EXPERIMENTAL
MATERIALS

Thymidine monophosphate, calf thymus DNA, nuclease S1, bovine serum albumin, anti-human and anti-rabbit IgG-alkaline phosphatase conjugates, ethidium bromide, Coomassie Brilliant Blue G250 and R250, Tween 20, Freund’s complete and incomplete adjuvants, polydeoxyribonucleotides and carbodiimide were from Sigma Chemical Company, U.S.A. Synthetic polynucleotides, Sepharose 4B, DEAE Sephadex A-25, Sephadex G-100, Ficoll 400 were obtained from Pharmacia Fine Chemicals, Sweden. Folin-Ciocalteu reagent, p-nitrophenyl phosphate and Blue Dextran 2000 were purchased from Centre for Biochemical Technology, New Delhi.

Polystyrene microtitre flat bottom ELISA plates having 96 wells (7mm diameter) were purchased from Nunc, Denmark. Acrylamide, bisacrylamide, ammonium persulphate, N,N,N′,N′-tetramethylethylenediamine (TEMED) were from Bio-Rad Laboratories, U.S.A. EDTA (disodium salt), hydrogen peroxide, isoamyl alcohol, chloroform were from Qualigens, India. Diphenylamine and ethanol were chemically pure. All other chemicals were of highest analytical grade available.

Equipment

ELISA microplate reader MR-600 (Dynatech, U.S.A.), ELISA microplate washer (Denley, England), ELICO pH meter model L1-120, Shimadzu UV-240 spectrophotometer equipped with thermo-programmer and controller unit, gel scanner GSC-3A, ultraviolet lamp having maximum emission at 254 nm (Vilber Lourmat, France), Avanti 30 table top high speed centrifuge (Beckman, U.S.A.). Polyacrylamide gel electrophoresis assembly (Bio-Rad, U.S.A.). UV trans-illuminator (Vilber
Lourmat, France), Agarose gel electrophoresis assembly GNA-100 (Pharmacia, Sweden), Beckman ultracentrifuge were the major equipments used in this study.

Sera Specimens

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 collected, showed high titre anti-DNA antibodies and fulfilled the American College of Rheumatology criteria for the diagnosis of SLE (Arnett et al., 1988). The sera of cancer patients were collected from Department of Radiotherapy, J.N. Medical College, A.M.U., Aligarh. Complement was inactivated at 56 °C for 30 minutes and stored at -80°C with 0.1% sodium azide.

METHODS

Purification of DNA

Commercially available, highly polymerized calf thymus DNA was purified free of proteins and single stranded regions (Ali et al., 1985). DNA was dissolved in 0.1 X SSC (15 mM sodium citrate and 150 mM sodium chloride), pH 7.3. Solutions of DNA (2 mg/ml) was mixed with equal volume of chloroform-isoamyl alcohol (24:1) in a stoppered cylinder and extracted gently for 1 hr. The DNA present in the aqueous layer was separated from the organic layer and re-extracted with chloroform-isoamyl alcohol. The DNA was precipitated with two volumes of cold 95% ethanol and collected on a glass rod. Traces of water was removed by rinsing the rod with ethanol and DNA dried by pressing against the wall of the container. The DNA was then dissolved in 30 mM acetate buffer, pH 5.0 containing 30 mM zinc chloride and treated with
nuclease S1 (150 units/mg DNA) at 37°C for 30 min to remove single stranded regions. The reaction was terminated by adding one-tenth volume of 200 mM EDTA, pH 8.0. The purified DNA was extracted twice with chloroform-isoamyl-alcohol and finally precipitated with 95% ethanol. The precipitate was dissolved in PBS (10 mM sodium-phosphate, containing 150 mM NaCl), pH 7.4.

**Estimation of DNA by Diphenylamine**

Colorimetric estimation of DNA was carried out by the method of Burton (1956) using diphenylamine reagent.

**(a) Preparation of diphenylamine reagent**

Recrystallized diphenylamine (750 mg) was dissolved in 50 ml of glacial acetic acid containing 0.75 ml concentrated sulphuric acid. The reagent was prepared immediately before use.

**(b) Procedure**

To varying amounts of DNA in 1.0 ml of 1N perchloric acid was mixed and incubated at 70°C in water bath for 15 min. One hundred microlitre of 5.43 mM acetaldehyde was added followed by 2.0 ml of diphenylamine reagent. The contents were mixed and allowed to stand at room temperature for 16-20 hrs. Absorbance was read at 600 nm and the concentration of DNA in unknown samples was determined from the standard plot of purified calf thymus DNA.

**Protein Estimation by Bradford Method**

Protein was estimated by the method of Bradford (1976). This assay is based on colour change that occurs when Coomassie Brilliant Blue G250, in acidic solution, binds strongly to protein hydrophobically and at
positively charged groups. In the environment of these positively charged groups, protonation is suppressed and a blue colour is observed \(\lambda_{\text{max}} = 595\,\text{nm}\).

**(a) Dye preparation**

Coomassie Brilliant Blue G250 (100 mg) was dissolved thoroughly in 50 ml of 95% ethanol. One hundred ml of 85% (v/v) orthophosphoric acid was added to this solution. The resulting solution was diluted to a final volume of 1 litre. On every use the dye solution was filtered, to remove undissolved particles.

**(b) Procedure**

Solutions containing 10-100 \(\mu\)g protein in a volume of upto 0.1 ml was pipetted into test tubes. The volume was adjusted to 1.0 ml with appropriate buffer. Five ml of dye solution was added and the contents were vortexed. The absorbance was read at 595 nm after 2 min and before 1 hr against a reagent blank prepared from 0.1 ml of buffer and 5.0 ml of dye solution.

**ROS Modification of TMP**

Aqueous solution of native TMP (0.31 mM) in PBS, pH 7.4 was irradiated under 254 nm light for 30 min at room temperature in the presence of hydrogen peroxide (3.10 mM). Native TMP samples exposed to hydrogen peroxide or UV light alone were used as corresponding controls.

**Spectroscopic Analysis**

The ultraviolet spectra of modified and unmodified TMP conjugate were recorded in the wavelength range of 200-400 nm on Shimadzu UV-240 spectrophotometer.
**Preparation of the Antigen**

The hapten-protein conjugate was prepared by the carbodiimide conjugation procedure of Halloran and Parker (1966). BSA (19 mg) and thymidine 5'-monophosphate (50 mg) were dissolved in 5.0 ml of distilled water. The pH was adjusted to 7.5 with 0.5 M NaOH, and ethyl ((dimethylamino) propyl) carbodiimide (20 mg) was added. The reaction mixture was incubated in the dark at room temperature for 24 hrs. The solution was then dialyzed extensively against 10 mM Tris-HCl buffer, pH 7.6, followed by extensive dialysis against distilled water.

The ultraviolet spectra of native TMP, BSA and TMP-BSA conjugate were recorded in the range of 200-400 on Shimadzu UV-240 spectrophotometer.

**Polyacrylamide Gel Electrophoresis**

BSA and TMP-BSA conjugate were subjected to polyacrylamide slab gel electrophoresis according to the method of Laemmli (1970).

(a) **Stock solutions**

(i) *Acrylamide-bisacrylamide (30:0.8):*

Prepared by dissolving in distilled water 30.0 g of acrylamide and 0.8 g of bisacrylamide in a total volume of 100.0 ml. The solution was stored at 4°C in a brown bottle.

(ii) *TEMED:* Used as supplied.

(iii) *Ammonium persulphate (1.5% w/w):*

0.15 g of ammonium persulphate was dissolved in 10.0 ml water. The solution was made fresh just before use.
(iv) **Resolving gel buffer (3M Tris-HCl, pH 8.8):**

Prepared by dissolving 36.0g Tris in 48.0 ml of 1 N HCl. The contents were mixed thoroughly, pH brought to 8.8 and final volume made upto 100.0 ml with distilled water.

(v) **Stacking gel buffer (0.5 M Tris-HCl, pH 6.8):**

Tris (6.05g) was dissolved in 40.0 ml distilled water, titrated to pH 6.8 with 1N HCl and volume made upto 100.0 ml with distilled water.

(vi) **Reservoir buffer (25 mM Tris, 192 mM glycine):**

3.03 g Tris and 14.4 g glycine were dissolved in distilled water and final volume made upto one litre.

(b) **Recipe for 7.5% Resolving gel**

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<th>Volume</th>
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</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide</td>
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<tr>
<td>Resolving gel buffer</td>
<td>3.75 ml</td>
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<tr>
<td>1.5% Ammonium persulphate</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>16.95 ml</td>
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<tr>
<td>TEMED</td>
<td>15.0 μl</td>
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c) **Recipe for 2.5% stacking gel**

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<tbody>
<tr>
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<tr>
<td>Stacking gel buffer</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>1.5% Ammonium persulphate</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>11.3 ml</td>
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<tr>
<td>TEMED</td>
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</table>

(d) **Procedure**

The resolving gel mixture was prepared by mixing the components in the appropriate volumes as listed above and poured into the space between the glass plates leaving sufficient space (about 3.5 cm) at the
top for the stacking gel. After the polymerization of separating gel, stacking gel mixture was poured and allowed to solidify. Samples containing 10% glycerol and 0.002% bromophenol blue were applied and electrophoresis was carried out at 24 mA for 2-3 hr. Staining of the gel was achieved with 0.1% Coomassie Brilliant Blue R 250 (in 25% isopropanol and 10% glacial acetic acid) and the gel was destained using a mixture of 10% glacial acetic acid and 30% methanol.

**ROS-Modification of TMP-BSA Conjugate**

Aqueous solution of TMP-BSA conjugate (6.20 mM) in PBS, pH 7.4 was irradiated under 254 nm light for 30 min. at room temperature in the presence of hydrogen peroxide (62 mM). Excess of hydrogen peroxide was removed by extensive dialysis against PBS, pH 7.4. The ultraviolet spectra of TMP and ROS-modified TMP were recorded.

**Absorption-Temperature Scan**

Thermal denaturation analysis of native and ROS-modified conjugates were accomplished in a Shimadzu UV-240 spectrophotometer coupled with a temperature programmer and controller assembly. Native and ROS-modified TMP-BSA conjugates were subjected to heat denaturation (Hasan and Ali, 1990). 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 $\lambda_{\text{max}}$ was recorded with increasing temperature. Simultaneously, UV spectra were also recorded at 30°C and 95°C in the same wavelength range.
Sephadex G-100 Gel Chromatography

Gel filtration was performed on Sephadex G-100 column. Five gm of gel was swollen in distilled water for 5 hr in a boiling water bath. The degassed gel slurry was poured into the column (42 cm x 1 cm) and left overnight at room temperature to settle under gravity. The flow rate was increased gradually and adjusted to 20 ml/hr. Uniform packing of column was checked by passing 0.2% (w/v) solution of Blue Dextran 2000. The column was equilibrated with PBS, pH 7.4.

One ml each of native and ROS-TMP-BSA conjugates were applied separately onto the column. The sample was allowed to percolate through the upper surface of the gel. The column was connected to a reservoir containing equilibrating buffer. Fractions of 3.0 ml were collected and absorbance monitored at 269 nm.

Densitometric Scanning

Samples of native and ROS-modified conjugates were subjected to polyacrylamide gel electrophoresis on 7.5% native gel. After electrophoresis, the lanes each of native and ROS-modified conjugates were cut and scanned on gel scanner GSC-3A of Shimadzu UV-240 spectrophotometer. Gel was scanned at the rate of 10 mm/min and the spectra was recorded simultaneously at a fixed wavelength of 269 nm.

Agarose Gel Electrophoresis

1% agarose in 30 ml TAE, pH 7.9, was dissolved by heating in boiling water bath. The solution was allowed to cool at 50°C and poured onto the gel tray and left at room temperature for complete solidification. Samples mixed with one-tenth volume of stop mix dye (30% Ficoll, 0.025% xylene cyanole FF in gel buffer), was loaded in the wells of the
submerged gel and electrophoresed for 2 hr at 30 mA. The gels were stained with Coomassie Brilliant Blue R 250 and destained in tap water.

**Immunization Schedule**

Female rabbits (8-12 months, weight 1-1.5 kg) were immunized with native and ROS-modified TMP-BSA conjugates. The immunizing antigen (100 µg/rabbit) was emulsified with equal volume of Freund's complete adjuvant (FCA) for the first injection given subcutaneously at multiple sites. Subsequent injections were given in incomplete adjuvant (IFA) intramuscularly. Each animal received a total of 700 µg of antigen during the course of seven injections. Booster dose was administered after a fortnight from the last injection. Blood was collected by cardiac puncture and serum separated. The separated serum was decomplemented by heating at 56°C for 30 min. Preimmune sera were collected before immunization. The sera were stored in small aliquots at -80°C with 0.1% sodium azide as preservative.

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

Serum IgG was isolated by affinity chromatography on protein A-Sepharose CL-4B column. 0.5 ml serum diluted with equal volume of PBS, pH 7.4 was applied to column (15 cm x 0.9 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 280 nm. The concentration was determined considering 1.4 O.D. 280 =1.0 mg IgG/ml. The IgG was then dialyzed against PBS, pH 7.4 and stored at -80°C with 0.1% sodium azide.
Immunological Techniques

Immune, SLE and cancer sera were tested for antibodies by immunodiffusion, counterimmunoelectrophoresis and enzyme linked immunosorbent assay.

(a) Immunodiffusion

Immunodiffusion (ID) was carried out by Ouchterlony double immunodiffusion system using glass petri dishes as described by Tan et al. (1966). Six ml of 0.4% molten agarose in PBS, pH 7.4 containing 0.1% sodium azide was poured onto glass petri dishes and allowed to solidify at room temperature and then kept at 4°C for 4 hrs. Wells 5 mm in diameter separated by 8 mm in distance, were cut into hardened gel. Antigen and antibody were loaded and kept in moist chamber for 24-48 hrs. The petri dishes were washed with 5% sodium citrate to remove non-specific precipitin lines, if any. The precipitin lines were analyzed visually and photographed.

(b) Counterimmunoelectrophoresis

Counterimmunoelectrophoresis was performed by the method of Kurata and Tan (1976). Molten agarose (0.6%) in 25 mM barbital buffer, pH 8.4, containing 0.1% sodium azide was poured onto 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). The slides were then electrophoresed for 45-60 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 (ELISA)**

ELISA was performed using polystyrene (96 wells) flat bottom microtitre plates as described by Aotsuka *et al.* (1979).

**(i) Buffers and substrate**

*Tris buffered saline (TBS)*

10 mM Tris, 150 mM sodium chloride, pH 7.4.

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

20 mM Tris, 144 mM sodium chloride, 2.68 mM potassium chloride (KCl), pH 7.4, containing 500 μL Tween-20/L

*Carbonate-bicarbonate buffer*

15 mM sodium carbonate, 35 mM sodium bicarbonate, 2 mM magnesium chloride, pH 9.6.

*Substrate*

500 μg p-nitrophenyl phosphate/ml of carbonate-bicarbonate buffer.

**(ii) Procedure**

Polystyrene microtitre plates were coated with 100 μl of antigen (2.5 μg/ml in TBS, pH 7.4) for 2 hr at room temperature and overnight at 4°C. Unbound antigen was removed by washing thrice with TBS-T. Unoccupied sites were blocked with 200 μL of 1.5% BSA in TBS for 4-6 hr at room temperature. The plates were washed once with TBS-T. The antibody 1:100 diluted in TBS was coated in each well. After incubating the plates for 2 hrs. at room temperature and at 4°C overnight, the plate was extensively washed with TBS-T. The bound antibodies were then assayed by an appropriate anti-immunoglobulin alkaline phosphatase conjugate using p-nitrophenyl phosphate as substrate. The plates were incubated at 37°C for 1 hr and then read at 410 nm. Each sample was
coated in duplicate and the results were expressed as a mean of $A_{\text{test}} - A_{\text{control}}$.

(d) Competition ELISA

The antibody specificity was determined by competition ELISA (Hasan et al., 1991). Varying amounts of inhibitors (0-20 µg/ml) were incubated with a constant amount of antibody for 2 hrs. at room temperature and overnight at 4°C. The resulting immune complex was coated in wells instead of the serum IgG. The remaining steps were the same as in direct binding ELISA. The results were expressed as percent inhibition.

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

$A_{\text{inhibited}} =$ Absorbance at 20 µg/ml inhibitor concentration

$A_{\text{uninhibited}} =$ Absorbance at zero inhibitor concentration.