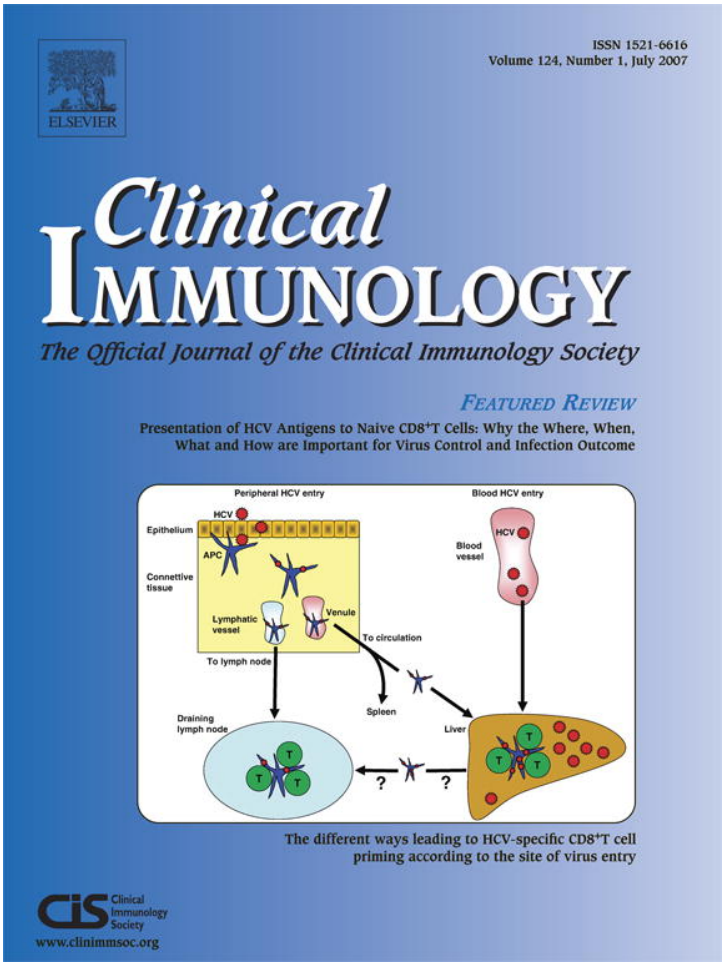

Clinical Immunology (2007) **124**, 83–89

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yclim

Immunogenicity of singlet oxygen modified human DNA: Implications for anti-DNA antibodies in systemic lupus erythematosus

Abdurahman Saud Al Arfaj[a,*], **Abdul Rauf Chowdhary**[b], **Najma Khalil**[b], **Rashid Ali**[c,1]

[a] *Division of Rheumatology, Department of Medicine, College of Medicine, King Saud University, Riyadh, Saudi Arabia*
[b] *Research Center, College of Medicine, King Saud University, Riyadh, Saudi Arabia*
[c] *Department of Biochemistry, College of Science, King Saud University, Riyadh, Saudi Arabia*

Received 21 November 2006; accepted with revision 29 March 2007
Available online 30 May 2007

KEYWORDS
Systemic lupus erythematosus (SLE);
Anti-dsDNA autoantibodies;
Reactive oxygen species (ROS);
Singlet oxygen ($^1O_2$)

Abstract Reactive oxygen species (ROS)-modified DNA has been shown to be a better and more discriminating immunogen than native DNA (nDNA) for the production of anti-DNA autoantibodies in SLE (systemic lupus erythematosus). Among ROS, the role of hydroxyl radical (·OH) in the induction of damage and modification of nDNA has been extensively studied while such documentation implicating singlet oxygen ($^1O_2$) in inducing immunogenicity in nDNA leading to the production of anti-double-stranded (ds) DNA autoantibodies in SLE is not yet available. This prospective study was undertaken to evaluate the immunogenicity of healthy human dsDNA modified with $^1O_2$ generated by methylene blue plus radiant light. Female rabbits were immunized with $^1O_2$-modified human dsDNA to raise anti-dsDNA antibodies. $^1O_2$-modified anti-dsDNA rabbit immune sera and the $^1O_2$-modified anti-dsDNA rabbit purified immunoglobulin G (IgG) were tested against a variety of dsDNA antigenic substrates through direct enzyme-linked immunosorbent assay (ELISA). The immunogenicity of $^1O_2$-modified human dsDNA was further evaluated by studying its immunoreactivity with SLE patients' sera and SLE patients' purified anti-dsDNA IgG. As compared to healthy human sera, $^1O_2$-modified anti-dsDNA rabbit immune sera as well as the $^1O_2$-modified anti-dsDNA rabbit purified IgG demonstrated a strong affinity towards $^1O_2$-modified human dsDNA. $^1O_2$-modified human dsDNA proved to be potentially more immunogenic against SLE patients' whole sera and SLE patients' purified IgG as compared to healthy human sera. Our findings suggest that $^1O_2$ may also be inducing immunogenicity in native dsDNA resulting in the production of anti-dsDNA autoantibodies as seen in SLE patients.

* Corresponding author. Internal medicine and Rheumatology, College of Medicine, King Saud University, P.O. Box 34471, Riyadh 11468, Saudi Arabia. Fax: +966 1 4671563.
E-mail address: asarfaj@ksu.edu.sa (A.S. Al Arfaj).
[1] Present address: Department of Biochemistry, Faculty of Medicine, J.N. Medical College, A.M.U., Aligarh, India.

1521-6616/$ – see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.clim.2007.03.548

Introduction

Systemic lupus erythematosus (SLE), a prototypic autoimmune disease, is characterized by the presence of autoantibodies against double-stranded (ds) deoxyribonucleic acid (DNA) [1,2]. Despite several studies in animal (mice) models, the triggering antigen or the mechanism that causes the production of anti-dsDNA autoantibodies in SLE patients is not fully understood [1,3–5]. It has however been well established that native DNA per se is not immunogenic [6,7]. Reactive oxygen species (ROS) have been implicated in causing immunogenic modifications in the native dsDNA (nDNA). They are known to cause oxidative damage to DNA leading to various autoimmune and degenerative diseases [8–10]. In vivo ROS are generated by oxidant enzymes, phagocytic cells, ionizing radiations, etc. [11]. It has been proposed that ROS generated from activated phagocytes penetrate cellular membranes and react with nuclear DNA, resulting in subsequent release of anti-DNA autoantibodies which contribute to development of SLE [8]. Hydroxyl radical (·OH), a very highly reactive oxygen species, has been shown to impart antigenicity to DNA in SLE [10–14]. ·OH produces strand breaks, base modifications and conformational changes in DNA. The principal oxidative products of ·OH include thymine glycols, cytosine glycols, 8-hydroxydeoxyguanosine (8-OHdG), 8-hydroxyadenine, etc. [11].

Singlet oxygen ($^1O_2$) has been implicated in DNA damage leading to mutagenesis, carcinogenesis and aging [15,16]. While ·OH generates a multiplicity of products from all four DNA bases, $^1O_2$ selectively attacks guanine base of DNA resulting in formation of 8-hydroxydeoxyguanosine (8-OHdG), a biomarker of oxidative damage [16,17]. Reports indicate the presence of increased levels of 8-OHdG in DNA of patients with SLE and rheumatoid arthritis [8,18]. While there are many reports on the role of ·OH in the etiology of SLE, our literature survey revealed that so far there are no reports on the possible role of $^1O_2$ in mediating production of anti-dsDNA autoantibodies and SLE pathogenesis. Hence, the present prospective study was undertaken to evaluate the immunogenicity of healthy human DNA modified with $^1O_2$ in order to gain insight into mechanism of production of anti-dsDNA autoantibodies in SLE.

Materials and methods

After the collection of demographic and clinical data, blood samples were obtained from SLE patients attending the Rheumatology clinics at King Khalid University Hospital (KKUH), Riyadh. The diagnosis of SLE was based upon the American College of Rheumatology (ACR) 1982 revised criteria [19]. Antibodies to dsDNA in SLE patients were detected by enzyme-linked immunosorbent assay (ELISA) (QUANTA Lite™ dsDNA ELISA test). Normal blood samples were obtained from healthy subjects recruited through blood bank at KKUH. SLE patients were matched for age and sex to the healthy subjects. Full informed consent was obtained prior to the blood extraction from SLE patients and healthy subjects.

Methods consisted of the following steps:

(i) *Blood collection*: 7–10 ml of whole blood was collected from SLE patients and healthy subjects in 10 cc plain silicone coated Vacutainers® and Potassium EDTA blood collection tubes for the preparation of serum and plasma respectively. The buffy coat, plasma and serum obtained from the blood samples were stored at −80 °C until further investigations. Serum samples from SLE patients were used for anti-dsDNA antibodies immunoassay as well as for the isolation and purification of anti-dsDNA immunoglobulin G (IgG) antibodies. nDNA and SLE patients' cellular fraction dsDNA were prepared from the buffy coats of healthy subjects and SLE patients respectively.

(ii) *Extraction of dsDNA from buffy coats*: QIAamp® DNA Blood Mini Kit (QIAGEN, Inc., 28159 Avenue, Stanford, Valencia, CA 91355, USA) was used. Briefly, 20 μl of QIAGEN protease or Proteinase K was dispensed into the bottom of 1.5 ml microcentrifuge tube, followed by 200 μl of buffy coat and 200 μl of lysis buffer. After centrifugation, the contents were transferred to a QIAamp spin column with 2 ml collection tube. The spin column was centrifuged at 6000×*g* (8000 rpm) for 2 min and then washed twice with washing buffers (AW1 and AW2). The dsDNA adsorbed onto the spin column was finally eluted with elution buffer. For the extraction of SLE patients' plasma fraction dsDNA also, QIAamp® DNA Blood Mini Kit was used as per the manufacturers instructions.

(iii) *Purification of dsDNA*: Extracted dsDNA was further purified through ethanol precipitation. To the dsDNA taken in a 10-ml centrifuge tube, sodium acetate was added to adjust its salt concentration (pH 5.2, 3 M), and then 2.5 volumes of ice-cold absolute ethanol was added and placed at −30 °C for 45 min for cryo-precipitation of the DNA. The tube was then centrifuged and the precipitated DNA was washed twice with 70 % ice-cold ethanol. The pellet was air dried, resuspended in TE buffer (10 mM Tris–HCl, 10 mM EDTA, pH 7.5–8.0), quantified using RNA/DNA calculator GeneQuant II (Pharmacia Biotech, Cambridge, UK) and then stored at −80 °C until the digestion.

(iv) *Fragmentation of dsDNA*: A marked increase in the binding of DNA fragments of size ranging from 50 to 800 bp with monoclonal antibodies has been observed in autoimmune diseases [20]. Hence, DNA was fragmented to obtain 70–800 bp fragments as per the method described by Ali et al. [20]. Briefly, purified DNA (2 mg/ml in 100 mM NaCl, 6 mM TRIS, 2 mM $CaCl_2$, pH 8.0) was digested with micrococcal nuclease (40 U/mg DNA) (Worthington Biochemical Corp., Lakewood, NJ 08701, USA) at 37 °C in a shaking water-bath for 2.5, 3.5 and 6 min to obtain DNA fragments. Digested fragments were extracted twice with chloroform-isoamylalcohol (24:1), precipitated with ethanol and air-dried. Finally, the DNA fragments were resuspended in Tris-buffered saline (TBS), pH 8.0, and stored at −30 °C until the separation and characterization of the fragments by gel filtration.

(v) *Separation and characterization of DNA fragments*: (a) *Separation of DNA fragments on the basis of size*: Fragments of digested DNA were separated on the basis of size by gel filtration on a Sepharose 4B column (Amersham Biosciences Int., HVD Biotech, Gmbh Glyfada, Athens). (b) *Determination of size of DNA fragments*: The size of separated dsDNA fragments was determined by polyacrylamide gel electrophoresis using GeneGel Clean 15/24 (Pharmacia Biotech AB, Uppsala, Sweden). The gel was rehydrated and run according to the manufacturer's instructions. (c) *Detection of DNA fragments*: Separated dsDNA fragments

and the DNA ladder were visualized using ultra-sensitive silver staining method (PlusOne DNA Silver Staining Kit; Pharmacia Biotech AB, Uppsala, Sweden).

(vi) *Modification of DNA by* $^1O_2$: Purified and fragmented healthy human dsDNA (350–450 bp) recovered from Sepharose 4B gel filtration was modified by exposure to methylene blue (MB) plus light according to the procedure described by Floyd et al. [16]. Under our experimental conditions, the predominant free radical species that imparted the damage to native fragmented human dsDNA was $^1O_2$.

(vii) *Characterization of* $^1O_2$*-induced modifications in the dsDNA*: The $^1O_2$-induced changes in the secondary structure of the dsDNA were evaluated by thermal denaturation according to the procedure described by Arif and Ali [21].

(viii) *Immunization of rabbits with* $^1O_2$*-modified dsDNA*: Five female rabbits were immunized with $^1O_2$-modified dsDNA according to the protocol described by Ashok and Ali [22]. Briefly, after obtaining the pre-immune sera from the rabbits, 100 μg of $^1O_2$-modified dsDNA was complexed with an equal amount of (W/W) methylated bovine serum albumin (BSA) (Sigma, USA) and emulsified with 1 ml of Freunds' complete adjuvant (Sigma-Aldrich Inc., Saint Louis, Missouri, USA) and injected subcutaneously at multiple sites in rabbits. Subsequent injections were given in Freunds' incomplete adjuvant. Each animal received ≈550–600 μg of dsDNA. Animals were bled by cardiac puncture to obtain the immune sera. Serum was decomplemented by heating at 56 °C for 30 min and stored in small aliquots at −40 °C with 0.02 % sodium azide till further use. Two female rabbits were processed as controls. These animals were immunized only with methylated BSA and Freunds' complete/incomplete adjuvant.

(ix) *Isolation and purification of* $^1O_2$*-modified anti-DNA IgG from the immunized rabbit sera*: The isolation of IgG from pooled (n=5) $^1O_2$-modified rabbit immune serum sample was performed by ammonium sulphate precipitation method. From the gamma-globulin fraction so obtained, IgG was isolated by DEAE Sepharose fast flow chromatography (Amersham Bioscience, UK), isolated IgG was then dialyzed in TEGD buffer (50 mM Tris, 1 mM EDTA, 5% glycerol, 0.1 mM dithiothreitol and 0.1 M NaCl, pH 7.9) for 24 h at 4 °C. Dialyzed IgG was affinity purified by using $^1O_2$-modified DNA-poly-lysyl-Sepharose 4B as matrix [23]. Finally, the resulting IgG was dialyzed against PBS (0.02 M, pH 7.2) for overnight at 4 °C and the homogeneity of purified IgG was verified by 7.5% SDS–polyacrylamide gel electrophoresis.

(x) *Isolation and purification of anti-dsDNA IgG from SLE patients sera*: High titer anti-dsDNA antibody serum samples (>100 IU/ml) from SLE patients (n=43) were pooled to give an approximate quantity of 20–25 ml. The method described above for isolation of rabbit IgG was employed for isolation of SLE IgG by DEAE Sepharose fast flow chromatography and the resulting IgG was affinity purified on Protein A-Sepharose CL-4B column (Amersham Biosciences, Uppsala, Sweden).

To evaluate the immunogenicity of $^1O_2$-modified human dsDNA and its role in pathogenesis of SLE, five different antigenic substrates; rabbit native dsDNA, healthy human dsDNA, $^1O_2$-modified human dsDNA, calf thymus dsDNA and SLE patients' plasma fraction dsDNA were tested against $^1O_2$-modified rabbit anti-dsDNA antibodies (immune rabbit sera) and immune rabbit purified IgGs through direct enzyme-linked immunosorbent assay (ELISA).

The immunogenicity of $^1O_2$-modified human dsDNA was further evaluated by studying and quantifying its immunoreactivity with SLE patients' whole sera and SLE patients' purified IgG. A total of five dsDNA antigens, healthy human dsDNA, $^1O_2$-modified human dsDNA, SLE patients' cellular fraction dsDNA, SLE patients' plasma fraction dsDNA and calf thymus dsDNA, were challenged with healthy human sera, SLE patients' sera and SLE patients' purified IgG.

(xi) *ELISA*: ELISA was performed on flat bottom 96 well, polystyrene microplates (Greiner Labortechnik™, GmbH, D-7440 Nurtingen, Germany) using Anthos 2001 automatic Microplate Washer and Reader equipped with software-support (Labtec Instruments, GmbH, Lagerhausstr, Wals, Austria). The microtiter wells were coated with 100 μl of the $^1O_2$-modified human dsDNA (50 μg/ml in TBS; pH 7.4) for 2 h at room temperature and overnight at 4 °C. Unbound antigen was washed thrice with Tris-buffered saline (TBST-20) buffer (20 mM Tris, 2.68 mM KCl, 150 mM NaCl, pH 7.4, containing 0.05% Tween-20). Unoccupied sites were then blocked by incubating with 150 μl of 1.5% BSA in TBS (10 mM Tris, 1.34 mM KCl, 150 mM NaCl, pH 7.4) for 3 min at 37 °C and then 4–6 h at room temperature. Plates were then washed 3 times with TBS, pH 7.4, and pat dried. Pooled rabbit antiserum (n=5) containing $^1O_2$-modified anti-dsDNA antibodies was serially diluted (1/10, 1/100 and 1/1000 in TBS-T20) and added in 100 μl quantities to each and adsorbed for 2 h at room temperature and then overnight at 4 °C. Following washing 3 times with TBS-T20 and pat-drying, the secondary antibody, an alkaline phosphatase-labeled goat anti-rabbit immunoglobulin (IgG, IgM, IgA, DAKO) at a dilution of 1/5000 in TBS-T20, was applied in 100 μl quantities and incubated for 2 h at room temperature. The plate was washed 3 times with TBS-T20 and then 100 μl of the substrate solution, p-nitrophenyl phosphate (p-NP), was added to each well and incubated for 15 min at room temperature. The reaction was stopped by adding 25 μl of 2 M H_2SO_4 in each well. The resulting absorbance was read at 492 nm using an Anthos 2001 plate reader. Each sample was tested in duplicate and the mean of two observations was used in the analysis.

Statistical analysis

The data were analyzed using SPSS version 12.0. Results were expressed as mean ± SD (standard deviation). The level of difference between the means was assessed by using two-tailed t-test and p value <0.05 was considered significant.

Results

The study group comprised of 34 healthy subjects and 43 SLE patients. The group of healthy subjects consisted of 29 (85.3%) females and 5 (14.7%) males with mean age of 31.09 ± 11.78 (range 19–47) years. Of the 43 SLE patients 41 (95.3%) were females and 2 (4.7 %) were males with mean age of 29.98 ± 10.58 (range 14–60) years. SLE patients were matched for age and sex to the healthy subjects. The difference between the healthy subjects and SLE patients with respect to the mean age and proportion of males and females was not statistically significant (p>0.05). The mean

anti-dsDNA antibody titer of healthy subjects was 8.95 ± 2.36 (4.0–15.0) IU/ml and that of SLE patients was 805 ± 582.0 (200.0–2311.0) IU/ml.

The outcome of coating the microplate with 5 types of dsDNA antigens and then challenging with pre-immune (negative) control rabbit sera ($n=2$), immunized rabbit whole sera ($n=5$), purified anti-$^1O_2$-modified dsDNA IgG in rabbit antisera ($n=5$) and healthy human sera ($n=34$) through direct ELISA is presented in Fig. 1.

Overall mean absorbance

At 492 nm, the overall mean absorbance of ELISA binding of pre-immune (control) rabbit sera against various types of dsDNA antigens was 0.077 ± 0.0099 (negative cut-off range 0.059–0.091), indicating almost negative binding between control rabbit sera and the tested dsDNA antigens.

Control (pre-immune) rabbit whole sera vs. dsDNA antigens

$^1O_2$-modified human dsDNA as well as all other dsDNA antigens did not show any type of binding with control rabbit sera. Pre-immune rabbit sera served as a negative control against immunized rabbit sera.

Immune ($^1O_2$-modified) rabbit whole sera vs. dsDNA antigens

The binding pattern of immune rabbit sera with different dsDNA antigens showed the maximum amount of binding with $^1O_2$-modified human dsDNA (0.683 ± 0.0122) followed by SLE patients' plasma fraction dsDNA (0.386 ± 0.069). There was very little binding of immune rabbit sera with rabbit nDNA and healthy human dsDNA. However, the reaction between CTD and immune rabbit sera did show some binding. When SLE patient's cellular dsDNA was coated onto the microtiter plates and challenged with immune rabbit sera, a weak binding was observed (0.112 ± 0.0098) as compared to the SLE plasma fraction dsDNA (0.386 ± 0.069) ($p<0.0001$).

Purified immune ($^1O_2$-modified) rabbit IgG vs. dsDNA antigens

The maximum binding of purified anti-$^1O_2$-modified rabbit IgG was recorded with $^1O_2$-modified human dsDNA (0.901 ± 0.061) which was significantly higher than binding of immune rabbit sera with $^1O_2$-modified human dsDNA (0.683 ± 0.0122) ($p=0.0001$). The binding of SLE patients' plasma fraction dsDNA showed a significant enhancement from 0.386 ± 0.069 (with rabbit immune serum) to 0.539 ± 0.067 with purified immune rabbit IgG ($p=0.0074$). The reactivity between CTD and the purified immune rabbit IgG increased insignificantly from 0.169 ± 0.013 with the immune rabbit sera to 0.203 ± 0.0065 with the purified immune rabbit IgG ($p=0.575$).

Healthy human whole sera vs. dsDNA antigens

A slight insignificant antigen–antibody reaction of healthy human whole sera was observed with SLE patients' plasma fraction dsDNA (0.127 ± 0.032) and healthy human dsDNA (0.100 ± 0.0183). This reaction was most likely due to the infiltration of some of the plasma (extracellular) fraction of dsDNA into cellular (chromosomal) dsDNA during the isolation and purification procedures.

In brief, Fig. 1 shows that, as compared to pre-immune (control) rabbit and healthy human sera, anti-$^1O_2$-modified dsDNA immune rabbit sera as well as the $^1O_2$-modified immune rabbit purified IgG demonstrated a strong binding towards $^1O_2$-modified human dsDNA and SLE patients' plasma

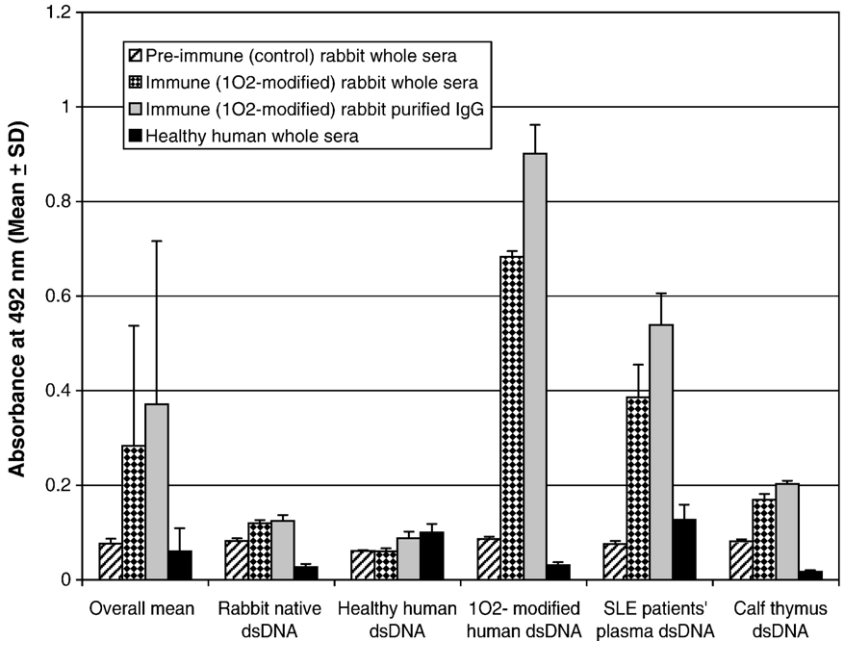

Figure 1 Direct ELISA binding of control rabbit whole sera, $^1O_2$-modified anti-dsDNA antibodies in rabbit whole sera, immune($^1O_2$-modified) rabbit purified IgG and healthy human whole sera to $^1O_2$-modified human dsDNA and other antigenic dsDNA.

fraction dsDNA ($p<0.001$ in all these comparisons). Rabbit native dsDNA and healthy human dsDNA did not show any appreciable immunobinding while minor reactivity of CTD was observed with rabbit immune sera as well as immune rabbit purified IgG. This cross reactivity of CTD needs further evaluation.

The results of direct ELISA binding of $^1O_2$-modified human dsDNA and the other four dsDNA antigenic substrates with healthy human whole sera ($n=34$), SLE patients' whole sera ($n=43$) and SLE patients' purified anti-dsDNA IgG ($n=43$) are depicted in Fig. 2.

Overall mean absorbance

The overall mean absorbance of direct ELISA binding of healthy human sera to $^1O_2$-modified human dsDNA and 4 other dsDNA antigens was 0.098 ± 0.0271, showing a negative antigen–antibody reaction (negative cut-off range 0.029–0.260).

Direct ELISA binding of healthy human sera with dsDNA antigens

The binding of healthy human sera with all the dsDNA antigens was in negative range. However, it was slightly higher with SLE patients' plasma fraction dsDNA than healthy human dsDNA and CTD.

Binding of SLE patients' sera with dsDNA antigens

SLE patients' sera reacted strongly against $^1O_2$-modified human dsDNA showing the maximum binding absorption of 0.536 ± 0.149, followed by SLE patients' plasma fraction dsDNA (0.413 ± 0.198) ($p=0.0015$). Among other dsDNA antigens, SLE patients' cellular fraction dsDNA had slightly higher binding (although within the negative range), than the healthy human dsDNA and CTD. As compared to healthy human serum (0.061 ± 0.0325), $^1O_2$-modified human dsDNA proved to be potentially more immunogenic against SLE patients' whole serum (0.536 ± 0.149) ($p<0.0001$).

Binding of SLE patients' purified IgG with dsDNA antigens

The binding between $^1O_2$-modified human dsDNA and SLE patients' purified IgG increased significantly to 0.795 ± 0.163 as compared to its binding with SLE patients' sera (0.536 ± 0.149) ($p<0.0001$). Enhancement of the same magnitude was also seen in the binding between SLE patients' plasma fraction dsDNA and SLE patients' purified IgG as compared to its binding with SLE patients' sera (0.601 ± 0.114 vs. 0.413 ± 0.198; $p<0.0001$). Interestingly, the immunobinding of healthy human dsDNA and CTD with SLE patients' purified IgG decreased significantly compared to binding with SLE patients' sera ($p<0.0001$ in both cases) indicating that the binding was nonspecific. A slight, insignificant increase in the ELISA binding between SLE patients' cellular fraction dsDNA and SLE patients' purified IgG compared to its binding with SLE patients' sera was also observed ($p=0.166$).

In brief, the results depicted in Fig. 2 show that significantly higher ELISA binding of $^1O_2$-modified human dsDNA and SLE patients' plasma fraction dsDNA was observed with SLE patients' whole sera and as well as with SLE patients' purified IgG when compared to binding of these dsDNA antigens with healthy human whole sera ($p<0.0001$ in all these comparisons). SLE patients' cellular fraction dsDNA did not

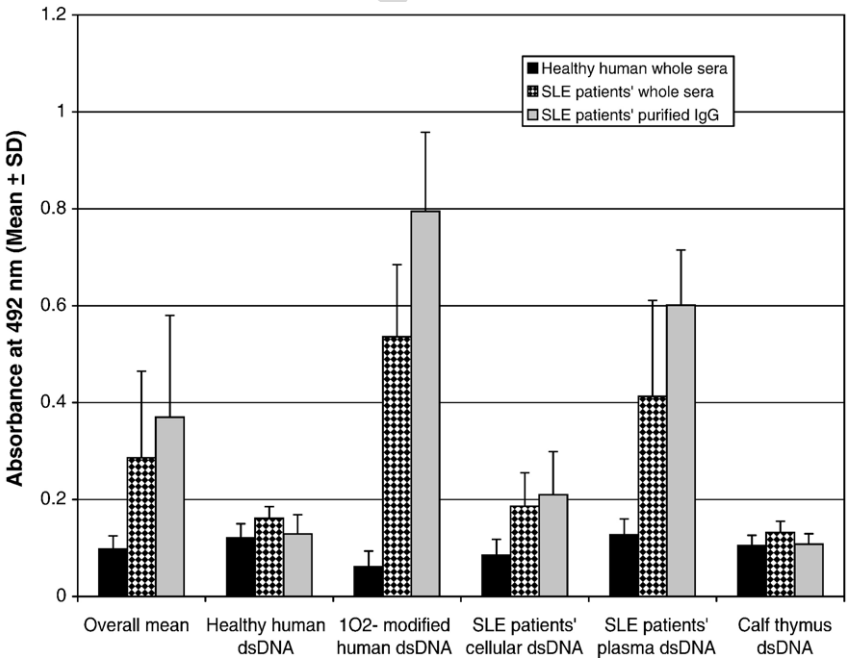

Figure 2 Direct ELISA binding of healthy human whole sera, SLE patients' whole sera, SLE patients' purified IgG to $^1O_2$-modified human dsDNA and other dsDNA antigens.

show significant affinity or immunogenicity towards SLE sera or SLE purified IgG.

Discussion

The possible role of reactive oxygen species (ROS) in eliciting immunogenic modifications in the native dsDNA molecule has been widely investigated. Studies have demonstrated ROS–DNA and ROS-modified bases and polynucleotides to be a better and more discriminating immunogen than nDNA for the production of human anti-dsDNA autoantibodies [9,11,13,24,25]. Among ROS, the possible role of $^1O_2$ in eliciting immunogenicity in the nDNA and pathogenesis of SLE has not yet been investigated. Earlier studies have shown that $^1O_2$ generated by photo- or chemi-excitation is capable of inducing genotoxic, carcinogenic and immunogenic effects like ·OH and other oxygen free radicals [26–28]. In vivo, $^1O_2$ is generated by photosensitization mechanisms induced by ultraviolet (UV) radiations. UVA exposure has been reported to cause skin aging mainly by $^1O_2$ dependent pathways [29]. In view of this, $^1O_2$ could possibly be implicated in causing UV-induced exacerbations in SLE.

In our study, the strong ELISA binding of $^1O_2$-modified rabbit immune sera as well as of $^1O_2$-modified immune rabbit purified IgG to the $^1O_2$-modified human dsDNA suggests that, in addition to ·OH, $^1O_2$ might also be an etiological agent causing in vivo alterations in the native dsDNA in SLE patients which results in the production of anti-dsDNA autoantibodies. This was further strengthened from the strong ELISA binding of SLE patients' sera and SLE patients' purified IgG to the $^1O_2$-modified human dsDNA.

In the present study, $^1O_2$ was generated by irradiation with visible light in the presence of MB. The immune response elicited by $^1O_2$-modified human dsDNA in the production of anti-dsDNA autoantibodies in our study supports the findings that $^1O_2$ so generated is capable of modifying nDNA to immunogenic form. This is in agreement with the study of Suzuki et al. [28] who showed that $^1O_2$ generated by MB photo oxidation is capable of inducing immunogenic effects.

We found significant immunobinding ($p<0.001$) between SLE patients' plasma fraction dsDNA and immunized rabbit sera and immunized rabbit purified IgG. This was most probably due to the presence of circulating dsDNA as free fragments or as antibody-dsDNA immune complexes which play an important role in SLE pathogenesis [8,30,31]. Lunec et al. [8] postulated that reactive oxygen species or free oxygen radicals may induce pathology in SLE patients by maintaining the presence of an antigenic form of DNA in the circulation of SLE patients. In a recent report, Pisetsky [3] has shown that single or double-stranded DNA in the peripheral blood of SLE patients can arise from both apoptotic and/or necrotic cells. This DNA circulates in the blood in the form of autoantibody-DNA immune complexes which can play a role in the pathogenesis of lupus. The generation of circulating DNA may be targeted for therapy to reduce pathogenic immune complexes [3].

As compared to SLE patients' plasma dsDNA, SLE patients' cellular DNA showed a very weak binding with $^1O_2$-modified rabbit immune sera, as well as with SLE sera, suggesting the extra cellular presence of altered or immunogenic form of dsDNA in SLE patients. This poor binding of the native or the cellular fraction dsDNA supports the findings that the native human dsDNA is not antigenic [6,7]. The role of extra cellular DNA in the pathogenesis of SLE is well established; however, there has been no report yet suggesting the presence of altered or modified dsDNA in the cellular limb. Moreover, it is also evident from this observation that either the degraded DNA resulting from cell apoptosis, or in the form of immunoreactive circulating antigen–antibody complex form, provided the antigenic epitopes for the positive reaction between SLE patients sera and the plasma fraction DNA.

SLE patients' purified IgG showed lower reactivity toward healthy human dsDNA and CTD as compared to SLE patients' sera's reactivity with these dsDNA ($p<0.001$) (Fig. 2). The reactivity decreased with the isolation of purified IgG. This kind of variation in binding could be due to the presence of anti-dsDNA autoantibodies other than IgG in SLE patients' sera which were removed during purification process to obtain SLE patients' IgG. This finding is in agreement with previous studies which reported that anti-dsDNA autoantibodies in SLE sera are of heterogeneous nature [32]. The immunogenicity of $^1O_2$-modified human dsDNA enhanced significantly when immune rabbit serum was replaced by immune rabbit purified IgG suggesting that dsDNA autoantibodies elicited by $^1O_2$-modified human dsDNA are of IgG class.

In conclusion, our results have shown that exposure of purified human dsDNA to the reactive $^1O_2$ produced such conformational changes in the primary structure of the dsDNA that rendered it highly immunogenic when injected into rabbits. The strong ELISA binding of $^1O_2$-modified rabbit immune sera, $^1O_2$-modified immune rabbit purified IgG, SLE patients' sera, SLE patients' purified IgG with the $^1O_2$-modified human dsDNA when compared to their binding with healthy human dsDNA suggests that, in addition to ·OH, $^1O_2$ might also be an etiological agent causing *in vivo* alterations in the native dsDNA in SLE patients. $^1O_2$ might also be one of those highly ROS which initiate or precipitate the phenomenon causing the oxidative damage to the nDNA leading to the production of anti-dsDNA autoantibodies in SLE. Ours is a pilot study and further studies are needed to evaluate the role of $^1O_2$ in SLE pathogenesis.

References

[1] A. Rahman, Autoantibodies, lupus and the science of sabotage, Rheumatology 43 (2004) 1326–1336.
[2] I.E. Hoffman, I. Peene, L. Meheus, T.W. Huizinga, et al., Specific antinuclear antibodies are associated with clinical features in systemic lupus erythematosus, Ann. Rheum. Dis. 63 (2004) 1155–1158.
[3] D.S. Pisetsky, The immune response to cell death in SLE, Autoimmun. Rev. 3 (2004) 500–504.
[4] H. Li, Y.Y. Zhang, Y.N. Sun, X.Y. Huang, Y.F. Jia, D. Li, Induction of systemic lupus erythematosus syndrome in BALB/C mice by immunization with active chromatin, Acta Pharmacol. Sin. 25 (2004) 807–811.
[5] O.P. Rekvig, M. Kalaaji, H. Nossent, Anti-DNA antibody subpopulations and lupus nephritis, Autoimmun. Rev. 3 (2004) 1–6.
[6] D.S. Pisetsky, J.P. Grudier, G.S. Gilkeson, A role for immunogenic DNA in the pathogenesis of systemic lupus erythematosus, Arthritis Rheum. 33 (1990) 153–159.
[7] T.N. Marion, N.R. Krishnan, D.D. Desai, N.T. Jou, D.M. Tillman,

Monoclonal anti-dsDNA antibodies: structure, specificity and biology, Methods 11 (1997) 3–9.
[8] J. Lunec, K. Herbert, S. Blount, H.R. Griffiths, P. Emery, 8-Hydroxydeoxyguanosine. A marker of oxidative DNA damage in systemic lupus erythematosus, FEBS Lett. 348 (1994) 131–138.
[9] M.S. Cooke, N. Mistry, C. Wood, K.E. Herbert, J. Lunec, Immunogenicity of DNA damaged by reactive oxygen species—implications for anti-DNA antibodies in Lupus, Free Radic. Biol. Med. 22 (1997) 151–159.
[10] B.T. Ashok, R. Ali, Binding of human anti DNA autoantibodies to reactive oxygen modified DNA and probing oxidative DNA damage in cancer using monoclonal antibody, Int. J. Cancer 78 (1998) 40–409.
[11] H. Hasan, A. Ali, R. Ali, Oxygen free radicals and systemic autoimmunity, Clin. Exp. Immunol. 131 (2003) 398–404.
[12] J. Ara, R. Ali, Reactive oxygen species modified DNA fragments of varying size are preferred antigen for human anti-DNA autoantibodies, Immunol. Lett. 34 (1992) 195–200.
[13] D.K. Garg, R. Ali, Reactive oxygen species modified polyguanylic acid: immunogenicity and implications for systemic autoimmunity, J. Autoimmun. 11 (1998) 371–378.
[14] D.K. Gark, Moinuddin, R. Ali, Hydroxyl radical modification of polyguanylic acid: role of modified guanine in circulating SLE anti-DNA autoantibodies, Immunol. Invest. 32 (2003) 187–199.
[15] A. KortenKamp, M. Casadevall, S.P. Faux, A. Jenner, et al., A role for molecular oxygen in the formation of DNA damage during the reduction of the carcinogen chromium (VI) by glutathione, Arch. Biochem. Biophys. 329 (1996) 199–207.
[16] R.A. Floyd, M.S. West, K.L. Eneff, J.E. Schneider, Methylene blue plus light mediates 8-Hydroxyguanine formation in DNA, Arch. Biochem. Biophys. 273 (1989) 106–111.
[17] C.G. Fraga, M.K. Shigenaga, J.W. Park, et al., Oxidative damage to DNA during aging: 8-hydroxy-2-deoxyguanosine in rat organ DNA and urine, Proc. Natl. Acad. U. S. A. 87 (1990) 4533–4537.
[18] P.V. Passen, J. Damoiseaux, J.W.C. Tervaert, Laboratory assessment in musculoskeletal disorders, Best Pract. Res., Clin. Rheumatol. 17 (2003) 475–494.
[19] E.M. Tan, A.S. Cohen, J.F. Fries, A.T. Masi, D.J. McShane, et al., The 1982 revised criteria for the classification of systemic lupus erythematosus, Arthritis Rheum. 25 (1982) 1271–1277.
[20] R. Ali, H. DerSimonian, B.D. Stollar, Binding of monoclonal anti-native DNA autoantibodies to DNA of varying size and conformation, Mol. Immunol. 22 (1985) 1415–1422.
[21] Z. Arif, R. Ali, Antigenicity of Poly(dA-dT). Poly(dA-dT) photocrosslinked with 8-methoxypsoralen, Arch. Biochem. Biophys. 329 (1996) 191–198.
[22] B.T. Ashok, R. Ali, Antigen binding characteristics of experimentally-induced antibodies against hydroxyl radical modified native DNA, Autoimmunity 29 (1999) 11–19.
[23] K. Alam, A. Ali, R. Ali, The effect of hydroxyl radical on the antigenicity of native DNA, FEBS Lett. 319 (1993) 66–70.
[24] S. Blount, H.R. Griffiths, J. Lunec, Reactive oxygen species modify human DNA. Eliciting a more discriminating antigen for the diagnosis of systemic lupus erythematosus, Clin. Exp. Immunol. 81 (1990) 384–389.
[25] S. Tasneem, R. Ali, Binding of SLE autoantibodies to native poly (I),ROS-poly(I)and Native DNA: a comparative study, J. Autoimmun. 17 (2000) 199–205.
[26] H. Sies, C.F. Menck, Singlet oxygen induced DNA damage, Mutat. Res. 275 (1992) 367–375.
[27] H. Sies, Damage to plasmid DNA by singlet oxygen and its protection, Mutat. Res. 299 (1993) 183–191.
[28] T. Suzuki, M.D. Friesen, H. Ohshima, Formation of a diiminoimidazole nucleoside from 2′-deoxyguanosine by singlet oxygen generated by methylene blue photooxidation, Bioorg. Med. Chem. 11 (2003) 2157–2162.
[29] B.S. Grether, H.S. Olaizola, H. Schmitt, et al., Activation of transcription factor AP-2 mediates UVA-radiation and singlet oxygen induced expression of the human intercellular adhesion molecule 1 gene, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 14586–14591.
[30] H. Sano, C. Morimoto, Isolation of DNA from DNA/anti-DNA antibody immune complexes in systemic lupus erythematosus, J. Immunol. 126 (1981) 538–539.
[31] A. Bengtsson, R. Nezlin, Y. Shenfeld, G. Sturfelt, DNA levels in circulating immune complexes decrease at severe SLE flares—correlation with complement component C1q, J. Autoimmun. 13 (1999) 111–119.
[32] M. Gripenberg, T. Helve, Anti-DNA antibodies of IgA class in patients with systemic lupus erythematosus, Rheumatol. Int. 6 (1986) 53–55.