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Plasmid DNA Acquires Immunogenicity on Exposure to Singlet Oxygen

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Abstract—In the present study, the effect of singlet oxygen ($^1O_2$) (generated by ultraviolet (UV) irradiation of methylene blue) on plasmid DNA has been analyzed by UV spectroscopy, fluorescence spectroscopy, and S_1 nuclease digestibility. Both native and $^1O_2$-modified plasmid DNA were treated with a number of restriction enzymes to map out the sites damaged by $^1O_2$. It was also observed that, on exposure to $^1O_2$, native plasmid DNA that is non-immunogenic acquired the ability to elicit an immune response in experimental animals. However, the induced antibodies exhibited appreciable cross reactivity with various polynucleotides and nucleic acids. The data indicate that the antibodies, though cross-reactive, preferentially bind $^1O_2$-modified epitopes on plasmid DNA. Gel retardation assay further substantiated the enhanced recognition of $^1O_2$-modified plasmid DNA over the native form. The antibodies developed were then subjected to competition ELISA with sera from various diseases such as systemic lupus erythematosus, rheumatoid arthritis, and cancer. These results suggest that upon exposure of DNA to $^1O_2$, neo-epitopes are generated, which may be one of the factors for the induction of circulating autoantibodies in the three diseases.

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Key words: plasmid DNA, reactive oxygen species, singlet oxygen, autoantibodies, autoimmune disease

Reactive oxygen species (ROS), formed exogenously and endogenously, play a major role in mediating antibacterial functions of phagocytic cells, but excessive ROS production can cause oxidative stress and tissue damage. Uncompensated ROS release is implicated in the etiology of a number of diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis, tissue ischemia, inflammation, and even cancer [1-3]. These free radicals generated in proximity to DNA react to form unique DNA base products. The detection of these products in biological fluids is used as evidence of damage by free radicals [4]. The damage to DNA by ROS includes single-strand breaks, base modifications, and conformational changes exposing base residues with DNA backbone and the minor regions of single-stranded DNA, rendering it highly immunogenic. Monoclonal anti-DNA antibodies react more strongly with denatured DNA than with native DNA. Thymine and guanine are most susceptible to modifications, followed by cytosine and adenine. Thymine glycol is the major oxidation product, and its presence in urine serves as an indicator of endogenous DNA damage. Cytosine glycols are also formed, which can undergo deamination to form uracil derivatives that base pair preferentially with adenine instead of guanine [5, 6]. Oxidative modifications of DNA bases can lead to mutations if left unrepaired or repaired with errors before replication, and these are usually related to modifications of GC base pairs (bp) rather than AT bp [7].

Singlet molecular oxygen ($^1O_2$) has also been implicated in several biological processes that may lead to genetic damage. DNA is one of the main targets of $^1O_2$. The biological consequences include a loss of transforming activity as well as mutagenicity and genotoxicity [8]. DNA modifications produced by singlet oxygen are almost exclusively oxidized purines [9]. Mammalian DNA per se is non-immunogenic, and all efforts to replicate disease by immunization of normal animals with high doses of native DNA (nDNA) have failed. In contrast to native mammalian DNA, bacterial DNA is a stronger immuno-

Abbreviations: RA) rheumatoid arthritis; ROS) reactive oxygen species; SLE) systemic lupus erythematosus; $^1O_2$) singlet oxygen.
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gen [10]. The immunogenicity of bacterial DNA has been attributed to the presence of nucleotide hexamers containing unmethylated CpG motifs. Similarly, the role of unmethylated CpG dinucleotide sequences in the immunogenicity of plasmid DNA is well recognized [11]. Other modified forms of DNA and polynucleotides [12, 13], modified determinants [14], and antibodies to self proteins [15] have been reported to be immunogenic, and the antibodies thus generated are cross reactive with native DNA. It appears that a fundamental defect in the immune system plays an initiating role to contribute to the autoimmune response seen in SLE and RA patients [16]. Increased levels of circulating antibodies and autoantibodies have also been reported in the serum of patients with malignancies.

In the present study, attempts have been made to ascertain the extent of damage caused by singlet oxygen to plasmid DNA by various physicochemical techniques and restriction enzyme mapping. The immunogenicity induced in plasmid DNA on exposure to singlet oxygen was also studied. The polyspecific nature of induced antibodies was analyzed through cross-reactivity experiments and comparative immunobinding of autoantibodies present in the sera of SLE, RA, and various cancer patients with native and $^1O_2$-modified plasmid DNA.

MATERIALS AND METHODS

Materials. Plasmid DNA (pUC18) was isolated from *E. coli* $DH_5\alpha$ using a plasmid Maxi Prep Kit supplied by Qiagen (USA). Various restriction enzymes, methylated bovine serum albumin, calf thymus DNA, anti-rabbit IgG–alkaline phosphatase conjugate, *p*-nitrophenyl phosphate, Freund's complete and incomplete adjuvants, and agarose were purchased from Sigma (USA). Synthetic polynucleotides were purchased from Pharmacia (Sweden). Tryptone, yeast extract, and bacto-agar were procured from Difco (USA). Polystyrene microtiter flat bottom ELISA plates having 96 wells (7 mm diameter) were purchased from Nunc (Denmark). All other reagents/chemicals were of the highest analytical grade available.

Modification of DNA by singlet oxygen. Aqueous solution of pUC18 plasmid DNA (20 μg/ml) in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) was irradiated by 254 nm UV light for 30 min at room temperature in the presence of methylene blue (50 μM). The samples were dialyzed extensively with PBS, pH 7.4, to remove the dye [17]. The modified form of plasmid DNA was characterized through UV absorption, fluorescence spectroscopy, and nuclease S_1 digestion [18].

Restriction enzyme digestion of plasmid DNA. One microgram each of both native and modified plasmid DNA was digested with 2 units of various restriction enzymes (*Eco*RI, *Sma*I, *Ssp*I, *Pvu*I, *Pvu*II, *Pst*I, *Sal*I, *Xba*I, *Xho*II, *Rsa*I, *Sac*I, *Kpn*I, *Hpa*II, *Hind*III, *Hinc*II, *Hinf*I, *Taq*I, *Bam*HI, and *Dra*I) at 37°C for 2 h. The reaction was stopped by adding one-tenth volume of stop mix dye, and the samples were loaded in the wells of submerged agarose gel and electrophoresed for 4 h at 30 mA.

Immunization schedule. Random bred female, New Zealand white rabbits ($n = 2$) were immunized subcutaneously at multiple sites with 50 μg of antigen complexed with methylated BSA in the ratio of 1 : 1 (w/w) and emulsified with an equal volume of Freund's complete adjuvant. The animals were boosted intramuscularly in Freund's incomplete adjuvant at weekly intervals for five weeks with the same amount of antigen. Test bleeds were performed seven days post boost, which gave appropriate titer of the antibody. Each animal received a total of 300 μg of antigen in the course of six injections. Blood was collected from the marginal vein of the ear; and serum was separated and decomplemented by heating at 56°C for 30 min. Pre-immune serum was collected *prior to* immunization. The sera were stored in small aliquots at −80°C followed by affinity purification of IgG on protein A-Sepharose CL-4B [19].

Serum samples. Sera were collected from 30 SLE and 25 RA patients that satisfied the American College of Rheumatology criteria for its diagnosis [20] showing high titer anti-DNA antibodies (>1 : 12,800). None of the patients had an active infection, known malignancy, tuberculosis, pregnancy, or cirrhosis. Serum samples of various cancer patients (of different age and sex) were obtained after careful clinical examination of the patients with proven histopathological diagnosis. Samples were collected from the Medical College Hospital (Aligarh, India) and the All India Institute of Medical Sciences (New Delhi, India). Normal human sera (used as negative control) were obtained from healthy individuals ($n = 15$). All serum samples were decomplemented by heating at 56°C for 30 min and stored at −80°C.

Linearity of ELISA method. To check the linearity of the ELISA, we performed titrations of five serum samples from each disease, with anti-$^1O_2$-modified plasmid DNA antibody levels diluted with PBS (1 : 150 to 1 : 5000). The curves obtained for the sera were plotted for linear regression analysis. The slopes of all curves were similar to each other, and thus, we may assume that the behavior of sera did not differ. In all, six different dilutions of each serum sample were tested, and the regression lines were created with five points (data not shown).

Enzyme linked immunosorbent assay. ELISA was performed on flat-bottomed 96-well polystyrene microtiter plates [21]. The plates were coated with 100 μl of the antigen (2.5 μg/ml) and left for 2 h at room temperature (25°C) and overnight at 4°C. The plates were washed with TBS-T (20 mM Tris, 2.68 mM KCl, 150 mM NaCl, pH 7.4, containing 0.05% Tween-20). Unoccupied sites were blocked with 150 μl of 1.5% BSA in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) for 6 h at room temperature.

Test serum (100 μl), serially diluted in TBS, was used to coat the wells, which were left for 2 h at room temperature and overnight at 4°C for proper adsorption, followed by washing with TBS-T. Bound antibodies were assayed with anti-human IgG– or anti-rabbit IgG–alkaline phosphatase conjugates and color development was initiated by the addition of 100 μl of 2.2 mM *p*-nitrophenyl phosphate substrate in carbonate-bicarbonate buffer, pH 9.6. The plate was allowed to develop for up to 30 min at room temperature and terminated by the addition of 100 μl of 4 M sulfuric acid. Absorbance was measured at 410 nm on an automatic microplate reader. Half of the plate, devoid of the coating antigen, served as control. The results were expressed as the mean of differences in absorbance values in test and control wells.

Competition ELISA. Specific antigen–antibody interaction was ascertained by competitive binding assay. Various concentrations of inhibitors (0-20 μg/ml) were incubated with a constant amount of antibody (1 : 100 serum or 50 μg/ml IgG) for 2 h at room temperature and overnight at 4°C. The mixture was added to antigen-coated plates and inhibition of antibody activity was detected as detailed for the direct binding ELISA.

Band-shift assay. Antigen–antibody binding and immune complex formation was visually detected by band-shift assay [22]. A constant amount of antigen (1 μg) was incubated with increasing amounts (0-60 μg) of IgG for 2 h at room temperature and overnight at 4°C. Samples were electrophoresed on 1%-agarose gel in 40 mM TAE (Tris-acetate buffer), pH 8.0, for 2 h at 30 mA. Gels were stained with ethidium bromide (0.5 μg/ml) and photographed under UV light.

RESULTS

The $^1O_2$-modified plasmid DNA exhibited a marked hyperchromic effect (37%) over the entire UV range and most noticeably at 260 nm when compared to the native form. The UV absorbance ratio (A_{260}/A_{280}) of $^1O_2$-modified plasmid DNA decreased to 1.4 from the usual 1.8 for pure native DNA. The generation of singlet oxygen was confirmed by the use of specific $^1O_2$ quencher (α-carotene) during exposure, which resulted in decrease in hyperchromic effect (from 37 to 4%). Fluorescence emission analysis of native and $^1O_2$-modified plasmid DNA, using ethidium bromide (2.5 μg/ml) as external fluor, showed a decrease in the fluorescence intensity in the case of the modified plasmid DNA signifying perturbations in the double helix as a result of singlet oxygen modification (Table 1). Native and $^1O_2$-modified plasmid DNA along with proper controls was subjected to nuclease S_1 digestion (2 units/μg of DNA for 1 h and electrophoreses was performed on 1% agarose) to confirm the generation of single strand breaks in $^1O_2$-modified plasmid DNA. On comparison to native plasmid DNA, the supercoiled form of $^1O_2$-modified plasmid DNA showed complete disappearance and the relaxed form showed appreciable loss of fluorescence intensity. Almost no change in the linear form could probably be due to compromised ethidium bromide fluorescence as a result of damage to all forms of plasmid DNA on exposure to singlet oxygen. After many nicks, the supercoiled DNA becomes linear and further nicks lead to fragmentation of the linear form, rendering the molecule too small to appear as a band. Whereas on nuclease S_1 digestion, it was observed that the single strand specific enzyme was not able to digest the native form. However, the digestion was more pronounced in the case of $^1O_2$-modified plasmid DNA on the basis of substantial decrease in intensity (Fig. 1).

Table 1. Characteristics of native and $^1O_2$-modified plasmid DNA

Parameter	Native plasmid DNA	$^1O_2$-modified plasmid DNA
A_{260}/A_{280}	1.8	1.4
Increase in hyperchromicity, %	—	37
Decrease in fluorescence intensity, %	—	29

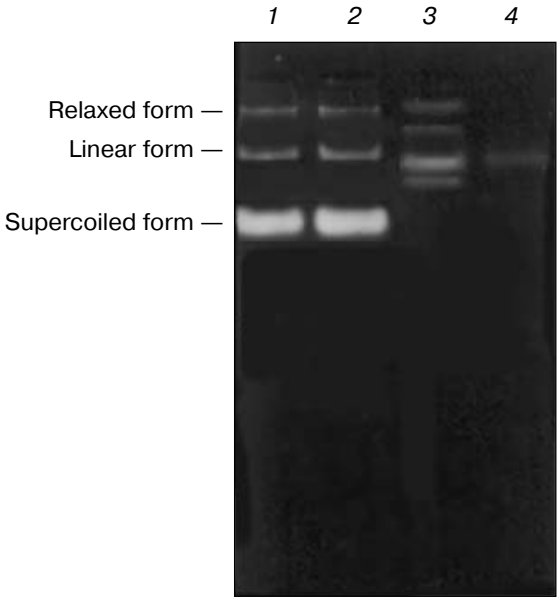

Fig. 1. Agarose gel electrophoresis pattern of nuclease S_1 digestion of native and modified plasmid DNA. Electrophoresis was carried out on 1% agarose gel for 2 h at 30 mA. Lanes: *1*) native plasmid DNA; *2*) native plasmid DNA treated with S_1 nuclease; *3*) modified plasmid DNA; *4*) modified DNA treated with nuclease S_1.

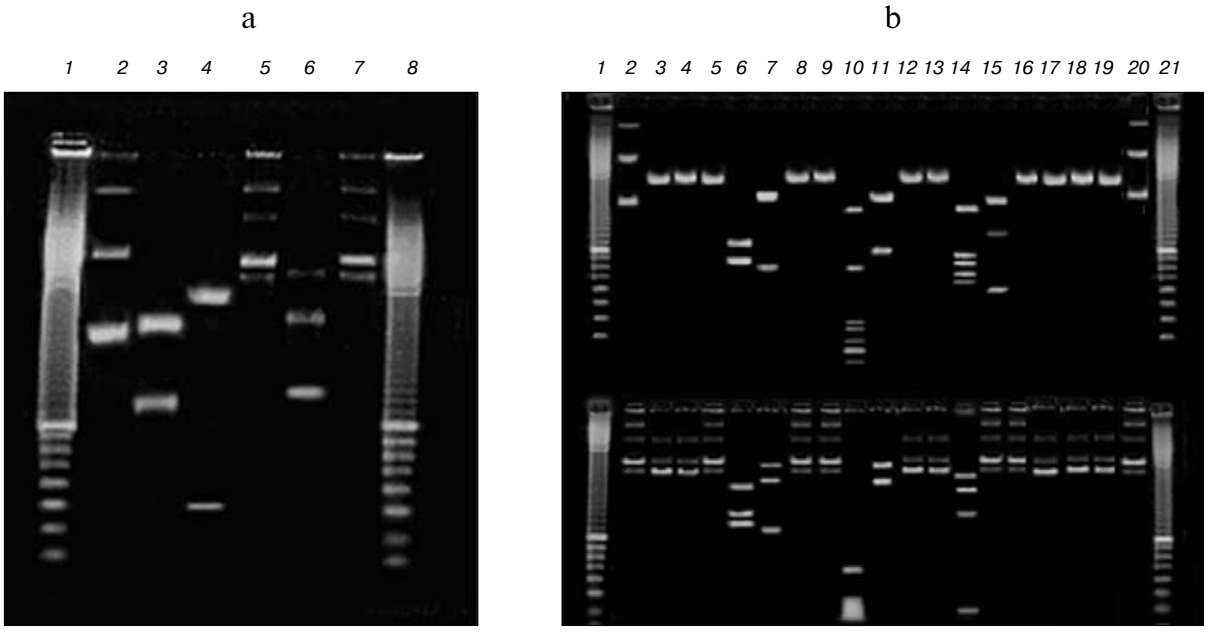

Fig. 2. a) *Pvu*I and *Pvu*II digestion of native and $^1O_2$-modified plasmid DNA. Lanes: *1*, *8*) 100 bp ladder; *2*) native plasmid DNA; *3*, *4*) native plasmid DNA treated with *Pvu*I and *Pvu*II, respectively; *5*) $^1O_2$-modified plasmid DNA; *6*, *7*) *Pvu*I- and *Pvu*II-treated $^1O_2$-modified plasmid DNA, respectively. b) Restriction enzyme digestion of native and $^1O_2$-modified plasmid DNA. Top row, lanes: *1*, *21*) 100 bp ladder; *2*, *20*) native plasmid DNA; *3-19*) native plasmid DNA treated with *Pst*I, *Sal*I, *Xba*I, *Xho*II, *Rsa*I, *Sac*I, *Kpn*I, *Hpa*II, *Dra*I, *Hind*III, *Hinc*II, *Hinf*I, *Taq*I, *Bam*HI, *Eco*R1, *Ssp*I, and *Sma*I, respectively. Bottom row, lanes: *1*, *21*) 100 bp ladder; *2*, *20*) $^1O_2$-modified plasmid DNA; *3-19*) $^1O_2$-modified plasmid DNA treated with *Pst*I, *Sal*I, *Xba*I, *Xho*II, *Rsa*I, *Sac*I, *Kpn*I, *Hpa*II, *Dra*I, *Hind*III, *Hinc*II, *Hinf*I, *Taq*I, *Bam*HI, *Eco*R1, *Ssp*I, and *Sma*I, respectively.

Both native and $^1O_2$-modified plasmid DNAs were treated with a number of restriction enzymes to find out what sites of the pUC 18 plasmid genome had been damaged by singlet oxygen (Fig. 2). No change in the electrophoretic profile of $^1O_2$-modified plasmid DNA was seen when it was treated with *Pvu*II (CAG↓CTG), *Xba*I (T↓CTAGA), *Sac*I (GAGCT↓C), *Kpn*I (GGTAC↓C), *Taq*I (T↓CGA), and *Bam*HI (G↓GATCC) indicating that singlet oxygen resulted in the modification of base(s) at the restriction site. The enzyme is thus unable to digest the plasmid.

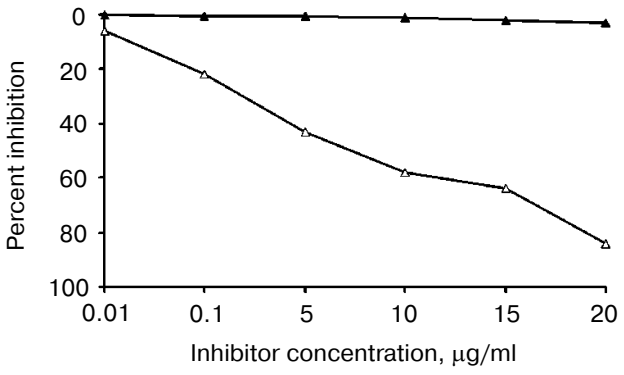

Fig. 3. Inhibition ELISA of immune (△) and pre-immune (▲) sera against $^1O_2$-modified plasmid DNA. Modified plasmid DNA was used as inhibitor. The microtiter plate was coated with $^1O_2$-modified plasmid DNA (2.5 μg/ml).

The immunogenicity of singlet oxygen modified plasmid DNA was determined by inducing antibodies in rabbits, which were found to be non-precipitating as assessed by double immunodiffusion. The modified form was found to be a potent immunogen and showed a titer > 1 : 12,800 by direct binding ELISA, whereas native plasmid DNA did not elicit appreciable immune response. The IgG isolated from the immune serum exhibited higher reactivity with the immunogen over pre-immune IgG. The specificity of the purified IgG was evaluated by competitive inhibition assay. A maximum of 84% inhibition of anti-$^1O_2$-modified plasmid DNA IgG binding to immunogen was observed (Fig. 3). The anti-$^1O_2$-modified plasmid DNA antibodies exhibited a wide range of heterogeneity as demonstrated by inhibition assays, using nucleic acids, synthetic polynucleotides, and nuclear proteins as inhibitors. Binding diversity of anti-$^1O_2$-modified plasmid DNA IgG with an array of nucleic acid antigens is given in Table 2.

Band shift assay was employed to visualize and confirm the interaction of native and $^1O_2$-modified plasmid DNA with anti-$^1O_2$-modified plasmid DNA IgG. An

Table 2. Antigenic binding specificity of anti-$^1O_2$-modified plasmid DNA antibody to various inhibitors by competition ELISA

Inhibitor	Maximum inhibition at 20 μg/ml, %	Concentration for 50% inhibition, μg/ml	Relative affinity, %
Native plasmid DNA	43	—*	
$^1O_2$-modified plasmid DNA	84	9.6	100
Native 400 bp DNA	36	–	
ROS-modified 400 bp DNA	57	14	69
Native calf thymus DNA	40	—	
$^1O_2$-modified calf thymus DNA	61	12.8	75
Guanine	48	—	
$^1O_2$-modified guanine	66	11.3	84
Adenine	23	—	
$^1O_2$-modified adenine	28	—	
Thymine	8	—	
$^1O_2$-modified thymine	23	—	
Cytosine	13	—	
$^1O_2$-modified cytosine	33	—	
PolydT	14	—	
PolydC	23	—	
PolydA	21	—	
Cardiolipin	38	—	
Chondroitin sulfate	26	—	

Note: The microtiter plates were coated with $^1O_2$-modified plasmid DNA (2.5 μg/ml).
* 50% inhibition was not achieved.

increase in the formation of high molecular weight immune complexes with increasing amount of IgG, which resulted in retarded mobility and substantial decrease in the fluorescence of unbound plasmid, was observed between $^1O_2$-modified plasmid DNA and immune IgG (Fig. 4). Retarded mobility was seen in the case of the modified form of DNA and not in the native form.

Singlet oxygen modified plasmid DNA exhibited higher inhibition in various disease conditions like SLE, RA, and cancer when compared to normal sera, indicating the presence of better epitopes for the circulating autoantibodies formed during the disease conditions (Table 3). All results are significant at $p < 0.001$.

DISCUSSION

Reactive oxygen species (ROS) are produced as by-products of cellular metabolic pathways and function as critical second messenger in a variety of intracellular signaling pathways. Thus, a defect or deficiency in the antioxidant defense system on one hand and/or the excessive intracellular generation of ROS on the other renders a cell oxidatively stressed. As a consequence, direct or indirect involvement of ROS in numerous diseases has been documented [10]. Singlet oxygen is known to be involved in several biological processes and is capable of interacting with any cellular component it encounters including macromolecules such as DNA, proteins, membrane

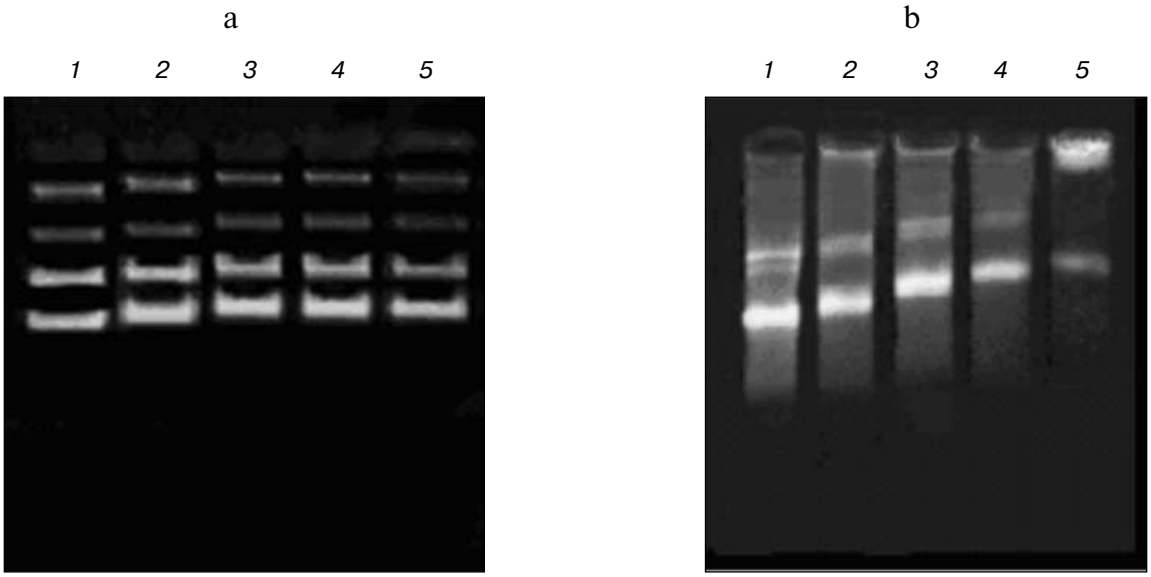

Fig. 4. Band shift assay of anti-$^1O_2$-modified plasmid DNA IgG binding to native plasmid DNA (a) and $^1O_2$-modified plasmid DNA (b). Native or modified plasmid DNA (0.5 μg each) was incubated with immune IgG for 2 h at 37°C and overnight at 4°C followed by electrophoresis performed on 1% agarose gel for 2 h at 30 mA. Lanes: *1*) native or $^1O_2$-modified plasmid DNA alone; *2-5*) native or $^1O_2$-modified plasmid DNA with 5, 15, 30, and 60 μg of immune IgG, respectively.

lipids, and carbohydrates. Singlet oxygen produces a large number of sugar/base-derived products in DNA. This species can directly induce single and double strand breaks in DNA, cleaving the sugar phosphate bond [23-25].

In the present study, it is observed that singlet oxygen caused extensive damage to plasmid DNA as was evident from the 37% hyperchromicity of the spectral curve of $^1O_2$-modified plasmid DNA as compared to native plasmid DNA. This can be attributed to the formation of strand breaks, disruption of hydrogen bonds, and a consequent helix destabilization due to single stranded regions in the DNA. Appreciable evidence for the generation of strand breaks (single and double) in the plasmid DNA as a consequence of $^1O_2$ modification was resolved by agarose gel electrophoresis. The supercoiled form of plasmid DNA becomes linearized upon $^1O_2$ modification. Thus, confirming that, generation of singlet oxygen results in strand breaks which convert supercoiled form to linear. Earlier studies demonstrated that the structural alteration in DNA, following damage by various agents, may at times be large enough to act as substrate for single strand specific nucleases [26-29]. Native and $^1O_2$-modified plasmid DNA was subjected to nuclease S_1 digestion in order to confirm the generation of single strand breaks. The data shows almost total digestion of $^1O_2$-modified plasmid DNA on treatment with S_1, while native plasmid DNA remained undigested. These observations clearly demonstrate that sufficient distortions are caused in the helical structure of DNA by $^1O_2$, rendering it susceptible to digestion by single strand specific nuclease S_1.

As a result of oxidative stress, ROS cause oxidative DNA damage *in vitro*. They damage the double-stranded plasmid DNA into open and linear forms [30]. When our native and $^1O_2$-modified plasmid DNA was digested with several restriction enzymes, native plasmid DNA was digested at the specific sites as expected, whereas $^1O_2$-modified plasmid DNA was not digested by *Pvu*II, *Xba*I, *Sac*I, *Kpn*I, *Taq*I, and *Bam*HI. All these five restriction enzymes involved cytosine at the restriction site except for *Bam*HI, which involved guanosine directly. From these data, we can predict that $^1O_2$ may have modified sites involving cytosine so that the enzyme is unable to digest it. Or, as it is known, $^1O_2$, when generated, reacts most specifically with the guanosine moiety forming 7,8-dihydro-8-oxoguanine (8-oxoguanine; 8-oxo-G), inducing lesions and disrupting the triple hydrogen bonds that bind cytosine to guanine; this destroys the integrity of plasmid DNA and in turn makes it impossible for the restriction enzymes to digest the plasmid DNA at their respective sites [8, 31-34]. Base alteration and conformational changes have been shown to influence the immunogenicity of DNA [4]. The results derived from the restriction enzyme digestion is consistent with the binding of induced antibodies against $^1O_2$-modified plasmid DNA with an array of nucleic acid antigens, showing preferential recognition of $^1O_2$-modified epitopes on guanine (54%), over $^1O_2$-modified cytosine (33%).

The origin of autoantibodies remains an enigma, and the production of anti-DNA antibodies is even more complicated. Even though nucleic acid antigens are by

Table 3. Inhibition in the binding of serum antibodies in normal, SLE, RA, and various cancers by native and $^1O_2$-modified plasmid DNA by competition ELISA

Type of serum	Mean maximum percent inhibition	
	native plasmid DNA	$^1O_2$-modified plasmid DNA
Normal human serum (n = 15)	2.2 ± 0.92 (41.8%)*	5.4 ± 1.41 (26.1%)*
SLE (n = 30)	28.2 ± 13.3 (47.1%)*	62.8 ± 10.4 (16.5%)*
Rheumatoid arthritis (n = 25)	24.6 ± 9.7 (39.4%)*	59.4 ± 6.8 (11.4%)*
Type of cancer:		
breast (n = 11)	21.4 ± 4.5 (21.0%)*	39.6 ± 6.3 (15.9%)*
lung (n = 9)	29.2 ± 10.5 (35.9%)*	51.9 ± 12.9 (24.8%)*
cervical (n = 7)	18.6 ± 2.22 (11.9%)*	33.1 ± 7.4 (22.3%)*
head and neck (n = 5)	24.5 ± 7.8 (31.8%)*	42.3 ± 8.5 (20.0%)*
oral (n = 7)	20.3 ± 8.70 (42.8%)*	36.8 ± 6.5 (17.6%)*
liver (n = 9)	31.4 ± 14.2 (45.2%)*	45.6 ± 9.6 (21.0%)*

Note: The results represent mean ± SD values. Serum dilution was 1 : 100 in all the cases. The microtiter plates were coated with $^1O_2$-modified plasmid DNA (2.5 μg/ml).

* The value in the bracket represents precision/coefficient of variation.

themselves poorly immunogenic, their antigenicity can be enhanced by modification with ROS [15]. $^1O_2$-modified plasmid DNA was found to be a potent immunogen when rabbits were immunized by it, inducing high-titer antibodies. The antigenic specificity of $^1O_2$-modified plasmid DNA was ascertained by direct binding and inhibition ELISA. The IgG was found to have high affinity for the immunogen, as only 8.6 μg/ml of immunogen was required to inhibit 50% of the antibody activity. Band shift assay further confirmed the high affinity of anti-$^1O_2$-modified plasmid DNA IgG towards the immunogen. The data indicate higher specificity of the immune IgG towards the $^1O_2$-modified epitopes. Exposure to $^1O_2$ might have generated potential epitopes against which the antibodies were raised. It is therefore postulated that in chronic inflammatory disease, ROS generated by phagocytic cells may cause damage to DNA generating neo-epitopes leading to the production of antibodies cross reacting with native DNA. Binding of induced antibodies against $^1O_2$-modified plasmid DNA, with an array of nucleic acids antigens demonstrates their heterogeneous antigen binding characteristics. It can be mentioned here that SLE anti-DNA autoantibodies as well exhibit polyspecificity with respect to antigen binding [10, 35].

SLE and rheumatoid arthritis are characterized by varied clinical manifestations and the production of high titer of autoantibodies. Oxidative damage to DNA serves several pathogenic functions in SLE and RA, thereby implying the role of oxidative stress in the etiology of both these rheumatic diseases [36, 37]. ROS are tumorigenic by virtue of their ability to increase cell proliferation, survival, cellular migration, and also by inducing DNA damage leading to genetic lesions that initiate tumorigenicity and sustain subsequent tumor progression. Carcinoma cells produce ROS at elevated rates *in vitro*, and *in vivo* many tumors appear persistent to oxidative stress [25]. The higher recognition of SLE, RA, and cancer autoantibodies by $^1O_2$-modified plasmid DNA as compared to the native analog clearly demonstrates that the modified form is a better antigen for the naturally occurring autoantibodies in these diseases, suggesting the role of $^1O_2$-induced neo-epitopes in the modified plasmid DNA. This data also confirms the overproduction of ROS in SLE, RA, and various cancers and points out $^1O_2$-modified plasmid DNA as a new diagnostic tool for studying the role of ROS in various other diseases where production of ROS is prevalent.

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