OXYGEN FREE RADICAL MODIFIED DNA: IMPLICATIONS IN THE ETIOPATHOGENESIS OF SYSTEMIC LUPUS ERYTHEMATOSUS

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ABSTRACT

The present study was designed to probe the possible role of singlet oxygen and superoxide anion radical modified DNA in the etiopathogenesis of Systemic lupus erythematosus. These species were generated by the exposure of riboflavin to 365 nm UV light. Modified DNA showed single strand breaks, hyperchromicity at 260nm and decrease in T_m. The modified DNA induced high titer antibodies in experimental animals. The antibodies showed reactivity with various nucleic acid polymers, a property commonly associated with Systemic lupus erythematosus anti-DNA autoantibodies. Systemic lupus erythematosus sera showed preferential binding of modified DNA over native DNA in direct binding and competitive binding solid phase immunoassays and band shift assays. The results suggest for the possible involvement of the singlet- superoxide modified DNA as a potential trigger for anti- DNA autoantibody production in SLE and thus in the etiopathogenesis of the disease.

KEY WORDS

Systemic lupus erythematosus, Singlet oxygen species, Superoxide radical, Anti-DNA autoantibodies.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease involving both humoral and cellular aspects of the innate and acquired immune systems and is characterized by autoantibodies with a spectrum of specificities that participate in disease pathogenesis (1). Sera of SLE patients contain a variety of autoantibodies of which a subset may be responsible for an array of clinical symptoms (2). Antibodies to dsDNA serve as a serological marker for the diagnosis of SLE. It has, however, been well established that native dsDNA or B-conformation *per se* is not immunogenic (3-5). Modified forms of DNA and polynucleotides (6,7), chromatin (8), modified self determinants (9) have been reported to be immunogenic and the antibodies thus generated are cross-reactive with native DNA.

Reactive oxygen species (ROS) are implicated in the inflammatory, autoimmune and connective tissue diseases particularly in respect of processes leading to the formation of pathogenic anti-DNA antibodies in SLE. Superoxide generated *in vivo* by several enzymatic and non-enzymatic pathways in the mammalian tissue (10-12) leading to unfavorable alteration of biomolecules have been implicated in several diseased states (13-15). Electronically excited molecular oxygen (singlet oxygen) is a potent biological toxin. It is one of the major cytotoxic species in eukaryotic cells (16-17). It oxidatively damages a variety of biological molecules including DNA (18-20). The radical generated by photoexcitation or by chemiexcitation, selectively reacts with the guanine residues (21) and causes fewer strand breaks (15). The biological consequences of singlet oxygen include loss of transforming activity as well as mutagenicity and genotoxicity. The study probes the possible role of singlet oxygen species ($^1\text{O}_2$) and superoxide radical ($\text{O}_2^{\cdot-}$) anion radical in the production of anti-DNA autoantibodies in SLE.

In the present study, calf thymus DNA (200bp) has been modified with $^1\text{O}_2$ and $\text{O}_2^{\cdot-}$ generated by photo-illumination of riboflavin under 365nm UV light. Antibodies induced in rabbits against modified DNA has been characterized with respect to antigen binding specificities. The cross-reactivity of naturally

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occurring anti-DNA autoantibodies with $^1\text{O}_2$ and $\text{O}_2^{\cdot-}$ modified DNA has also been examined.

MATERIALS AND METHODS

Calf thymus DNA, nuclease S1, superoxide dismutase, riboflavin, anti-human and anti-rabbit alkaline phosphatase conjugates were purchased from Sigma Chemical Company (U.S.A.). Nitroblue tetrazolium (NBT), sodium azide was from Loba Chemie, India. Polystyrene flat bottom ELISA plates (96 wells) were from NUNC, Denmark. All other chemicals were of analytical grade.

Preparation of 200 bp DNA fragments: Commercially available calf thymus DNA was purified free of proteins, single stranded regions, contaminations of RNA as described earlier (22) and subjected to digestion with micrococcal nuclease to obtain fragments varying from 70 to 800 bp. DNA fragments of about 200 bp were obtained by passing the above digested sample on a Sepharose 4B-column (46.0 cm x 1.2 cm). Fractions were subjected to polyacrylamide gel electrophoresis and their size was ascertained by PAGE using 100 bp ladder as standard marker.

Modification of DNA: Purified 200 bp calf thymus DNA was modified by the method of Waris et al (23). 100 µg/ml of DNA in 50 mM potassium phosphate buffer (pH 7.8), containing 0.1 mM EDTA, 0.06% TritonX-100 and 40 µM riboflavin in a total volume of 3.0ml was exposed to 365 nm UV light at room temperature for 1h.

Formation of singlet oxygen ($^1\text{O}_2$) was quantitated in aqueous solution by monitoring the bleaching of para nitrosodimethyl aniline (pRNO) at 440nm (24). While the formation of superoxide ($\text{O}_2^{\cdot-}$) radical was confirmed by the reduction of nitroblue tetrazolium (NBT), which was measured at 560nm (25). The samples were extensively dialyzed to remove riboflavin and TritonX-100. UV absorption characteristics of native and modified DNA were recorded on a Shimadzu UV-240 spectrophotometer. Fluorescence emission spectra of native and modified DNA samples using EtBr were also recorded at an emission wavelength of 300nm.

Thermal denaturation: Thermal denaturation of native and modified 200bp DNA under identical conditions was evaluated by a temperature scan from 30°C to 95°C at an increment of 1°C/min on a Shimadzu UV-240 spectrophotometer equipped with a temperature programmer and controller assembly. The change in absorbance at 260nm was recorded and melting temperature (T_m) of the samples calculated (26).

Nuclease S1 digestion: Modified DNA was also characterized by nuclease S1 treatment (27). One microgram, each of native and modified 200bp DNA, incubated with nuclease S1 (20 units/µg DNA) in acetate buffer (30mM each of sodium acetate and zinc chloride, pH 5.0), for 30min at 37°C. The reaction was stopped by adding one-tenth volume of 0.2M EDTA, pH 8.0. Samples were electrophoresed on a 1% agarose gel for 2h at 30mA and visualized under UV light after staining with ethidium bromide.

Immunization schedule: The immunization of random bred, female, New Zealand white rabbits was performed as described previously (27). Briefly, rabbits were immunized intramuscularly at multiple sites with 50mg of antigen complexed with methylated BSA in the ratio of 1:1 (w/w) and emulsified with an equal volume of Freund's adjuvants. Test bleeds were performed 7 days post boost which gave appropriate titer of the antibody. The animals were bled and the serum separated from the blood (preimmune and immune) was decomplexed by heating at 56°C for 30min.

Immunological techniques: Immunoglobulin G was isolated on a Protein-A agarose column. The isolated IgG migrated as a single band in SDS-PAGE. Enzyme linked immunosorbent assay (ELISA) was performed on flat bottom 96 well polystyrene immunoplates (Maxisorp) as described previously (28). Antibody specificity was ascertained by competitive binding assay (28). Varying concentrations of inhibitors (0-20 µg/ml) were allowed to interact with a constant quantity of antibody (50 µg/ml IgG or 1:100 diluted serum) for 2h at room temperature and overnight at 4°C. The mixture was added to antigen coated plates and the bound antibody was assayed as described in the direct binding ELISA. Band shift assay was performed for the visual detection of antigen-antibody binding and immune complex formation (29). Electrophoresis was carried out on 1% agarose gel for 2h at 30mA in 40mM Tris-acetate, 9mM EDTA buffer (TAE), pH 8.0. A constant amount of antigen (1 µg) was incubated with increasing concentrations (0-80 µg) of SLE IgG for 2h at room temperature and overnight at 4°C before loading onto the gel. Gels were stained with ethidium bromide (0.5 µg/ml) and photographed under UV illumination.

RESULTS

Singlet oxygen and superoxide radicals were generated by the UV (365nm) illumination of riboflavin. Its generation was detected by NBT. For optimal generation of the radical species, time of illumination and variable concentration of riboflavin and Triton X-100 were studied. The ability of riboflavin to form

$^1\text{O}_2$ was determined by monitoring the bleaching of pRNO. A near complete inhibition of $^1\text{O}_2$ production was observed in presence of NaN_3 , a specific quencher of $^1\text{O}_2$.

Characterization of modified DNA: UV absorption spectrum of the modified DNA exhibited 30% hyperchromicity with a shift of approximately 10nm towards longer wavelength, both at λ_{max} and λ_{min} (Figure 1).

Fig 1: Ultraviolet absorption spectra of 200 bp native DNA (—) and modified DNA (----).

Table 1: UV and thermal denaturation characteristics of native and modified DNA under identical experimental conditions

Parameter	Native DNA	Modified DNA
Absorbance ratio (A_{260}/A_{280})	1.8	1.6
λ_{max} (nm)	260	270
λ_{min} (nm)	230	240
Percent hyperchromicity at 95°C	36.7	17.2
Melting temperature (T_m), °C	86	78
Onset of duplex melting °C	65	58

Fig 2: Thermal melting profile of native DNA (o) and modified DNA (●).

Thermal denaturation characteristics of native and modified 200 bp DNA were monitored between 30°C and 95°C. T_m of native and modified DNA was found to be 86°C and 78°C, respectively (Figure 2). The physicochemical characteristics of native and modified DNA have been presented in Table 1.

Samples of native and modified DNA were also subjected to fluorometric analysis after intercalation of EtBr. The decrease in the fluorescence intensity was observed due to decreased amount of ethidium bromide intercalation in the modified DNA as compared to native DNA (Figure3), indicating perturbation in the DNA helix/ generation of ss regions.

Fig 3: Fluorescence emission spectra of native DNA (—) and modified DNA (---). The samples were excited at 300 nm.

The generation of single stranded regions in modified DNA was probed by nuclease S1 digestion. Results showed substantially decreased intensity in case of S1 treated modified 200 bp DNA compared to the untreated control on agarose gel electrophoresis. However, the unmodified native 200 bp DNA was not appreciably digested under similar experimental conditions (Figure 4).

Fig 4: Nuclease S1 digestibility of native and modified DNA. Lane 1 contained native 200 bp DNA, while lane 2 contained native 200 bp DNA treated with nuclease S1. Lane 3 contained modified 200 bp DNA, while lane 4 contained modified 200 bp DNA treated with nuclease S1. Electrophoresis was carried out on 1% agarose gel for 2h at 30 mA.

Antigenicity of modified DNA: Modified DNA was found to be a potent immunogen. Induced antibodies showed a titer of $\geq 1:25600$ in direct binding ELISA (Figure 5). The isolated IgG also exhibited higher recognition with the immunogen (Figure 6) while the preimmune serum/IgG gave negligible binding.

Table 2: Antigenic specificity of modified DNA IgG

Inhibitor	Maximum % inhibition at 20µg/ ml of inhibitor
Modified DNA (200 bp)	84.5
nDNA	47.8
nDNA (200 bp)	32.3
ROS-DNA (200 bp)	50.25
Poly(dA-dU).poly(dA-dU)	12.7
Poly (dI-dC).poly(dI-dC)	38.7
Poly (dA-dT).poly (dA-dT)	44.4
Chondroitin sulfate	48.0

The microtitre plates were coated with modified DNA (2.5µg/ ml).

Fig 5: Direct binding ELISA of modified DNA with preimmune (Δ) and immune (▲) sera. Microtitre plate was coated with modified DNA (2.5µg/ml).

Fig 6: Direct binding ELISA with affinity purified preimmune (Δ) and immune IgG (▲). The microtitre plate was coated with modified DNA (2.5µg/ml).

Specificity of the induced antibodies for antigenic determinants on modified DNA was evaluated by competitive binding assay (Figure 7). Antibodies against the modified DNA exhibited broad spectrum reactivity with nucleic acids and synthetic polynucleotides in competitive inhibition analysis. A maximum of 84.5% inhibition in the binding of modified DNA IgG was observed for the immunogen. Fifty percent inhibition was achieved at a concentration of 1.9µg/ml. Induced antibodies showed lesser binding to native DNA. The data indicates

Fig 7: Inhibition ELISA of immune (●) and preimmune (Δ) serum antibodies with modified DNA. The microtitre plate was coated with modified DNA (2.5μg/ml) and the serum dilution was 1:100.

higher specificity of the immune IgG towards ROS modified epitopes. Native DNA and 200bp DNA showed 47.8% and 32.3% inhibition respectively, whereas ROS-DNA showed an inhibition of 50.25%. Antibodies against the modified DNA were

Table 3: Competitive inhibition data of SLE sera

Sera No.	Maximum percent inhibition at 20 μg/ml	
	Native 200 bp DNA	Modified 200 bp DNA
1.	39.0	70.1
2.	28.3	68.6
3.	29.0	40.0
4.	46.0	66.5
5.	48.0	77.0
6.	23.7	58.7
7.	35.4	61.6
8.	16.0	57.7
9.	23.5	59.0
10.	26.0	52.0
11.	50.0	75.9
12.	30.0	41.5
13.	34.7	39.7
14.	46.0	59.0
15.	23.3	69.1
16.	14.4	55.6
17.	27.0	37.0
Mean± SD	31.78 ± 10.9	58.18 ± 12.7

Microtitre plates were coated with modified DNA (2.5 μg/ ml)

probed for their antigen binding characteristics using different nucleic acid polymers. (Table2). Synthetic polymers, Poly (dA-dU).poly (dA-dU).poly (dI-dC).poly (dI-dC) and poly (dA-dT).poly (dA-dT) showed an inhibition of 12.7%, 38.7% and 44.4% respectively. Besides binding to various nucleic acids and synthetic polynucleotides, the immune IgG also showed cross reactivity towards chondroitin sulphate.

Recognition of modified DNA by SLE anti-DNA

autoantibodies: To probe the possible role of singlet oxygen and superoxide anion in the etiopathogenesis of SLE, sera showing high titer ($\geq 1:3200$) anti-DNA autoantibodies were selected for their binding to modified DNA. These sera showed substantially higher binding with the modified DNA relative to native DNA in competition ELISA. The modified DNA showed greater inhibition (37.0-77.0%) compared to native DNA (14.4-50.0%) in all the serum samples (Table 3).

The formation of immune complex by modified DNA and SLE

Fig 8: Band shift assay of SLE IgG binding to (a) native DNA and (b) $^1\text{O}_2\text{-O}_2^{\cdot-}$ DNA. Native DNA and $^1\text{O}_2\text{-O}_2^{\cdot-}$ DNA (1μg) each were incubated with ,10,20,40 and 80 μg of IgG for 2 hr at 37°C and overnight at 4°C. Electrophoresis was carried out on 1% agarose gel for 2 hr at 30 mA. Lane 1 contains native or $^1\text{O}_2\text{-O}_2^{\cdot-}$ DNA, while lanes 2,3,4, and 5 contain native or $^1\text{O}_2\text{-O}_2^{\cdot-}$ DNA with increasing concentrations of SLE IgG.

IgG was visualized by band shift assay. As clearly evident, with an increase in the amount of IgG an increase in the formation of high molecular weight immune complex, having retarded mobility was observed (Figure 8a). The recognition of native DNA by SLE IgG was similarly demonstrated by the shift in electrophoretic mobility upon immune complex formation (Figure 8b).

DISCUSSION

ROS, including hydroxyl radical (OH), hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), singlet oxygen (1O_2) and peroxy radicals are produced during normal aerobic metabolism as well as under many pathologic conditions, such as tissue ischemia, degenerative and inflammatory diseases etc. These reactive species are also induced by ionizing radiations. Besides, many carcinogenic chemicals can cause DNA damage, including generation of single strand breaks and base modifications.

Superoxide is the most abundant reactive species generated *in vivo* by several enzymatic pathways in mammalian tissue (10-12) and has been implicated in several disease states via unfavorable alteration of biomolecules (13-15). It is known that $O_2^{\cdot-}$ itself can exert deleterious effects in biological systems (10). The superoxide radical produced in excessive amounts might be responsible for DNA damage in autoimmune diseases. Singlet oxygen, which is also generated predominantly in photosensitization reactions, is of particular physiologic significance because of its relatively long life in aqueous solution, its ability to cross cell membrane barrier and high reactivity towards biomolecules. Increasing evidence indicates that 1O_2 is capable of inducing oxidative DNA damage (30,31). Sodium azide is considered to be a relatively specific 1O_2 scavenger and is shown to decrease 1O_2 induced genotoxicity and oxidative damage (11).

In the present study, 200 bp calf thymus DNA was modified by the combined effect of singlet oxygen and superoxide anion radicals. This study shows that the damage observed by illumination of riboflavin system is due to the production of singlet oxygen (1O_2) and superoxide anion radical ($O_2^{\cdot-}$). Their respective production were confirmed by the use of specific quenchers, sodium azide and SOD. The increase in absorption at 260 nm together with a net decrease of 8°C in Tm could be attributed to single strand breaks and perturbation of the double helical structure of DNA. The fluorescence spectra of modified DNA showed decrease in fluorescence intensity as compared to native DNA which indicates the generation of strand breaks as lesser amount of ethidium bromide gets intercalated in

modified DNA as compared to native DNA. Formation of single stranded regions in DNA was studied by nuclease S1 digestion. The results showed partial digestion of modified DNA on treatment with nuclease S1, while native DNA remained undigested. This observation clearly demonstrates that sufficient single strand breaks are generated in DNA by ROS rendering it susceptible to digestion by single strand specific nuclease S1. Induction of high titer antibodies against modified DNA is indicative of conformational/ structural alterations in DNA following exposure to ROS as native B-helix is known to be non-immunogenic. The induced antibodies were found to be highly specific towards the immunogen. Native and modified DNA did cause appreciable inhibition in the antibody activity but it was substantially less as compared to the immunogen.

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by various immunologic disorders, including production of autoantibodies, formation of immune complexes, decreased serum complement levels, and lymphocytopenia (32). The presence of anti-DNA autoantibodies in the sera of SLE patients has long been considered as a marker of the disease as well as the pathogenic factor for its renal manifestations. It has been reported that denaturation of dsDNA by ROS results in an increased binding of anti-DNA antibodies present in the sera of SLE patients (33) and reactive oxygen species (ROS)-modified DNA is a better and more discriminating immunogen than native DNA (nDNA) for the production of anti-DNA autoantibodies in SLE (34). The detection of 8-hydroxydeoxyguanosine the DNA isolated from the serum of SLE patient (35) reinforces the evidence that ROS may be involved in SLE. Thus, it appears that ROS modification exposes epitopes on DNA that are recognized by circulating anti-DNA autoantibodies. Increased apoptosis and decreased clearance of apoptotic cells as observed in systemic lupus erythematosus (SLE) might well be a contributory factor in systemic autoimmunity. Anti-DNA antibodies may combine with circulating antigen and which contribute to the deposition of immune complexes in renal glomeruli (36). Preferential binding of SLE autoantibodies to modified DNA as compared to native DNA in both direct binding and inhibition ELISA indicates that singlet oxygen and superoxide anion radical causes DNA damage rendering it highly immunogenic. The role of modified DNA in the production of SLE anti-DNA autoantibodies has been discussed.

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