Experimental
MATERIALS

Calf thymus DNA, nuclease S1, riboflavin, superoxide dismutase, catalase, bovine serum albumin, agarose, anti-human/anti-rabbit IgG-alkaline phosphatase, anti-rat-IgG-HRP and FITC conjugates, ethidium bromide, Coomassie Brilliant Blue G 250 and R 250, standard protein markers, sodium dodecyl sulphate, Freund’s complete and incomplete adjuvants, methylated bovine serum albumin, Tween-20, Triton X-100 and nitroblue tetrazolium were purchased from Sigma Chemical Company, U.S.A. Synthetic polynucleotides, Protein A-Sepharose CL-4B, DEAE Sephadex A-25 and Ficoll 400 were obtained from Pharmacia Fine Chemicals, Sweden. EDTA (disodium salt), chloroform, isoamyl alcohol and hydrogen peroxide were from Qualigens, India.

Polystyrene microtitre flat bottom ELISA plates having 96 wells (7 mm diameter) were purchased from Nunc, Denmark. p-nitrophenyl phosphate and Folin-Ciocalteau reagent were obtained from Centre for Biochemical Technology, New Delhi. Acrylamide, ammonium persulphate, bisacrylamide, hydroxyapatite, N,N,N’,N’-tetramethylethylene diamine (TEMED) were from Bio-Rad Laboratories, U.S.A. Diphenylamine and ethanol were chemically pure. All other chemicals were of highest analytical grade available.

Equipment

Shimadzu UV-240 spectrophotometer equipped with thermo-programmer and controller unit, ELISA microplate reader MR-600 (Dynatech, U.S.A.), ELISA microplate washer (Denley, England), ELICO pH meter model L1-120, gel scanner GSC-3A, ultraviolet lamp (Vilber Lourmat, France), agarose gel electrophoresis assembly (GNA-100) and gradient mixer GM-1 (Pharmacia, Sweden), Beckman Ultracentrifuge, Avanti 30 table top high speed refrigerated centrifuge (Beckman, U.S.A.), polyacrylamide gel electrophoresis assembly (Bio-Rad, U.S.A.), and fluorescent microscope
(Nikon, Japan) were the major equipment used in this study.

Collection of Sera

SLE sera were obtained from patients 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 patients with cancer proven with histopathological diagnosis were obtained from J.N. Medical College Hospital of 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

Highly polymerized calf thymus DNA was purified free of proteins, RNA and single stranded regions as described by Ali et al. (1985). The DNA (2 mg/ml) was dissolved in 0.1X SSC (15 mM sodium citrate and 150 mM sodium chloride), pH 7.3 and extracted with a mixture of chloroform-isoamyl alcohol (24:1) in a stoppered measuring cylinder for 1 hr. The aqueous layer containing DNA was separated from the organic layer and re-extracted with chloroform-isoamyl 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 30 mM acetate buffer, pH 5.0 containing 30 mM zinc chloride and treated with nuclease S1 (200 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. Extraction procedure was repeated as before and final preparation of DNA was dissolved in phosphate-buffered saline (PBS), pH 7.4.
**Determination of DNA Concentration**

The concentration of DNA was determined by the method of Burton (1956).

(a) *Crystallization of diphenylamine*

Diphenylamine (2 gm) was dissolved in 200 ml boiling hexane. Approximately 0.5 gm of activated animal charcoal was added and filtered through Whatman No. 1 filter paper. The filtrate was kept overnight at 4°C. Diphenylamine crystals so obtained, were again filtered to remove hexane and finally dried at room temperature.

(b) *Preparation of diphenylamine reagent*

Recrystallized diphenylamine (750 mg) was dissolved in 50 ml glacial acetic acid containing 0.75 ml concentrated sulphuric acid.

(c) *Procedure*

One ml of 1 N perchloric acid was added to 1.0 ml of DNA sample and incubated in a thermostat water bath at 70°C for 15 min. One hundred microlitre 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. and absorbance was recorded at 600 nm. The DNA concentration in unknown samples was determined using calf thymus DNA as standard.

**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

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 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 in 100 mM sodium hydroxide

(ii) 0.5 percent copper sulphate in 1.0 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 1.0 ml of protein sample was added 5.0 ml of alkaline copper reagent. The samples were allowed to stand at room temperature for 10 min. Working Folin-Ciocalteau reagent (1.0 ml) was added to the tubes, mixed and incubated for 30 min at room temperature. The absorbance was read at 660 nm. The concentration of protein in unknown sample was determined from a standard plot of bovine serum albumin.

Protein estimation by Bradford method

This assay is based on colour change that occurs 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 colour is observed ($\lambda_{\text{max}} = 595$ nm).

(a) Dye preparation

One hundred mg Coomassie Brilliant Blue G 250 was dissolved in 50 ml of 95% ethanol. To this solution was added 100 ml of 85% (v/v) orthophosphoric acid. The resulting solution was diluted to a final volume of 1 litre.

(b) Protein assay

To solutions containing 10-100 $\mu$g protein in a volume of up to 0.1 ml was added 5 ml of dye solution and contents mixed by vortexing. The absorbance was taken at 595 nm after 5 min against a reagent blank.

Modification of Calf Thymus DNA by Superoxide Anion Radicals

Superoxide radical was detected by photosensitized reduction of nitroblue tetrazolium (NBT), leading to the formation of a blue coloured product, nitroblue formazan (Nakayama et al., 1983). In order to identify superoxide radical ($O_2^{-}$), superoxide dismutase and other reactive oxygen species scavengers were added to the buffer before introducing the chemicals.

Native calf thymus DNA was 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, 0.033 $\mu$M NBT, 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 white fluorescent light at room temperature. The samples were dialyzed extensively to remove riboflavin and Triton X-100.
Spectroscopic Analysis

The ultraviolet spectra of modified and unmodified DNA were recorded in the wavelength range of 200-400 nm on a Shimadzu UV-240 spectrophotometer.

Circular Dichroism Measurements

The CD spectra were taken on a Jasco J-700 spectropolarimeter using quartz cell of 1 cm path length. Circular dichroic (CD) spectra of native and superoxide modified DNA were performed in the wavelength range from 220 nm to 350 nm. Scans were recorded at 0.2 nm intervals. The concentration of DNA was 151 μM base pair. Molar ellipticities were calculated in terms of the base pair concentration according to following equation

\[ \theta = \frac{[\theta]}{10c} \]

where, \( \theta \) = Measured ellipticity (mdeg)

\( c \) = Molar concentration of DNA

\( l \) = Path length in cm

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 \(T^\circ \text{C}\)
\(A_{\text{max}}\) = Final maximum absorbance on the completion of denaturation (95°C)
\(A_{30}\) = Initial absorbance at 30°C

**Hydroxyapatite Column Chromatography**

Hydroxyapatite column chromatography was employed to differentiate nucleic acids endowed with different secondary structures. DNA and superoxide modified DNA (in 10 mM Na-phosphate buffer, pH 6.8) were chromatographed on hydroxyapatite column as described by Dardalhon and Averback (1988). Batch elution was carried out with 125 mM and 250 mM Na-K-phosphate buffer, pH 6.8 at a flow rate of 15 ml/hr and fractions of 3 ml were collected and absorbance monitored at 260 nm.

**Detection of Strand Breaks**

The damage to DNA induced by superoxide anion radical was ascertained by alkaline sucrose density ultracentrifugation and nuclease \(S_1\) digestibility.

**Alkaline Sucrose Density Gradient Ultracentrifugation**

Native and superoxide modified DNA (0.2 ml) were treated by the addition of an equal volume of 0.2 N NaOH. After 10 min to allow...
denaturation of DNA, 0.4 ml sample was layered on top of a linear 4.6 ml alkaline sucrose gradient (5-20% sucrose in 0.1 N NaOH and 10 mM EDTA) (Ali and Sauerbier, 1978). The gradients were centrifuged at 30,000 rpm for 1 hr at room temperature in swinging bucket rotor of Beckman ultracentrifuge. The bottom of the tubes were pierced and six drop fractions were collected, diluted to about 0.5 ml with distilled water and their absorbance recorded at 260 nm.

(b) Nuclease S<sub>1</sub> Digestibility

Native and superoxide modified DNA were characterized by nuclease S<sub>1</sub> 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 S<sub>1</sub> (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.

(i) 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 the gel tray and allowed to solidify at room temperature.

(ii) Sample preparation and loading

Native and superoxide modified DNA samples treated with nuclease S<sub>1</sub> 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.
Separation of DNA Bases

The separation and quantitation of bases in native and superoxide modified DNA was carried out according to Hasan and Ali (1990).

(a) Acid hydrolysis of DNA samples

Native and superoxide modified DNA samples were treated with 70% perchloric acid. The samples were heated at 100°C for 1 hr to release the bases. The hydrolyzate was neutralized and the bases were separated on DEAE Sephadex A-25 column.

(b) DEAE Sephadex A-25 column chromatography

The swollen ion exchanger was mixed with equilibrating buffer (1 mM Tris-HCl, pH 7.6) and packed in a column (40 cm x 0.75 cm). The column was equilibrated with the same buffer and 3 ml of sample was applied onto the column and eluted with a linear gradient of 1-20 mM Tris-HCl, pH 7.6, at a flow rate of 40 ml/hr. Three ml fractions were collected and absorbance read at 260 nm. The control experiments were carried out with individual bases and hydrolyzed native DNA in order to locate the pattern of unmodified bases.

Immunization Schedule

Native and superoxide modified DNA (100 μg) were complexed with an equal amount (w/w) of methylated BSA and emulsified with an equal volume of complete Freund's adjuvant and injected intramuscularly in rabbits. Subsequent injections were given in incomplete Freund's adjuvant. Each animal received a total of 500 μg of antigen in the course of five injections. Blood was collected by cardiac puncture, 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 with 0.1% sodium azide as preservative.
Four female rats (Sprague-Dawley), were immunized with native and superoxide modified DNA (50 μg/rat) subcutaneously. The antigen was complexed with methylated BSA and emulsified in Freund's complete adjuvant. Three booster doses (each 25 μg/rat) were given intraperitoneally at weekly intervals with incomplete adjuvant. The control group did not receive any antigen. Animals were bled by cardiac puncture and blood was collected. Serum was separated and decomponented by heating at 56°C for 30 min and stored at -80°C with 0.1% sodium azide as preservative. Rats were sacrificed and their kidneys were removed and stored at -80°C.

**Immunofluorescence**

Kidney sections (approx. 5 μm thick) were sliced and fixed on slides with acetone and incubated with anti-rat IgG fluorescein isothiocyanate conjugate (1:100 diluted) for 30 min. The fixed sections on slides were washed three times with PBS and mounted with 50% glycerol. The slides were then viewed under a fluorescent microscope.

**Isolation of IgG by Protein A-Sepharose CL-4B**

Serum IgG was isolated by affinity chromatography on Protein A-Sepharose CL-4B column. Serum (0.5 ml) diluted with equal volume of PBS, pH 7.4 was applied to column (0.9 cm x 15 cm) 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 with 1 ml of 1 M Tris-HCl, pH 8.5. Three ml fractions were collected and read at 251 nm and 278 nm. The IgG concentration was determined considering 1.4 O.D_{280} = 1.0 mg IgG/ml. The isolated IgG was then dialyzed against PBS, pH 7.4 and stored at -20°C with 0.1% sodium azide.
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:

(i) *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.

(ii) *Resolving gel buffer*

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

(iii) *Stacking gel buffer*

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

(iv) *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.

(v) *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 in to 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 60 V for 6-8 hr. 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 mixture of 10% acetic acid and 10% 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>
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<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 µL</td>
</tr>
</tbody>
</table>

Immunological Detection of Antibodies

Sera were tested for antibodies by immunodiffusion, counterimmunoelectrophoresis, enzyme linked immunosorbent assay and gel retardation assay.

(a) Immunodiffusion

Immunodiffusion (ID) was carried out by Ouchterlony double diffusion system. Six ml of 0.4% molten agarose in PBS containing 0.1% sodium
azide was poured on to 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) Counterimmunoelectrophoresis

Counterimmunoelectrophoresis (CIE) was performed by the method of Kurata and Tan (1976) using 0.6% molten agarose solution in 25 mM barbital buffer, pH 8.4 containing 0.1% sodium azide. Agarose was poured on to 2.5 mm thick glass slides (7.5 cm x 2.5 cm) and allowed to harden at room temperature and then at 4°C. Wells each 3 mm in diameter were cut and loaded with antigen and antibodies (in anodal and cathodal wells, respectively). Electrophoresis was performed for 30 min in 50 mM barbital buffer, pH 8.4 with a current of 3-4 mA per slide. Non-specific precipitin lines were removed with 5% sodium citrate.

(c) 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

Substrates

(i) 500 µg p-nitrophenyl phosphate (p-NPP)/ml carbonate-bicarbonate buffer

(ii) 500 µg o-phenylene diamine (OPD)/ml citrate-phosphate buffer, containing 1 µl/ml of hydrogen peroxide

(ii) Procedure

Antibodies were detected by ELISA using polystyrene microtiter 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 hr at room temperature. The plates washed once with TBS-T and antibody (100 µl/well) to be tested, were diluted in TBS was 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 or HRP 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 or orthophenylenediamine (OPD/H$_2$O$_2$) substrate respectively. The plates were incubated for 1-2 hr and reaction stopped in
the case of OPD/H$_2$O$_2$ system with 50 µl of 5 N H$_2$SO$_4$ and read at 490 nm. In the case of p-NPP, the absorbance was read 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 amounts 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 superoxide 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 buffer, pH 7.9. The gels were stained with ethidium bromide (0.5 µg/ml), visualized under UV light and photographed.

**Quantitative Precipitin Titration**

The immunointeraction between antigen and antibody was investigated by precipitin analysis under equilibrium conditions. Increasing amounts of
antigens (0-40 μg) were mixed with 100 μg each of IgG in an assay volume of 0.2 ml. The mixture was incubated at 37°C for 2 hr and overnight at 4°C. The tubes were centrifuged at 10,000 rpm for 5 min. The precipitate was washed with cold PBS, pH 7.4 and dissolved in a fixed volume of 1 M NaCl. The amount of bound and unbound antigen and antibody in the dissociated immune complex supernatant was determined. For antigen diphenylamine reagent (Burton, 1956) and for antibody dye binding method (Bradford, 1976) was used. The binding data was analyzed and antibody affinity was calculated (Langmuir, 1918).