Experimental
MATERIALS

Calf thymus DNA, nuclease S1, riboflavin, superoxide dismutase, DNase 1 (RNase free), bovine serum albumin, anti-human/anti-rabbit IgG-alkaline phosphatase conjugate, p-nitrophenyl phosphate, ethidium bromide, Coomassie Brilliant Blue G-250 and R-250, sodium dodecyl sulphate, Tween-20, Triton X-100, Millipore filter (0.45 \( \mu \text{m} \) pore size), Freund's complete and incomplete adjuvants, polydeoxyribonucleotides, and agarose were purchased from Sigma Chemical Company, U.S.A. Synthetic polynucleotides, Ficoll 400, xylene cyanole FF were purchased from Pharmacia Fine Chemicals, Sweden. Folin-Ciocalteau reagent and Blue Dextran 2000 were purchased from Centre for Biochemical Technology, New Delhi. Protein A sepharose CL 4B was from Genei, India. Absolute ethanol was obtained from BDH Laboratory Supplies, England. Polystyrene microtitre flat bottom ELISA plates having 96 wells (7 mm diameter) were purchased from NUNC, Denmark. Acrylamide, ammonium persulphate, bisacrylamide, N, N, N', N'-tetramethylethylene diamine (TEMED) were from Bio-Rad Laboratory U.S.A. EDTA (disodium salt), hydrogen peroxide, sucrose, chloroform, isoamyl alcohol, methanol, glacial acetic acid were from Qualigens, India. Diphenylamine was chemically pure. All other reagents/chemicals were of the highest analytical grade available.

Equipments

Shimadzu UV-240 spectrophotometer equipped with thermo-programmer and controller unit, high speed tissue homogenizer (York Scientific industries, Delhi), ELISA microplate reader MR-600 (Dynatech, U.S.A.), ELICO pH meter model L1-120, ultraviolet lamp (Vilber Lourmat, France), UV-transilluminator (Vilber Lourmat, France), agarose gel electrophoresis assembly (GNA-100) and gradient mixer GM-1 (Pharmacia, Sweden), Avanti 30 table top high speed refrigerated centrifuge (Beckman, U.S.A.), polyacrylamide gel electrophoresis assembly (Bio-Rad, U.S.A.), fluorimeter (Hitachi, Japan) were the major equipments used in this study.
Collection of Sera and Blood Samples

Normal human sera were obtained from healthy subjects. SLE sera were obtained from outdoor and indoor patients of the Department of Medicine, All India Institute of Medical Sciences, New Delhi. The SLE sera showed high titre anti-DNA antibodies and fulfilled the American College of Rheumatology revised criteria for the classification of SLE (Arnett et al., 1988).

METHODS

Determination of DNA concentration

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

(a) Crystallization of diphenylamine

Diphenylamine (2g) 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 hrs. Absorbance was read at 600 nm and the concentration of DNA in unknown samples was determined from a standard plot of calf thymus DNA purified free of RNA and proteins.
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 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) 2 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 and incubated for 10 min at room temperature. 1 ml of working Folin-Ciocalteau 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 solutions containing 10-100 µg 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.

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 extracted with an equal volume of chloroform isoamyl alcohol (24:1) in a stoppered container 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 volume 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 isoamyl 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.

Poly acrylamide gel electrophoresis (PAGE) for proteins

PAGE was performed as described by Laemmli (1970).
(i) **Acrylamide-bisacrylamide (30:0.8)**

A stock solution was prepared by dissolving 30 gm of acrylamide and 0.8 gm bis-acrylamide 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 1N 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 6.8 with 1N 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 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 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>
<td>2.5 ml</td>
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<tr>
<td>Resolving gel buffer</td>
<td>-</td>
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<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 persulpahte</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>

Isolation of chromatin

Chromatin was isolated as described by Bonner et al (1968) with slight modifications. 10 g of fresh goat liver was homogenized with 200 ml of saline EDTA (0.075 M NaCl, and 0.024 M EDTA, pH 8.0). The homogenate was strained through 6-8 layers of cheesecloth. The filtrate was centrifuged at 1500 g for 15 minutes. The pellet was homogenized in 40 ml of Tris buffer, and then sedimented at 10,000 g for 15 minutes. This step was repeated once. The final pellet was suspended in 30 ml of Tris buffer (0.05 M, pH 8.0). Five-milliliter aliquots of the above suspension were layered on 25 ml portions of 1.7 M sucrose (0.01 M Tris buffer, pH 8.0) contained in centrifuge tubes. The upper two-thirds of each tube were gently mixed and the tubes were then centrifuged at 21,000 rpm for 3 hours in F0650 rotor (rotor temperature 4°C). The pellets were resuspended in 0.01 M Tris buffer, pH 8.0, and dialyzed against the same buffer overnight. The dialyzed suspension was sheared in Virtis homogenizer for 90 seconds, stirred for 30 minutes, and then centrifuged at 10,000 g for 30 minutes. The supernatant was taken out and UV spectra of the diluted supernatant was recorded. The supernatant is referred to as sheared liver chromatin.
Modification of chromatin by hydroxyl radical

Aqueous solution of chromatin in PBS, pH 7.4 was irradiated under 254 nm light for one hour at room temperature in the presence of hydrogen peroxide (15.1 mM). Excess of hydrogen peroxide was removed by extensive dialysis against PBS, pH 7.4.

Modification of chromatin by superoxide anion radical and singlet oxygen

Superoxide radical was detected by photosensitized reduction of nitroblue tetrazolium (NBT), leading to the formation of a blue coloured products, nitroblue formazan (Nakayama et al, 1983). Production of superoxide radical was confirmed by monitoring the inhibition of formation of blue coloured 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.

Isolated chromatin was modified by the method of Naseem et al (1988). A total volume of 3.0 ml of reaction mixture contained chromatin, $A_{260}=2$, 50 mM potassium phosphate buffer, pH 7.8, 1.1 mM EDTA, 0.06% Triton X-100 and 40 μM riboflavin. The reaction mixture was irradiated at 365 nm at room temperature followed by extensive dialysis to remove riboflavin and Triton X-100.

Spectroscopic analysis

The ultraviolet spectra of modified and unmodified chromatin samples were recorded in the wavelength range of 230-400 nm on a Shimadzu UV-240 spectrophotometer. The modifications incurred on native chromatin were also analyzed by UV-difference spectroscopy.
Fluorescence emission spectroscopy of native and modified chromatin samples using ethidium bromide was also performed.

**Absorption-temperature scan**

Thermal denaturation analysis of chromatin was performed in order to ascertain the degree of modification incurred on the chromatin by determining mid point melting temperature (Tm). Native and modified chromatin 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:

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

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

**Nuclease S1 digestibility**

Native and modified chromatin were characterized by nuclease S1 digestibility (Matsuo and Ross, 1987). One microgram each of native and modified chromatin 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.
(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 gel tray and allowed to solidify at room temperature.

(ii) **Sample preparation and loading**

Native and modified chromatin 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 chromatin (50 μg) were 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 300 μg of antigen in the course of 6 injections. Blood was collected from marginal vein of the ear. Serum was separated and decomplemented 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 (12mm X 45mm) 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 1M Tris-HCl, pH 8.5. Three ml fractions were collected and read
at 251 and 278 nm. The IgG concentration was determined considering $1.40 \, \text{OD}_{280} = 1.0$ mg IgG/ml. The isolated IgG was then dialyzed against PBS, pH 7.4 and stored at -20°C with 0.1% sodium azide.

**Immunological detection of antibodies**

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

(a) **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)**
  
  $10 \, \text{mM Tris}, \, 150 \, \text{mM NaCl}, \, \text{pH} \, 7.4$

- **Tris buffered saline-Tween 20 (TBS-T)**
  
  $20 \, \text{mM Tris}, \, 144 \, \text{mM NaCl}, \, 2.68 \, \text{mM KCl}, \, \text{pH} \, 7.4$, containing $500 \, \mu\text{l} \, \text{Tween 20/L}$.

- **Carbonate-bicarbonate buffer**
  
  $15 \, \text{mM sodium carbonate}, \, 35 \, \text{mM sodium bicarbonate}, \, \text{pH} \, 9.6$, containing $2 \, \text{mM magnesium chloride}$.

- **Citrate-phosphate buffer**
  
  $50 \, \text{mM citric acid}, \, 50 \, \text{mM Na}_2\text{HPO}_4$, pH 5.0.

**Substrates**

(i) $500 \, \mu\text{g} \, \text{p-nitrophenyl phosphate (p-NPP)/ml of carbonate-bicarbonate buffer}$.

(ii) **Procedure**

Antibodies were detected by ELISA using polystyrene microtitre plates as solid support. 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 150µl of 1.5% BSA in TBS for 4 - 5 hrs at room temperature. The plates were 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 induplicate. The control wells were treated similarly but were devoid of antigen. Results were expressed as a mean of $A_{\text{test}} - A_{\text{control}}$.

(b) 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

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

(c) 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 chromatin) 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 (0.5µg/ml), visualized under UV light and photographed.