as

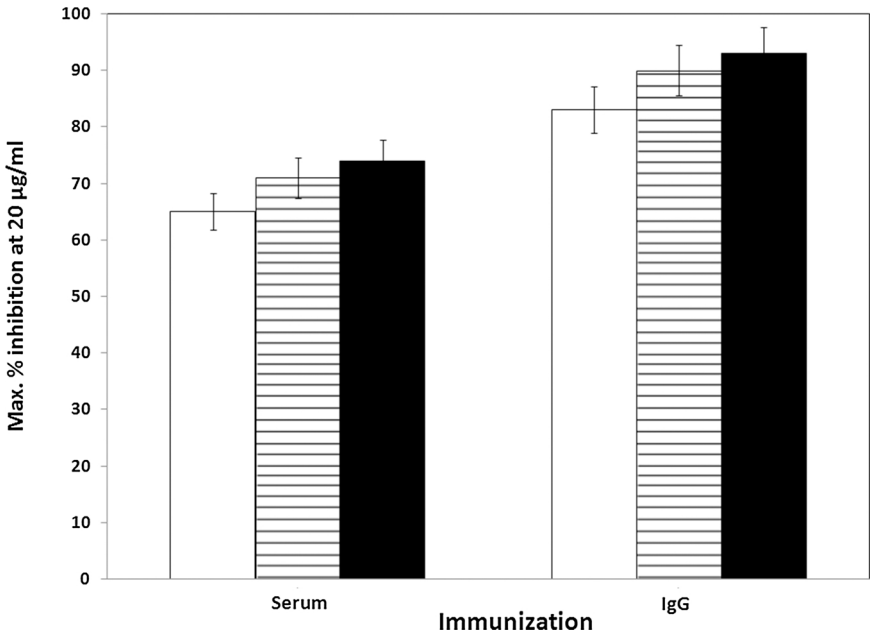

Fig. 6. Inhibition ELISA of anti-(16 α-OHE_1-H_1, H_1, 16 α-OHE_1) immune sera (■, ▒, □) and anti-(16 α-OHE_1-H_1, H_1, 16 α-OHE_1) IgG binding to 16 α-OHE_1-H_1 (■), H_1 (▒), 16 α-OHE_1 (□). Inhibition values for pre-immune sera and IgG with 16 α-OHE_1-H_1, H_1, 16 α-OHE_1, were negligible and are not shown. Microtire plates were coated with respective antigens (2.5 µg/ml).

an immunological biomarker for this disease. It is a female disease and estrogen concentration somehow influences the disease activity [22]. Anti-H_1 antibodies have been well described in SLE patients. The presence of these autoantibodies is not associated with any clinical parameters but was correlated with disease activity (87%) [23]. Measurement of these antibodies would help to present lab parameters for the progression and diagnosis of SLE [23]. Anti-H_1 antibodies can be used as a marker for SLE with high specificity in more than 90% of the patients [10]. In addition, these antibodies are also found in severe nephropathy in SLE patients [24]. Thus, this study determined whether the combining effects of 16 α-OHE_1 and H_1 might have any role in SLE or not.

To study this, the binding affinity of SLE autoantibodies with 16 α-OHE_1-H_1,H_1 and 16 α-OHE_1 was measured to determine whether this adduct (16 α-OHE_1-H_1) has any affinity with SLE autoantibodies or not. The specificity of autoantibodies in SLE from the sera of 60 patients and 30 control subjects was analyzed by direct binding, competition ELISA, and quantitative precipitin titration. The 16 α-OHE_1-H_1 adduct showed greater binding than either H_1 ($p < 0.05$) or 16 α-OHE_1 ($p < 0.001$). The combination of 16 α-OHE_1 and H_1 generates specific molecules/groups that might be better epitopes for SLE autoantibodies. This means that 16 α-OHE_1-H_1 can function as an effective inhibitor that showed substantial difference in the recognition of 16 α-OHE_1-H_1 over H_1 or 16 α-OHE_1. This study showed the same type of results that were already proven by earlier studies demonstrating high binding of catecholestrogen-modified antigen to SLE autoantibodies [3,17]. This preferable binding was not shown by either antigen (H_1 or 16 α-OHE_1). The specificities of SLE autoantibodies according to the medication showed that the patients who were not treated with any medication demonstrated the highest specificity of SLE autoantibodies. This might be because these patients already have high levels of autoantibodies - these were neither suppressed nor controlled by any drugs. Furthermore, drugs such as azathioprine, methotrexate, and cyclophasamide act as immunosuppressants and somehow control autoantibody production. Prednisolone also influences immune system by acting as an anti-inflammatory agent to control the release of antibodies.

Quantitative precipitin titration was performed for better understanding of the interaction of SLE autoantibodies with 16 α-OHE_1-H_1. The apparent association constant with an order of 10^{-7} M clearly indicated better recognition of 16 α-OHE_1-H_1 over H_1 or 16 α-OHE_1. The high recognition of 16 α-OHE_1-H_1 by SLE autoantibodies clearly showed the presence of this adduct or some of its epitopic regions involving in SLE pathogenesis. The epitopes generated by formation of this adduct are unique. They are easily recognized by SLE autoantibodies. Immuno-cross reactivity experiments clearly showed that anti-16 α-OHE_1-H_1 and anti-16 α-OHE_1 antibodies cross-react with each other's antigen. This gives us the opportunity to estimate 16 α-OHE_1 in the sera of SLE patients. These results are similar to earlier reports demonstrating cross-reacting autoantibodies in the sera of SLE patients [25]. Because of high binding shown by anti-16 α-OHE_1-H_1 antibodies towards 16 α-OHE_1, these antibodies can be used as a probe to estimate 16 α-OHE_1 in the serum of SLE patients. Significantly higher levels of 16 α-OHE_1 were observed in SLE patients versus controls that further demonstrate its important role in the pathogenesis of SLE. This study agrees with previous findings demonstrating a significantly higher concentration of 16 α-OHE_1 in the serum of SLE patients [26].

5. Conclusions

In conclusion, the present study clearly showed a possible antigenic role of 16 α-OHE_1-H_1 in the etiopathogenesis of SLE. Unique epitopes generated by this adduct might stimulate a specific response to produce SLE autoantibodies. These effectively recognized this antigen. However, the possible immune modulation role of estrogen metabolites cannot be ruled out. It might be possible that the estrogen metabolites generated during oxidation might produce SLE autoantibodies with high binding to 16 α-OHE_1-H_1 in the sera of different SLE patients.

Conflict of interests

The authors declare no conflict of interests

Financial disclosure

This work was supported by the Grant (No.: R.G.P.1/53/59) through research groups program from the Deanship of Scientific Research, King Khalid University, Abha, Kingdom of Saudi Arabia.

The author contribution

Study Design: Ali Khan Wahid.
Data Collection: Ali Khan Wahid.
Statistical Analysis: Ali Khan Wahid.
Data Interpretation: Ali Khan Wahid.
Manuscript Preparation: Ali Khan Wahid.
Literature Search: Ali Khan Wahid.
Funds Collection: Ali Khan Wahid.

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