MATERIALS

Calf thymus DNA, nuclease S1, micrococcal nuclease, riboflavin, superoxide dismutase, bovine serum albumin, agarose, anti-human/anti-rabbit IgG-alkaline phosphatase and FITC conjugates, ethidium bromide, Coomassie Brilliant Blue G-250 and R-250, sodium dodecyl sulphate, Freund’s complete and incomplete adjuvants, methylated bovine serum albumin, Tween-20, Triton X-100, nitroblue tetrazolium, sodium azide, para-nitrosodimethyl aniline were purchased from Sigma Chemical Company, U.S.A. Protein-A agarose from Genei, synthetic polynucleotides and Ficoll 400 were obtained from Pharmacia Fine Chemicals, Sweden. EDTA (disodium salt), chloroform and isoamyl alcohol were from Qualigens, India.

Polystyrene microtitre flat bottom ELISA plates having 96 wells (7 mm diameter) were purchased from NUNC, Denmark. QIAamp blood midi kit from Qiagen, p-nitrophenyl phosphate and Folin-Ciocalteau reagent were obtained from Centre for Biochemical Technology, New Delhi. Acrylamide, ammonium persulphate, bisacrylamide, N,N,N',N'-tetramethylethylene diamine (TEMED) were from Bio-Rad Laboratory U.S.A. EDTA (disodium salt), hydrogen peroxide, chloroform, isoamyl alcohol, methanol, glacial acetic acid were from Qualigens, India. Diphenylamine and ethanol were chemically pure. All other chemicals were of highest analytical grade available.

Equipments

Shimadzu UV-240 Spectrophotometer equipped with thermo-programmer and controller unit, ELISA microplate reader, Elico pH meter model L1-120, ultraviolet lamp (Vilber Lourmat, France), agarose gel electrophoresis assembly (GNA-100); Beckman ultracentrifuge, Avanti 30 table top high speed refrigerated centrifuge (Beckman, U.S.A.); polyacrylamide gel electrophoresis assembly (Bio-Rad, U.S.A.) and fluorimeter (Hitachi, Japan) were the major equipments used in this study.
Collection of sera

SLE sera were obtained from outdoor and indoor patients of the Department of Medicine, All India Institute of Medical Sciences, New Delhi, showing high titre anti-DNA antibodies and fulfilled the American College of Rheumatology criteria for the diagnosis of SLE (Arnett et al., 1988). Normal human sera were obtained from healthy individuals and stored in small aliquots at -20°C. Sera of cancer patients proven with histopathological diagnosis were obtained from J.N. Medical College Hospital A.M.U., Aligarh. Sera were decomplemented by heating at 56°C for 30 min and stored in aliquots at -20°C.

METHODS

Purification of calf thymus DNA

Commercially obtained calf thymus DNA was purified free of proteins and single stranded regions as described by Ali et al. (1985). DNA (2 mg/ml) was dissolved in 0.1 X SSC buffer (15 mM sodium citrate and 150 mM sodium chloride, pH 7.3) and then mixed with an equal volumes of chloroform-isooamyl alcohol (24:1) in a stoppered container and gently mixed occasionally for 1 hr. The aqueous layer containing DNA was separated from the organic layer and re-extracted with chloroform-isooamyl alcohol. The DNA was precipitated with two volumes of cold absolute ethanol and collected on a glass rod. After drying in air, the DNA was dissolved in acetate buffer (30 mM sodium acetate containing 30 mM zinc chloride, pH 5.0) and treated with nuclease S1 (150 units/mg DNA) at 37°C for 30 min to remove single stranded regions. The reaction was stopped by adding one-tenth volume of 200 mM EDTA, pH 8.0. The nuclease S1 treated DNA was extracted twice with chloroform-isooamyl alcohol and finally precipitated with two volumes of cold ethanol. The precipitate was dissolved in phosphate-buffered saline (PBS) (10 mM sodium phosphate containing 150 mM sodium chloride), pH 7.4.
Fragmentation of purified native calf thymus DNA

Micrococcal nuclease was used to digest purified DNA to obtain smaller fragments (Ali et al., 1985). Purified DNA (2 mg/ml) in 6 mM Tris, 100 mM NaCl and 2 mM CaCl₂, pH 8.0 was treated with 0.25 units of micrococcal nuclease per mg DNA at 37°C for 6 min. The reaction was stopped by adding one-tenth volume of 200 mM EDTA, pH 8.0. The mixture was extracted twice with chloroform-isooamyl alcohol and fragmented DNA was precipitated with cold absolute ethanol and dissolved in 30 mM acetate buffer, pH 5.0 containing 30 mM ZnCl₂. The DNA was digested with nuclease S1 (150 units/mg DNA) at 37°C for 30 min. The reaction was stopped with one-tenth volume of 200 mM EDTA, pH 8.0. The digested DNA fragments were extracted twice with chloroform-isooamyl alcohol and precipitated with cold absolute ethanol. The DNA fragments were then dissolved in TBS (0.01 M Tris, 0.15 M NaCl) pH 8.0 and separated on the basis of size by gel filtration on Sepharose 4B column (46.0 cm x 1.2 cm) equilibrated with TBS. Fractions of 4 ml were collected. The size of the DNA fragments was determined by PAGE.

Polyacrylamide gel electrophoresis

Nucleic acid samples were subjected to PAGE under non-denaturing conditions as described by Laemmli (1970). The following stock solutions were prepared:

(a) Acrylamide-bisacrylamide (30:0.8)

30 gm acrylamide and 0.8 gm bisacrylamide was dissolved in distilled water to a final volume of 100 ml. The solution was filtered and stored at 4°C in an amber colored bottle.

(b) Resolving gel buffer

36 gm Tris was dissolved in 48 ml of 1 N HCl, pH adjusted to 8.8 and final volume made upto 100 ml with distilled water.
(c) Electrode buffer

The electrophoretic buffer used was TAE, pH 7.9 (40 mM Tris, 1.14 ml glacial acetic acid and 1 mM EDTA).

(d) Procedure

The PAGE assembly was set up and the glass plates separated by 1.5 mm thick spacer were sealed with 1% agarose from the sides and bottom. The non-denaturing gel was prepared and poured between the glass plates and allowed to polymerize at room temperature. Nucleic acid samples were mixed with one-tenth volume of ‘stop mix’ (30% Ficoll, 0.025% xylene cyanol FF and 500 mM EDTA in 10 times concentrated TAE buffer) and applied onto the gel. The gel was electrophoresed for 8-10 hr at room temperature at 80 volts, stained with ethidium bromide (0.5 µg/ml) and visualised under UV light.

Recipe for 7.5% non-denaturing PAGE

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>1.5% ammonium persulphate</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>23.5 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>20.0 µl</td>
</tr>
</tbody>
</table>

Polyacrylamide gel electrophoresis for proteins

Polyacrylamide slab gel electrophoresis was performed under denaturing conditions as described by Laemmli (1970). The following stock solutions were prepared:
(a) Acrylamide-bisacrylamide (30:0.8)

A stock solution was prepared by dissolving 30 gm of acrylamide and 0.8 gm bisacrylamide in distilled water to a final volume of 100 ml.

(b) Resolving gel buffer

A stock solution was prepared by dissolving 36.3 gm Tris base in 48.0 ml of 1N HCl. The contents were mixed, pH adjusted to 8.8 and the final volume brought to 100 ml with distilled water.

(c) Stacking gel buffer

6.05 gm Tris was dissolved in 40 ml distilled water, pH titrated to 6.8 with 1N HCl and the final volume adjusted to 100 ml with distilled water.

(d) Electrode buffer

3.03 gm Tris, 14.4 gm glycine and 1.0 gm SDS were dissolved in distilled water, pH adjusted to 8.3 and final volume made up to one litre.

(e) Procedure

Glass plates, separated by 1.5 mm thick spacer were sealed with 1% agarose. The resolving gel mixture was prepared by mixing the components in the appropriate volume and poured into the space between the glass plates leaving sufficient space at the top for the stacking gel. After the polymerization of separating gel, stacking gel mixture was poured and allowed to solidify. Protein samples containing 10% glycerol and 0.002% bromophenol blue were applied and electrophoresis was carried out at 60V for 6-8 hrs. Staining of the gel was achieved with 0.1% Coomassie Brilliant Blue R-250 (in 25% isopropanol and 10% glacial acetic acid). Destaining was carried out in a 10% mixture each of acetic acid and methanol.
Recipe for 7.5% SDS-PAGE

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Stacking gel</th>
<th>Resolving gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide</td>
<td>1.25 ml</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>2.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>-</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>5.65 ml</td>
<td>16.95 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1 ml</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>1.5% ammonium persulphate</td>
<td>0.5 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.75 μl</td>
<td>15.0 μl</td>
</tr>
</tbody>
</table>

_Determination of DNA concentration_

DNA concentration was estimated colorimetrically by the method of Burton (1956) using diphenylamine reagent.

(a) Crystallization of diphenylamine

Diphenylamine (2 g) was dissolved in 200 ml boiling hexane. After adding 0.5 g of activated charcoal, the hot mixture was filtered through Whatman No 1 filter paper and the filtrate was kept overnight at 4°C and dried at room temperature before use.

(b) Preparation of diphenylamine reagent

750 mg of recrystallized diphenylamine was mixed with 50 ml of glacial acetic acid and 0.75 ml concentrated sulphuric acid. The reagent was prepared fresh before use.

(c) Procedure

One ml of DNA sample was mixed with 1.0 ml of 1N perchloric acid and incubated at 70°C for 15 min. 100 μl of 5.43 mM acetaldehyde was added followed by 2.0 ml of freshly prepared diphenylamine reagent. The contents were mixed and incubated at room temperature for 16-20 hr. Absorbance was read at 600 nm and the
concentration of DNA in unknown samples was determined from a standard plot of purified calf thymus DNA.

**Determination of protein concentration**

Protein was estimated by the methods of Lowry *et al.* (1951) and Bradford (1976).

**Protein estimation by Folin’s-phenol reagent**

The protein estimation by this method utilizes alkali (to keep the pH high), Cu$^{2+}$ ions (to chelate proteins) and tartarate (to keep the Cu$^{2+}$ ions in solution at high pH).

(a) **Folin-Ciocalteau’s reagent**

The reagent was purchased from Centre for Biochemical Technology, New Delhi and diluted 1:4 with distilled water before use.

(b) **Alkaline copper reagent**

The components of alkaline copper reagent were prepared as follows:

(i) Two percent sodium carbonate, 100 mM sodium hydroxide.

(ii) 0.5 percent copper sulphate in 1 percent sodium potassium tartarate.

The working reagent was prepared fresh before use by mixing components (i) and (ii) in the ratio of 50:1.

(c) **Procedure**

To 1 ml of protein sample was added 5 ml of alkaline copper reagent and incubated for 10 min at room temperature. 1 ml of working Folin-Ciocalteau’s reagent was added and the tubes were read at 660 nm after 30 min. The concentration of protein in unknown sample was determined from a standard plot of bovine serum albumin.

**Protein estimation by dye-binding method**

This assay is based on color change when Coomassie Brilliant Blue G-250 in acidic medium, binds strongly to protein hydrophobically and at positively charged
groups (Bradford, 1976). In the environment of these positively charged groups, protonation is suppressed and a blue color is observed ($\lambda_{\text{max}}$=595 nm).

(a) Dye preparation

100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol and 100 ml of 85% (v/v) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre and filtered through Whatman No. 1 filter paper to remove undissolved particles.

(b) Protein assay

To 1 ml of solution containing 10-100 $\mu$g protein was added 5 ml of dye solution and contents mixed by vortexing. The absorbance was read at 595 nm after 5 min against a reagent blank.

Modification of calf thymus DNA by free radicals

Purified calf thymus DNA was modified by superoxide anion radical and singlet oxygen. Superoxide anion radical was detected by photosensitized reduction of nitroblue tetrazolium (NBT), leading to the formation of a blue colored product, nitroblue formazan (Nakayama et al, 1983). Production of superoxide radical was confirmed by monitoring the inhibition of formation of blue colored product in the presence of superoxide dismutase (SOD).

Formation of singlet oxygen was measured in aqueous solution by monitoring the bleaching of p-nitrosodimethylaniline (pRNO) (Kraljic and Moshni, 1978). Production of singlet oxygen was confirmed by monitoring the bleaching of pRNO in presence of sodium azide (NaN₃), a specific quencher of singlet oxygen.

DNA fragments of around 200 bp were modified by the method of Naseem et al. (1988). A total volume of 3.0 ml contained, 100 $\mu$g/ml DNA, 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA, 0.06% Triton X-100 and 40 $\mu$M riboflavin. Immediately after mixing, the reaction was carried out in the presence of UV light (365 nm) at room temperature. The samples were dialyzed extensively to remove riboflavin and Triton X-100.
Spectroscopic analysis

(a) The ultraviolet spectra of modified and unmodified 200 bp DNA were recorded in the wavelength range of 200-400 nm on a Shimadzu UV-240 spectrophotometer.
(b) The modification incurred on DNA fragments was also analyzed by UV-difference spectroscopy.
(c) Fluorescence emission spectroscopy of native and modified 200 bp DNA samples using ethidium bromide was performed on a fluorimeter.

Absorption – temperature scan

Thermal denaturation analysis of nucleic acids was performed in order to ascertain the degree of modification incurred on the nucleic acids by determining mid point melting temperature (Tm). Native and modified samples were subjected to heat denaturation on a Shimadzu UV-240 spectrophotometer coupled with a temperature programmer and controller assembly (Hasan and Ali, 1990). All the samples were melted from 30°C to 95°C at a rate of 1.5°C / min after 10 min equilibration at 30°C. The change in absorbance at 260 nm was recorded with increasing temperature. Percent denaturation was calculated as follows:

\[
\text{Percent denaturation} = \frac{A_T - A_{30}}{A_{\text{max}} - A_{30}} \times 100
\]

Where, \(A_T\) = Absorbance at a temperature T°C.
\(A_{\text{max}}\) = Final maximum absorbance on the completion of denaturation (95°C).
\(A_{30}\) = Initial absorbance at 30°C.

Nuclease S1 digestibility

Native and modified DNA were characterized by nuclease S1 digestibility (Matsuo and Ross, 1987). One microgram each of native and modified DNA in acetate buffer (30 mM each of sodium acetate and zinc chloride, pH 5.0) were treated with nuclease S1 (20 units/μg DNA) for 30 min at 37°C. The reaction was stopped by adding
one-tenth volume of 200 mM EDTA, pH 8.0. The digested and control samples were subjected to agarose gel electrophoresis.

(a) Gel preparation

Agarose (1%) in TAE buffer (40 mM Tris-acetate, pH 8.0 containing 2 mM EDTA) was dissolved by heating. The solution was cooled to about 50°C and then poured into gel tray and allowed to solidify at room temperature.

(b) Sample preparation and loading

Native and modified DNA samples treated with nuclease S1 were mixed with one-tenth volume of sample buffer (0.125% bromophenol blue, 30% Ficoll 400, 500 mM EDTA in 10X electrophoresis buffer). The samples were loaded in the wells and electrophoresed for 2 hr at 30 mA. The gels were stained with ethidium bromide (0.5 μg/ml), viewed by illumination under UV light and photographed.

Immunization schedule

Native and modified DNA (100 μg) were complexed with an equal volume (w/w) of methylated BSA and emulsified with an equal volume of complete Freund’s adjuvant and injected intramuscularly in female rabbits. Subsequent injections were given in incomplete Freund’s adjuvant. Each animal received a total of 800 μg of antigen in the course of 8 injections. Blood was collected from marginal vein of the ear, serum was separated and decoated by heating at 56°C for 30 minutes. Pre-immune serum was collected prior to immunization. The sera were stored in small aliquots at -20°C with 0.1% sodium azide as preservative.

Isolation of IgG by Protein-A agarose

Serum IgG was isolated by affinity chromatography on Protein-A agarose column. Serum (0.3 ml) diluted with equal volume of PBS, pH 7.4 was applied to column (12 x 45 mm) equilibrated with the same buffer. The wash through was recycled 2-3 times. Unbound IgG was removed by extensive washing with PBS, pH 7.4. The bound IgG was eluted with 0.58% acetic acid in 0.85% sodium chloride (Goding, 1976) and
neutralized immediately with 1 ml of 1M Tris-HCl, pH 8.5. Three ml fractions were collected and read at 251 and 278 nm. The IgG concentration was determined considering $1.4 \ OD_{280} = 1.0 \ mg \ IgG/ml$. The isolated IgG was dialyzed against PBS, pH 7.4 and stored at -20°C with 0.1% sodium azide.

**Immunological detection of antibodies**

Level of antibodies was evaluated by immunodiffusion, enzyme linked immunosorbent assay and gel retardation assay.

(a) **Immunodiffusion**

Immunodiffusion (ID) was carried out by Ouchterlony’s double diffusion system. Six ml of 0.4% molten agarose in PBS containing 0.1% sodium azide was poured on to a glass petridishes and allowed to solidify at room temperature. Wells of 5 mm diameter were cut into hardened gel and an appropriate concentration of antigen and antibody was placed in the wells. The petridishes were allowed to stand in a moist chamber at room temperature for 48-72 hr. The gels were washed with 5% sodium citrate to remove non-specific precipitin lines. The result was analyzed visually.

(b) **Enzyme linked immunosorbent assay**

The following reagents were prepared in distilled water and used in enzyme immunoassay.

(i) **Buffers and reagents**

**Tris buffered saline (TBS)**

10 mM Tris, 150 mM NaCl, pH 7.4

**Tris buffered saline-Tween 20 (TBS-T)**

20 mM Tris, 144 mM NaCl, 2.68 mM KCl

pH 7.4, containing 500 µl Tween 20/L.

**Carbonate-bicarbonate buffer**

15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6, containing 2 mM magnesium chloride.
**Citrate-phosphate buffer**

50 mM citric acid, 50 mM Na$_2$HPO$_4$, pH 5.0.

**Substrate**

500 μg p-nitrophenyl phosphate (p-NPP)/ml carbonate-bicarbonate buffer, pH 9.6.

(ii) **Procedure**

Antibodies were detected by ELISA using polystyrene microtitre plates as solid support (Aotsuka et al., 1979). One hundred microlitre of 2.5 μg/ml antigen in TBS, pH 7.4 was coated in test wells of microtitre plates, incubated for 2 hr at 37°C and overnight at 4°C. The antigen coated wells were washed three times with TBS-T to remove unbound antigen. Unoccupied sites were blocked with 150 μl of 1.5% BSA in TBS for 4 - 5 hrs at room temperature. The plates washed once with TBS-T and antibody (100 μl/well) to be tested, were diluted in TBS and added to each well. After 2 hr incubation at 37°C and overnight at 4°C, the plates were washed four times with TBS-T and an appropriate anti-immunoglobulin alkaline phosphatase conjugate was added to each well. After incubation at 37°C for 2 hr, the plates were washed four times with TBS-T and three times with distilled water and developed using p-nitrophenyl phosphate substrate respectively. The absorbance was recorded at 410 nm on an automatic microplate reader. Each sample was run in duplicate. The control wells were treated similarly but were devoid of antigen. Results were expressed as a mean of $A_{\text{test}} - A_{\text{control}}$.

**Competition ELISA**

The antigenic specificity of the antibodies was determined by competition ELISA (Hasan et al., 1991). Varying amount of inhibitors (0-20 μg/ml) were mixed with a constant amount of antiserum or IgG. The mixture was incubated at room temperature for 2 hr and overnight at 4°C. The immune complex thus formed was coated in the wells instead of the serum. The remaining steps were the same as in direct binding ELISA.
Percent inhibition was calculated using the formula

\[
\text{Percent inhibition} = 1 - \frac{A_{\text{inhibited}}}{A_{\text{uninhibited}}} \times 100
\]

**Band shift assay**

For the visual detection of antigen-antibody binding and immune complex formation, gel retardation assay was performed (Sanford et al., 1988). A constant amount of antigen (native and modified DNA) was incubated with varying amounts of IgG in PBS, pH 7.4 for 2 hr at 37°C and overnight at 4°C. One-tenth volume of ‘stop mix’ dye was added to the mixture and electrophoresed on 1% agarose for 2 hr at 30 mA in TAE (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA) buffer, pH 7.9. The gels were stained with ethidium bromide (0.5 μg/ml), visualized under UV light and photographed.

**Isolation of human-DNA**

Human DNA was isolated from whole blood using QIAamp Blood Midi Kit supplied by QIAGEN, U.S.A. Blood was collected in presence of anticoagulant (EDTA).

**Procedure**

To 2 ml of whole blood was added 200 μl protease stock solution and 2.4 ml buffer AL. Contents were mixed by vortexing and incubated at 70°C for 10 min. Two ml ethanol (95-100%) was added the contents were again mixed by vortexing. Half of the solution (~3.3 ml) was transferred into the Midi column with centrifugation tube and was spinnned at 3000 rpm for 3 min. The filtrate was discarded. The remaining half of the sample was transferred to the same Midi Column and the whole procedure repeated. The Midi column was washed with 2 ml of buffer AW1 and centrifuged at 5000 rpm for 1 min. Two ml of buffer AW2 was added and the column centrifuged again at 5000 rpm for 15. The Midi column was placed in a fresh 15 ml centrifuge tube. 300 μl of buffer AE was added onto the Midi column, incubated at room temperature for 5 min and centrifuged at 5000 rpm for 5 min. This step was repeated once with 300 μl buffer AE. The pooled filtrate contain human DNA. The absorbance of DNA solution was read at 260 nm and 280 nm to ascertain its purity and concentration.
Photochemical generation of superoxide anion radical and singlet oxygen species

Singlet oxygen and superoxide anion radicals were generated by illumination of riboflavin with UV light (365 nm). The generation of superoxide anion was monitored by nitroblue tetrazolium (NBT) assay. In order to optimize the superoxide generation, the time of illumination and dose dependent response of riboflavin and Triton X-100 was studied. The results showed an increase in absorbance at 560 nm with increasing time period of illumination. It is evident from the curve that 20 min of illumination of UV light caused optimum generation of superoxide radical (Fig. 2). Similarly, dose dependent experiments were carried out with varying amounts of riboflavin (Fig. 3) and Triton X-100 (Fig. 4). The ability of riboflavin to form singlet oxygen (\(^{1}\text{O}_2\)) was determined by monitoring the bleaching of pRNO (Joshi, 1985; Fig. 5).

The generation of these radicals was further confirmed by the use of their respective quenchers i.e. SOD for superoxide anion radical (\(\text{O}_2^{-}\)) and sodium azide for singlet oxygen (\(^{1}\text{O}_2\)). The evidence for the formation of \(^{1}\text{O}_2\) was obtained by examining the progress of the reaction in presence of sodium azide (NaN\(_3\)), a specific quencher for \(^{1}\text{O}_2\). A near complete inhibition of \(^{1}\text{O}_2\) production was observed (Fig. 6). Dose dependent experiment was carried out with varying concentration of NaN\(_3\) (0, 10, 15, 25, 50, 75, 100 mM). Maximum inhibition was obtained at 100 mM (Fig. 7). Superoxide dismutase was used to further confirm the production of superoxide anion radical. No inhibition in the formation of blue coloured product (nitroblue formazan) was observed due to the presence of singlet oxygen in the system (Fig. 8). Inhibition in the formation of nitroblue formazan was observed when both SOD and NaN\(_3\), quencher of singlet were used (Fig. 9).

Commercially available calf thymus DNA was purified free of proteins, RNA and single stranded regions and digested with micrococcal nuclease to obtain DNA fragments. The digested DNA was subjected to gel filtration on Sepharose 4B column (Fig. 10). Alternate fractions on PAGE resulted in DNA of varying size (Fig. 10 inset). The fraction(s) of 200 bp (average size) was selected for these studies.
Fig. 2. Time dependent generation of superoxide ($O_2^-$) anion radical by riboflavin in light ($\Delta$), in dark (●), without Triton X-100 (○), without riboflavin (▲).
Fig. 3. Superoxide anion radical generation under varying concentrations of riboflavin.
Fig. 4. Superoxide anion radical generation under varying concentrations of Triton X-100.
Fig. 5. Time dependent production of singlet oxygen ($^1$O$_2$) by riboflavin in light (O), riboflavin in dark (●), pRNO alone in light (△), pRNO alone in dark (▲).
Fig. 6. Effect of sodium azide (NaN₃) on generation of $^1$O₂ by riboflavin in light (○) and in dark (□); with pRNO in light (△), and in dark (●); with NaN₃ in light (▲) and in dark (▼).
Fig. 7. Quenching of $^{1}$O$_{2}$ with increasing concentrations of sodium azide.
Fig. 8. Quenching of $\cdot O_2^-$ with increasing concentrations of SOD.
Fig. 9. Quenching of $^1O_2$ and $O_2^-$ with increasing concentrations of SOD, keeping NaN$_3$ constant.
Fig. 10. Elution profile of micrococcal nuclease digested calf thymus DNA on Sepharose 4B column. Inset: Polyacrylamide gel electrophoresis of fractionated DNA. Lanes (3-10) represent alternate fractions from 30-44, while lanes 1 contained 100 bp DNA ladder as marker.
Modification of DNA by superoxide anion radical and singlet oxygen species

DNA fragments of average size of 200 bp in 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA, 0.06% Triton X-100, 40 μM riboflavin was illuminated for 1 hr under 365 nm UV light at room temperature. The $^{1}\text{O}_2^{-}\text{O}_2^{-}$-DNA samples were dialyzed against PBS, pH 7.4 to remove riboflavin and Triton X-100.

Characterization of $^{1}\text{O}_2^{-}\text{O}_2^{-}$-DNA

Figure 11 shows the UV absorption spectra of superoxide radical and singlet oxygen species modified DNA with characteristic hyperchromicity. The spectra shows a shift of approximately 10 nm towards longer wavelength side at both $\lambda_{\text{max}}$ and $\lambda_{\text{min}}$ for $^{1}\text{O}_2^{-}\text{O}_2^{-}$-DNA relative to native DNA. The percent hyperchromicity was 30% in 1 hr illuminated sample.

The modifications incurred on nDNA fragments was also analyzed by UV difference spectroscopy (Fig. 11 inset). The spectral curve exhibited an appreciable negative inversion in absorption between 240-260 nm, followed by increased absorption at 270 nm. Moreover the spectral curve also exhibited a shoulder at around 290 nm. Elimination of the characteristic 260 nm peak is indicative for the loss of the double helical structure of DNA. These changes in the curve are also indicative of the possible generation of single stranded regions and ring opening of the nucleic acid bases as a consequence of the modification.

Native and $^{1}\text{O}_2^{-}\text{O}_2^{-}$-DNA was used to record fluorescence spectra. The decrease in the fluorescence intensity was observed probably indicating the generation of strand breaks due to lesser amount of ethidium bromide intercalation in modified DNA as compared to native DNA (Fig. 12).

DNA was modified in presence and absence of NaN₃. Figure 13 shows the UV absorption spectra of native DNA, $^{1}\text{O}_2^{-}\text{O}_2^{-}$-DNA and $^{1}\text{O}_2^{-}\text{O}_2^{-}$-DNA in presence of NaN₃. Approximately 50% quenching in the absorbance was observed.
Fig. 11. Ultraviolet absorption spectra of 200 bp native DNA and \(^1\text{O}_2-\text{O}_2^-\)-DNA. Native DNA (—), \(^1\text{O}_2-\text{O}_2^-\)-DNA (----). Inset: Ultraviolet difference spectroscopical scanning of \(^1\text{O}_2-\text{O}_2^-\)-DNA, native DNA served as control.
Fig. 12. Fluorescence emission spectra of EtBr (-----), native DNA (—) and $^{1}O_2$-$O_2^-$ DNA (---). The samples were excited at 300 nm.
Fig. 13. UV absorption spectra of 200 bp DNA modified in presence of sodium azide (100 mM), a specific quencher of $^1\text{O}_2$. 200 bp DNA (—), modified 200 bp DNA (—), modified DNA in presence of NaN₃ (— —).
Thermal melting of $^{1}$O$_2$-O$_2^{-}$-DNA

Thermally induced transitions were measured spectrophotometrically at 260 nm by heating nucleic acid samples at a rate of 1.5°C per min. Melting curves were recorded at temperatures from 30°C to 95°C. Increase in UV absorption at 260 nm was taken as a measure of denaturation. Figure 14 shows the thermal denaturation profile of native and $^{1}$O$_2$-O$_2^{-}$-DNA. The melting temperature of native DNA at which 50% of the double helical structure is lost was found to be 86°C, while in case of $^{1}$O$_2$-O$_2^{-}$-DNA it was found to be 78°C. The results exhibit a net decrease of 8°C in the Tm value for the $^{1}$O$_2$-O$_2^{-}$-DNA when compared to its unmodified native conformer. These findings indicate, therefore, that the decrease in Tm to the extent of 8°C is primarily due to structural alteration of DNA which occurs upon generation of single strand breaks and base modifications. The percent hyperchromicity and the values of native and $^{1}$O$_2$-O$_2^{-}$-DNA are shown in Table 4.

Nuclease S1 digestibility

Native and $^{1}$O$_2$-O$_2^{-}$-DNA were digested with nuclease S1 (20 units/μg DNA) for 30 min. The resulting DNA sample was subjected to agarose gel electrophoresis. The controls were the unmodified DNA samples with and without nuclease S1 treatment. The results showed substantially decreased intensity in case of S1 treated $^{1}$O$_2$-O$_2^{-}$-DNA compared to non-S1 treated control. However, some loss in fluorescence intensity was observed in unmodified native DNA (Fig. 15).

Antigenicity of $^{1}$O$_2$-O$_2^{-}$-DNA

The antigenicity of the $^{1}$O$_2$-O$_2^{-}$-DNA was probed by inducing antibodies in rabbit. The induction of antibodies and their specificity was assayed by direct binding and competition ELISA. The binding of these antibodies to the immunogen and native DNA was further substantiated by band shift assay.

The antibodies raised against $^{1}$O$_2$-O$_2^{-}$-DNA was found to be non-precipitating in immunodiffusion. Direct binding ELISA was used to characterize the immune response
Fig. 14. Thermal melting profile of native DNA (O) and $^{1}$O$_2$-O$_2$-DNA (●).
**TABLE – 4**

_Ultraviolet and thermal denaturation characteristics of native and $^{1}$O$_2$-O$_2^-$-DNA under identical experimental condition_

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Native DNA</th>
<th>$^{1}$O$_2$-O$_2^-$-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance ratio ($A_{260}/A_{280}$)</td>
<td>1.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Percent hyperchromicity at 95°C</td>
<td>36.7</td>
<td>17.2</td>
</tr>
<tr>
<td>Melting temperature (Tm), °C</td>
<td>86.0</td>
<td>78.0</td>
</tr>
<tr>
<td>Onset of duplex melting, °C</td>
<td>65.0</td>
<td>58.0</td>
</tr>
</tbody>
</table>
Fig. 15. Nuclease S1 digestibility of native and $^{1}\text{O}_2^{-}\text{O}_2^{-}$-DNA. Lane 1 contained native DNA, while lane 2 contained native DNA treated with nuclease S1. Lane 3 contained $^{1}\text{O}_2^{-}\text{O}_2^{-}$-DNA, while lane 4 contained $^{1}\text{O}_2^{-}\text{O}_2^{-}$-DNA treated with nuclease S1. Electrophoresis was carried out on 1% agarose gel for 2 hr at 30 mA.
in rabbits following immunization with $^1\text{O}_2\text{O}_2^{-}$-DNA. The anti-serum showed a titre of 1:25600 (Fig. 16). The antibodies against native DNA complexed with methylated BSA was also raised in rabbits. The results evaluated by direct binding ELISA showed negligible binding. Preimmune serum as control did not show appreciable binding with the immunogen. The specificity of the induced antibodies for antigenic determinants on $^1\text{O}_2\text{O}_2^{-}$-DNA was evaluated by competitive binding assays. A maximum of 70% inhibition in antibody binding was recorded at an inhibitor ($^1\text{O}_2\text{O}_2^{-}$-DNA) concentration of 20 $\mu$g/ml (Fig. 17).

(a) Purification and binding characteristics of immune IgG

Immunoglobulin G was isolated from preimmune and immune rabbit serum by affinity chromatography on Protein- A Agarose column (Fig. 18). The purity of the IgG was evaluated by SDS-polyacrylamide gel electrophoresis in absence of a reducing agent. The purified IgG migrated as a single band upon electrophoresis (Fig. 18 inset).

Direct binding ELISA of the purified IgG showed a strong reactivity with the immunogen (Fig. 19). Preimmune IgG as negative control showed negligible binding to $^1\text{O}_2\text{O}_2^{-}$-DNA.

(b) Antigenic specificity of anti-$^1\text{O}_2\text{O}_2^{-}$-DNA antibodies

The anti-$^1\text{O}_2\text{O}_2^{-}$-DNA antibodies exhibited a broad spectrum of reactivity as demonstrated by inhibition assay using the immunogen, nucleic acids, synthetic polynucleotides, chondroitin sulphate, cardiolipin and chromatin as inhibitors. A maximum of 84.5% inhibition of anti-$^1\text{O}_2\text{O}_2^{-}$-DNA IgG with immunogen as an inhibitor was observed (Fig. 20). Fifty percent inhibition was achieved at an inhibitor concentration of 1.9 $\mu$g/ml. Competition experiments with native DNA showed considerable inhibition in antibody activity. Pre-incubation of immune antibody with native DNA inhibited its binding to $^1\text{O}_2\text{O}_2^{-}$-DNA by 47.8% at 20 $\mu$g/ml (Fig. 20). ROS-DNA, 200 bp DNA showed a maximum inhibition of 50.25%, 32.3% at 20 $\mu$g/ml, respectively (Fig. 20).
Fig. 16. Direct binding ELISA of $^{1}O_{2}$-$O_{2}^{-}$-DNA with preimmune (△) and immune sera (▲). Microtitre plate was coated with $^{1}O_{2}$-$O_{2}^{-}$-DNA (2.5 μg/ml).
Fig. 17. Inhibition ELISA of immune (O) and preimmune (Δ) serum antibodies with $^{1}\text{O}_2-\text{O}_2^-$-DNA. The microtitre plate was coated with $^{1}\text{O}_2-\text{O}_2^-$-DNA (2.5 µg/ml) and the serum dilution was 1:100.
Fig. 18. Elution profile of anti-\(^{1}O_2\)-G\(^.-\)-DNA IgG on Protein - A Agarose column. Inset: SDS-PAGE of purified IgG on 7.5% polyacrylamide gel.
Fig. 19. Direct binding ELISA with affinity purified preimmune (△) and immune IgG (▲) with $^1O_2-O_2^-\text{-DNA}$. The microtitre plate was coated with $^1O_2-O_2^-\text{-DNA}$ (2.5 μg/ml).
Fig. 20. Inhibition of immune IgG binding to $^{1}$O$_{2}$ -O$_{2}^{−}$-DNA. The competitors were $^{1}$O$_{2}$ -O$_{2}^{−}$-DNA (●), native calf thymus DNA (△), 200 bp DNA (○), $^{·}$OH-modified DNA (▲). The microtitre plate was coated with $^{1}$O$_{2}$ -O$_{2}^{−}$-DNA (2.5 μg/ml).
Native chromatin, ROS-chromatin, cardiolipin and chondroitin sulphate were used as inhibitors of antibody activity. Native chromatin and ROS-chromatin showed a maximum inhibition of 7.9% and 12.5% respectively. Cardiolipin and chondroitin sulphate inhibited antibody binding to antigen to the extent of 37% and 48%, respectively (Fig. 21).

To further define the structural determinants recognized by anti-$^{2}$-O$_2$-O$_2$'-DNA antibodies, their interactions with various synthetic polymers were studied. Poly(dA-dU).poly(dA-dU) showed maximum inhibition of 12.7% at a concentration of 20 µg/ml, whereas, poly(dI-dC).poly(dI-dC) and poly(dA-dT).poly(dA-dT) showed maximum inhibitions of 38.7% and 44.4%, respectively (Fig. 22). Table 5 summarises the results of the inhibition studies of the anti-$^{2}$-O$_2$-O$_2$'-DNA antibodies with various inhibitors.

**Band shift assay**

The visual detection of antigen antibody interaction was performed by band shift assay. Constant amounts of native and $^{2}$-O$_2$-O$_2$'-DNA were incubated with varying amounts of immune IgG for 2 hr at room temperature and overnight at 4°C. The resulting immune complexes were then electrophoresed on 1% agarose for 2 hr at 30 mA. Figure 23 shows the binding of IgG to $^{2}$-O$_2$-O$_2$'-DNA. As clearly evident, with an increase in the amount of IgG, there was an increase in the formation of high molecular weight immune complexes, which resulted in retarded mobility with a subsequent decrease in the fluorescence intensity of antigen.

**Binding characteristics of human anti-DNA autoantibodies to $^{2}$-O$_2$-O$_2$'-DNA**

The binding pattern of SLE anti-DNA autoantibodies to native and $^{2}$-O$_2$-O$_2$'-DNA was determined by direct binding ELISA. All the 24 sera showed appreciable binding to native and $^{2}$-O$_2$-O$_2$'-DNA. Preferentially high binding was observed in case of $^{2}$-O$_2$-O$_2$'-DNA. Figures 24 and 25 shows the enhanced binding to SLE anti-DNA autoantibodies (1:100 serum dilution) with $^{2}$-O$_2$-O$_2$'-DNA as compared to native DNA. With normal human sera no appreciable binding was observed.
Fig. 21. Inhibition of immune IgG binding to $^{1}\text{O}_2$-$\text{O}_2^{-}$-DNA. The competitors were cardiolipin (●), chondroitin sulphate (△), chromatin (▲), \textsuperscript{1}OH-modified chromatin (O). The microtitre plate was coated with $^{1}\text{O}_2$-$\text{O}_2^{-}$-DNA (2.5 µg/ml).
Fig. 22. Inhibition of immune IgG binding to $^1\text{O}_2$-$\text{O}_2^-$-DNA. The competitors were poly(dI-dC).poly(dI-dC) (△), poly(dA-dU).poly(dA-dU) (▲), poly(dA-dT).poly(dA-dT) (O). The microtitre plate was coated with $^1\text{O}_2$-$\text{O}_2^-$-DNA (2.5 μg/ml).
# TABLE – 5

**Antigenic specificity of $^{1}O_2$-$O_2^-$-DNA IgG**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Maximum % inhibition at 20 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{1}O_2$-$O_2^-$-DNA</td>
<td>84.5</td>
</tr>
<tr>
<td>Native DNA</td>
<td>47.8</td>
</tr>
<tr>
<td>200 bp DNA</td>
<td>32.3</td>
</tr>
<tr>
<td>ROS-200 bp DNA</td>
<td>50.25</td>
</tr>
<tr>
<td>Poly (dA-dU). poly (dA-dU)</td>
<td>12.7</td>
</tr>
<tr>
<td>Poly (dI-dC). poly (dI-dC)</td>
<td>38.7</td>
</tr>
<tr>
<td>Poly (dA-dT). poly (dA-dT)</td>
<td>44.4</td>
</tr>
<tr>
<td>Chondroitin sulphate</td>
<td>48.0</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>37.0</td>
</tr>
<tr>
<td>Native chromatin</td>
<td>7.9</td>
</tr>
<tr>
<td>ROS modified chromatin</td>
<td>12.5</td>
</tr>
</tbody>
</table>

The microtitre plates were coated with modified DNA (2.5 μg/ml).
Fig. 23. Band shift assay of anti-\(^{1}\text{O}_2-\text{O}_2^+\)-DNA IgG binding to (a) native DNA and (b) \(^{1}\text{O}_2-\text{O}_2^+\)-DNA. Native DNA and \(^{1}\text{O}_2-\text{O}_2^+\)-DNA (1 µg) each were incubated with 10, 20, 40, and 60 µg of IgG for 2 hr at 37°C and overnight at 4°C. Electrophoresis was performed on 1% agarose for 2 hr at 30 mA. Lane 1, contains native or \(^{1}\text{O}_2-\text{O}_2^+\)-DNA, while lanes 2, 3, 4, and 5 contain native or \(^{1}\text{O}_2-\text{O}_2^+\)-DNA with increasing concentrations of IgG.
Fig. 24. Direct binding ELISA of SLE sera to native DNA (■□), and $^{1}\text{O}_2$-$\text{O}_2$-DNA (□□). Normal human sera (NHS) served as negative control. Serum dilution was 1:100.
Fig. 25. Direct binding ELISA of SLE sera to native DNA ( ), and $^1\text{O}_2-\text{O}_2^-$-DNA ( ). Normal human sera (NHS) served as negative control. Serum dilution was 1:100.
Antigenic specificity of SLE autoantibodies

Competition ELISA was also performed to further evaluate the specificity of each SLE sera for native and $^{1'}\text{O}_2$-$\text{O}_2^\cdot$-DNA (Figs 26-37). The antibody was incubated with increasing concentrations (0-20 $\mu$g/ml) of inhibitors (native or $^{1'}\text{O}_2$-$\text{O}_2^\cdot$-DNA). Out of 24 SLE sera tested $^{1'}\text{O}_2$-$\text{O}_2^\cdot$-DNA showed higher degree of recognition by 17 sera and inhibited their activity to a maximum of 77%. On the other hand native DNA was found to be less reactive for the same samples showing a maximum inhibition of 50%. However, one sample showed greater recognition towards native DNA as compared to $^{1'}\text{O}_2$-$\text{O}_2^\cdot$-DNA. The results of this study, summarised in Table 6 shows $^{1'}\text{O}_2$-$\text{O}_2^\cdot$-DNA as a better inhibitor for 23 SLE sera.

Purification of SLE and cancer sera IgG

SLE and cancer sera IgG were purified by affinity chromatography on Protein- A Agarose. Protein A has been known to bind IgG from most of the mammalian species. The purified IgG eluted in a single symmetrical peak (Fig. 38). Their purity was checked by SDS-PAGE under non-reducing conditions which resulted in a single band (Fig. 38 inset).

Band shift assay

Band shift assay was employed to visualize the interaction of native and $^{1'}\text{O}_2$-$\text{O}_2^\cdot$-DNA with SLE IgG. A constant amount (1 $\mu$g) of antigen was incubated with varying amounts of SLE IgG (0-80 $\mu$g) for 2 hr at 37°C and overnight at 4°C. These immune complexes were then electrophoresed on 1% agarose for 2 hr at 30 mA. An increase in the IgG concentration caused an increase in the immune complex formation which resulted in a relative increase in the molecular weight and consequently retarded mobility (Fig. 39).
Fig. 26. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (1 and 2). The inhibitors used were native DNA (Δ, O), and $^{1}O_2$-O$_2^-$-DNA (▲, ●). The microtitre plates were coated with $^{1}O_2$-O$_2^-$-DNA (2.5 μg/ml) and serum dilution was 1:100.
Fig. 27. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (3 and 4). The inhibitors used were native DNA (Δ,O), and $^{1}\text{O}_2^{-}\text{O}_2^{-}$-DNA (▲,●). The microtitre plates were coated with $^{1}\text{O}_2^{-}\text{O}_2^{-}$-DNA (2.5 μg/ml) and serum dilution was 1:100.
Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (5 and 6). The inhibitors used were native DNA (Δ, O), and $^{1}$O$_{2}$-O$_{2}^{-}$-DNA (▲, ●). The microtitre plates were coated with $^{1}$O$_{2}$-O$_{2}^{-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.
Fig. 29. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (7 and 8). The inhibitors used were native DNA (△, ○), and $\text{O}_2^-\text{O}_2^-$-DNA (△, ●). The microtitre plates were coated with $\text{O}_2^-\text{O}_2^-$-DNA (2.5 μg/ml) and serum dilution was 1:100.
Fig. 30. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (9 and 10). The inhibitors used were native DNA (Δ, ○), and \(^{1}O_{2}-O_2^-\)-DNA (▲, ●). The microtitre plates were coated with \(^{1}O_{2}-O_2^-\)-DNA (2.5 μg/ml) and serum dilution was 1:100.
Fig. 31. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (11 and 12). The inhibitors used were native DNA (Δ, O), and $^{1}$O$_{2}$-O$_{2}^{−}$-DNA (▲, ●). The microtitre plates were coated with $^{1}$O$_{2}$-O$_{2}^{−}$-DNA (2.5 μg/ml) and serum dilution was 1:100.
Fig. 32. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (13 and 14). The inhibitors used were native DNA (Δ, O), and $^{1}O_{2}^{-}$-DNA (▲, ○). The microtitre plates were coated with $^{1}O_{2}^{-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.
Fig. 33. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (15 and 16). The inhibitors used were native DNA (△, ○), and \( ^1\text{O}_2 - ^2\text{O}_2^- \)-DNA (▲, ●). The microtitre plates were coated with \( ^1\text{O}_2 - ^2\text{O}_2^- \)-DNA (2.5 µg/ml) and serum dilution was 1:100.
Fig. 34. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (17 and 18). The inhibitors used were native DNA (△, O), and \(^{1}O_{2}^{-}\)-DNA (▲, ●). The microtitre plates were coated with \(^{1}O_{2}^{-}\)-DNA (2.5 μg/ml) and serum dilution was 1:100.
Fig. 35. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (19 and 20). The inhibitors used were native DNA (Δ, O), and $^{1}\text{O}_2-\text{O}_2^-$-DNA (▲, ●). The microtitre plates were coated with $^{1}\text{O}_2-\text{O}_2^-$-DNA (2.5 μg/ml) and serum dilution was 1:100.
Fig. 36. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (21 and 22). The inhibitors used were native DNA (Δ, O), and $^{15}$O$_2$-O$_2^-$-DNA (▲, ●). The microtitre plates were coated with $^{15}$O$_2$-O$_2^-$-DNA (2.5 μg/ml) and serum dilution was 1:100.
Fig. 37. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (23 and 24). The inhibitors used were native DNA (△,○), and ¹⁰₂⁻O₂⁻-DNA (▲,●). The microtitre plates were coated with ¹⁰₂⁻O₂⁻-DNA (2.5 µg/ml) and serum dilution was 1:100.
<table>
<thead>
<tr>
<th>Sera no.</th>
<th>Maximum percent inhibition at (20 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native DNA</td>
</tr>
<tr>
<td>1</td>
<td>39.0</td>
</tr>
<tr>
<td>2</td>
<td>28.3</td>
</tr>
<tr>
<td>3</td>
<td>15.0</td>
</tr>
<tr>
<td>4</td>
<td>27.0</td>
</tr>
<tr>
<td>5</td>
<td>29.0</td>
</tr>
<tr>
<td>6</td>
<td>46.0</td>
</tr>
<tr>
<td>7</td>
<td>48.0</td>
</tr>
<tr>
<td>8</td>
<td>23.7</td>
</tr>
<tr>
<td>9</td>
<td>35.4</td>
</tr>
<tr>
<td>10</td>
<td>16.0</td>
</tr>
<tr>
<td>11</td>
<td>23.5</td>
</tr>
<tr>
<td>12</td>
<td>26.0</td>
</tr>
<tr>
<td>13</td>
<td>50.0</td>
</tr>
<tr>
<td>14</td>
<td>30.0</td>
</tr>
<tr>
<td>15</td>
<td>34.7</td>
</tr>
<tr>
<td>16</td>
<td>46.0</td>
</tr>
<tr>
<td>17</td>
<td>23.3</td>
</tr>
<tr>
<td>18</td>
<td>14.4</td>
</tr>
<tr>
<td>19</td>
<td>27.0</td>
</tr>
<tr>
<td>20</td>
<td>12.0</td>
</tr>
<tr>
<td>21</td>
<td>16.0</td>
</tr>
<tr>
<td>22</td>
<td>25.0</td>
</tr>
<tr>
<td>23</td>
<td>16.0</td>
</tr>
<tr>
<td>24</td>
<td>28.0</td>
</tr>
</tbody>
</table>

Microtitre plates were coated with ¹O₂-O₂⁻-DNA (2.5 μg/ml).
Fig. 38. Elution profile of SLE (●) and lung cancer IgG (△) on Protein-A Agarose column. Inset: Purity of (a) isolated SLE IgG and (b) cancer IgG on 7.5% SDS-PAGE.
Fig. 39. Band shift assay of SLE IgG binding to (a) native DNA and (b) $^{1}{\text{O}}_{2}^{-}$DNA. Native DNA and $^{1}{\text{O}}_{2}^{-}$-DNA (1 μg) 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 $^{1}{\text{O}}_{2}^{-}$-DNA, while lanes 2, 3, 4, and 5 contain native or $^{1}{\text{O}}_{2}^{-}$-DNA with increasing concentrations of SLE IgG.
Recognition of native and modified antigens by cancer antibodies

The study comprised 30 sera from patients suffering from cancer of different organ and organ systems. Sera from normal, healthy individuals served as control. Cancer sera were obtained after careful clinical examination of patients with histopathological diagnosis attending J.N. Medical College Hospital, A.M.U., Aligarh. The binding of circulating antibodies in cancer sera (at 1:100 serum dilution) with native and $^{1}$O$_2$-O$_2^-$-DNA was assessed by direct binding ELISA. All the sera tested showed higher recognition to $^{1}$O$_2$-O$_2^-$-DNA (Fig. 40). The binding specificity of antibodies in cancer sera were analysed by competitive binding assay (Table 7). The samples contained 5 sera of lung cancer. All the sera showed greater recognition of $^{1}$O$_2$-O$_2^-$-DNA than native DNA. The maximum inhibition observed was 60.8%, 61.3%, 65.3%, 66.8% and 70.7% with $^{1}$O$_2$-O$_2^-$-DNA (Fig. 41). The results indicated higher binding of antibodies in lung cancer sera towards $^{1}$O$_2$-O$_2^-$-DNA than native DNA.

Five sera were collected from patients with cancer of urinary bladder. All five sera showed higher inhibition of 59.3%, 52.6%, 55.4%, 55.4%, 68.75% with $^{1}$O$_2$-O$_2^-$-DNA over native DNA (Fig. 42). Four sera were from patients suffering from breast cancer. All sera showed greater inhibition with $^{1}$O$_2$-O$_2^-$-DNA 59%, 46%, 50.7%, 53.6% where as, with native DNA all four sera showed inhibition below 40% (Fig. 43).

Among four sera from cancer of head and neck, two sera showed higher inhibition of 65% and 67.2% with $^{1}$O$_2$-O$_2^-$-DNA while two sera showed a moderate inhibition of 45% and 48.5% (Fig. 44).

Four sera were collected from patients suffering from oral carcinoma. Among them, two sera showed 69.6% and 65.6% inhibition with $^{1}$O$_2$-O$_2^-$-DNA and one sera showed greater inhibition with native DNA 46% and 40% inhibition with $^{1}$O$_2$-O$_2^-$-DNA while one sera showed moderate inhibition with $^{1}$O$_2$-O$_2^-$-DNA 48% (Fig. 45).

Four sera from gall bladder cancer showed 39.66%, 42.8%, 45% and 41% inhibition with $^{1}$O$_2$-O$_2^-$-DNA and 20.8%, 25.6%, 27% and 28% inhibition with native DNA (Fig. 46).
Fig. 40. Binding of various cancer sera to native (■) and $^{1}O_2-O_2^{-}$-DNA (■). Normal human sera (NHS) serve as negative control. The histograms show mean absorbance values for binding of NHS and sera from patients with cancer of lung (1), urinary bladder (2), breast (3), head and neck (4), oral (5), gall bladder (6), prostrate (7), vulva (8), CML (9), Hodgkin’s (10).
Fig. 41. Detection of autoantibodies against native and $^{1}\text{O}_2-\text{O}_2^-$-DNA, in the sera of patients with cancer of lung. (a) Cancer sera 1, 2 and 3 by native DNA ($\triangle$, $\diamond$, $\Box$), and $^{1}\text{O}_2-\text{O}_2^-$-DNA ($\Delta$, $\bullet$, $\triangleleft$). (b) Cancer sera 4 and 5 by native DNA ($\triangle$, $\bigcirc$) and $^{1}\text{O}_2-\text{O}_2^-$-DNA ($\Delta$, $\bullet$). The microtitre plates were coated with $^{1}\text{O}_2-\text{O}_2^-$-DNA (2.5 $\mu$g/ml) and serum dilution was 1:100.
Fig. 42. Detection of autoantibodies against native and $^{1}O_2-O_2^{-}$-DNA, in the sera of patients with cancer of urinary bladder. (a) Cancer sera 1,2 and 3 by native DNA ($\triangle, O, \nabla$), and $^{1}O_2-O_2^{-}$-DNA ($\Delta, \bullet, \nabla$). (b) Cancer sera 4 and 5 by native DNA ($\triangle, O$) and $^{1}O_2-O_2^{-}$-DNA ($\Delta, \bullet$). The microtitre plates were coated with $^{1}O_2-O_2^{-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.
Detection of autoantibodies against native and $^{1}{O}_2$-$^2{O}_2$-DNA, in the sera of patients with breast cancer. (a) Cancer sera 1 and 2 by native DNA (Δ, O), and $^{1}{O}_2$-$^2{O}_2$-DNA (▲, ■). (b) Cancer sera 3 and 4 by native DNA (Δ, O) and $^{1}{O}_2$-$^2{O}_2$-DNA (▲, ■). The microtitre plates were coated with $^{1}{O}_2$-$^2{O}_2$-DNA (2.5 μg/ml) and serum dilution was 1:100.
Fig. 44. Detection of autoantibodies against native and $^{1}\text{O}_2$-$\text{O}_2^-$-DNA, in the sera of patients with cancer of head and neck. (a) Cancer sera 1 and 2 by native DNA ($\triangle$,O), and $^{1}\text{O}_2$-$\text{O}_2^-$-DNA ($\blacktriangle$,●). (b) Cancer sera 3 and 4 by native DNA ($\triangle$,O) and $^{1}\text{O}_2$-$\text{O}_2^-$-DNA ($\blacktriangle$,●). The microtitre plates were coated with $^{1}\text{O}_2$-$\text{O}_2^-$-DNA (2.5 μg/ml) and serum dilution was 1:100.
Fig. 45. Detection of autoantibodies against native and $^{1}O_{2}$-$O_{2}^{-}$-DNA, in the sera of patients with oral cancer. (a) Cancer sera 1 and 2 by native DNA ($\triangle$, $\bigcirc$), and $^{1}O_{2}$-$O_{2}^{-}$-DNA ($\Delta$, $\bullet$). (b) Cancer sera 3 and 4 by native DNA ($\triangle$, $\bigcirc$) and $^{1}O_{2}$-$O_{2}^{-}$-DNA ($\Delta$, $\bullet$). The microtitre plates were coated with $^{1}O_{2}$-$O_{2}^{-}$-DNA (2.5 $\mu$g/ml) and serum dilution was 1:100.
Fig. 46. Detection of autoantibodies against native and $^{1}\text{O}_2^\text{=}\text{O}_2^\text{=}$-DNA, in the sera of patients with cancer of gall bladder. (a) Cancer sera 1 and 2 by native DNA ($\triangle, O$), and $^{1}\text{O}_2^\text{=}\text{O}_2^\text{=}^{-}$-DNA ($\blacktriangle, \bullet$). (b) Cancer sera 3 and 4 by native DNA ($\triangle, O$) and $^{1}\text{O}_2^\text{=}\text{O}_2^\text{=}^{-}$-DNA ($\blacktriangle, \bullet$). The microtitre plates were coated with $^{1}\text{O}_2^\text{=}\text{O}_2^\text{=}^{-}$-DNA (2.5 $\mu$g/ml) and serum dilution was 1:100.
Four sera were collected from patients suffering from prostate cancer. Three sera showed maximum inhibition of 25.8%, 37%, 45.4% with \(^{1}\text{O}_2-\text{O}_2^-\)-DNA and one sera showed almost equal percent inhibition (32% and 31%) with native and \(^{1}\text{O}_2-\text{O}_2^-\)-DNA (Fig. 47).

One serum sample each from Hodgkin’s lymphoma and CML showed higher recognition for \(^{1}\text{O}_2-\text{O}_2^-\)-DNA with maximum inhibition of 65% and 45%. Two serum samples from carcinoma of vulva showed higher recognition for \(^{1}\text{O}_2-\text{O}_2^-\)-DNA with maximum inhibition of 68% and 54% as compared to native DNA 45% and 35% (Fig. 48). The binding data of cancer sera are shown in Table 7.

**Band shift assay**

The binding of native and \(^{1}\text{O}_2-\text{O}_2^-\)-DNA to lung cancer IgG was detected by band shift assay. Constant amounts of antigen were incubated with varying amounts of cancer IgG for 2 hr at room temperature and overnight at 4°C. The immune complexes were then electrophoresed on 1% agarose for 2 hr at 30 mA (Fig. 49). The gel visualized under UV light revealed formation of high molecular weight immune complexes which resulted in retarded mobility and decrease in fluorescence intensity of the nucleic acid on agarose gel.

**Detection of oxidative lesions in DNA**

DNA was isolated from lymphocytes of various cancer patients. The purity and concentration of the DNA preparations were ascertained by \(A_{260}\) and \(A_{280}\) measurements. DNA isolated from lymphocytes of normal healthy individuals were used as controls for this study.

Anti-\(^{1}\text{O}_2-\text{O}_2^-\)-DNA IgG was used as probe to detect oxidative lesions in the DNA from cancer patients. Immune complexes, formed by incubating a fixed amount (60 \(\mu\)g) of anti-\(^{1}\text{O}_2-\text{O}_2^-\)-DNA IgG with increasing concentrations (0 to 20 \(\mu\)g/ml) of DNA were coated on microtitre plates already coated with \(^{1}\text{O}_2-\text{O}_2^-\)-DNA (2.5 \(\mu\)g/ml).

Two DNA samples isolated from patients with lung cancer (Fig. 50) showed a high inhibition 64% and 68.6% of immune IgG binding to immunogen. Another two
Fig. 47. Detection of autoantibodies against native and $^{1}O_2-O_2^-$-DNA, in the sera of patients with prostate cancer. (a) Cancer sera 1 and 2 by native DNA (△,○), and $^{1}O_2-O_2^-$-DNA (▲,●). (b) Cancer sera 3 and 4 by native DNA (△,○) and $^{1}O_2-O_2^-$-DNA (▲,●). The microtitre plates were coated with $^{1}O_2-O_2^-$-DNA (2.5 μg/ml) and serum dilution was 1:100.
Fig. 48. Detection of autoantibodies against native and $^{1}O_2-O_2^-$-DNA, in the sera of patients with Hodgkin's lymphoma, CML, cancer of vulva. (a) Hodgkin's lymphoma and CML with native DNA (△,○), and $^{1}O_2-O_2^-$-DNA (▲,●). (b) Cancer of vulva (sera 1 and 2) by native DNA (△,○) and $^{1}O_2-O_2^-$-DNA (▲,●). The microtitre plates were coated with $^{1}O_2-O_2^-$-DNA (2.5 μg/ml) and serum dilution was 1:100.
TABLE – 7

Inhibition of the binding of antibodies in cancer sera to $^{1}\text{O}_2$-$\text{O}_2^-$ DNA by native and $^{1}\text{O}_2$-$\text{O}_2^-$ DNA

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>No. of sera tested</th>
<th>Maximum percent inhibition at 20 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Native DNA</td>
</tr>
<tr>
<td>Lung</td>
<td>5</td>
<td>32.3, 16.5, 39.3, 15, 20.4</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>5</td>
<td>38.6, 32.8, 35.6, 32.9, 45</td>
</tr>
<tr>
<td>Breast</td>
<td>4</td>
<td>39.7, 28.5, 24.5, 30.2</td>
</tr>
<tr>
<td>Head and neck</td>
<td>4</td>
<td>39.9, 35.7, 40.7, 43</td>
</tr>
<tr>
<td>Oral</td>
<td>4</td>
<td>34.4, 38.7, 46.42</td>
</tr>
<tr>
<td>Gall bladder</td>
<td>4</td>
<td>20.8, 25.6, 27, 28</td>
</tr>
<tr>
<td>Prostrate</td>
<td>4</td>
<td>32, 18.6, 23.7, 41.9</td>
</tr>
<tr>
<td>Vulva</td>
<td>2</td>
<td>45, 35</td>
</tr>
<tr>
<td>CML</td>
<td>1</td>
<td>28</td>
</tr>
<tr>
<td>Hodgkin’s</td>
<td>1</td>
<td>50</td>
</tr>
</tbody>
</table>

The microtitre plates were coated with $^{1}\text{O}_2$-$\text{O}_2^-$ DNA (2.5 μg/ml) and serum dilution was 1:100.
Fig. 49. Band shift assay of cancer IgG binding to (a) native DNA and (b) $^{1}\text{O}_2 - \text{O}_2^-$-DNA. Native DNA and $^{1}\text{O}_2 - \text{O}_2^-$-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 $^{1}\text{O}_2 - \text{O}_2^-$-DNA, while lanes 2,3,4, and 5 contain native or $^{1}\text{O}_2 - \text{O}_2^-$-DNA with increasing concentrations of cancer IgG.
Fig. 50. Inhibition of binding of anti-$^{1}O_{2}$-$O_{2}^{-}$-DNA IgG by genomic DNA isolated from lymphocytes of patients with cancer of lung. The microtitre plates were coated with $^{1}O_{2}$-$O_{2}^{-}$-DNA (2.5 μg/ml). The curves (△) and (▲) represent DNA isolated from two different individuals with cancer of lung.
samples of DNA were from oral cancer (Fig. 51). These DNA samples showed a high inhibition of 61.1% and 60% in antibody binding. Two DNA samples isolated from cancer of prostrate showed inhibition 42.7% and 37% of antibody activity (Fig. 52). On the contrary, DNA isolated from two normal healthy individuals showed negligible inhibition of 20% and 25% with anti-\(^{1}\)O\(_2\)-O\(_2\)–DNA IgG binding to the immunogen (Fig. 53). These results have been summarised in Table 8.
Fig. 51. Inhibition of binding of anti-\(^{1}\text{O}_2-\text{O}_2^*-\)DNA IgG by genomic DNA isolated from lymphocytes of patients with oral cancer. The microtitre plates were coated with \(^{1}\text{O}_2-\text{O}_2^*-\)DNA (2.5 µg/ml). The curves (△) and (▲) represent DNA isolated from two different individuals with oral cancer.
Fig. 52. Inhibition of binding of anti-$^1\mathrm{O}_2-\cdot\mathrm{O}_2^-$-DNA IgG by genomic DNA isolated from lymphocytes of patients with prostrate cancer. The microtitre plates were coated with $^1\mathrm{O}_2-\cdot\mathrm{O}_2^-$-DNA (2.5 $\mu$g/ml). The curves (△) and (▲) represent DNA isolated from two different individuals with prostrate cancer.
Fig. 53. Inhibition of binding of anti-\( ^{1}O_2-O_2^{*}\)-DNA IgG by genomic DNA isolated from lymphocytes of normal individuals. The microtitre plates were coated with \( ^{1}O_2-O_2^{*}\)-DNA (2.5 \( \mu \)g/ml). The curves (\( \triangle \)) and (\( \blacktriangle \)) represent DNA isolated from two different individuals.
<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>No. of sera tested</th>
<th>Maximum percent inhibition at 20 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>64.8</td>
</tr>
<tr>
<td>Lung</td>
<td>2</td>
<td>68.0</td>
</tr>
<tr>
<td>Oral</td>
<td>2</td>
<td>61.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate</td>
<td>2</td>
<td>42.8</td>
</tr>
</tbody>
</table>

The microtitre plates were coated with $^{1}$O$_2$O$_2^-$-DNA (2.5 µg/ml)