Experimental
MATERIALS

Human serum albumin (HSA) essentially fatty acid free, anti-human/anti-rabbit IgG-alkaline phosphatase conjugate, p-nitrophenyl phosphate, Tween-20, SDS-molecular weight protein markers, calf thymus DNA, nuclease S1, ethidium bromide, Coomassie Brilliant Blue G-250 and R-250, sodium dodecyl sulphate, Millipore filter, Freund’s complete and incomplete adjuvants, agarose, bovine serum albumin, ampicillin and mannitol were purchased from Sigma Chemical Company, U.S.A. Protein-A Sepharose CL-4B was purchased from Genei, India. Sephacryl S-200 HR, Ficoll 400 and 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, India. Absolute ethanol was obtained from BDH Laboratory Supplies, England. Polystyrene microtitre flat bottom ELISA plates and modules were purchased from NUNC, Denmark. Qiagen plasmid mega kit, acrylamide, ammonium persulphate, bisacrylamide, N,N,N,N-tertramethylethlenediamine (TEMED) were from Bio-Rad Laboraties U.S.A. EDTA (disodium salt), hydrogen peroxide, sucrose, chloroform, isoamylalcohol, methanol, glacial acetic acid were from Qualigens, India. All other reagents/chemicals were of the 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.), Hitachi F-200 spectrofluorophotometer (Japan), Amershan Biosciences akta purifier column resource Q anion exchanger, and Jasco spectropolarimeter (model J-720) were the major equipments used in this study.
Collection of Sera and Blood Samples

Normal human sera (n = 22) were obtained from healthy subjects. SLE serum samples were obtained from outdoor and indoor patients of the Department of Medicine, All India Institute of Medical Sciences, New Delhi. SLE sera from 54 female of age ranging from 35-75 years (mean 48.2 ± 9.5) were studied. All SLE sera showing high titre anti-DNA antibodies that satisfied the American College of Rheumatology (ACR) criteria for the diagnosis of SLE (Arnett et al., 1988) were used in this study.

Sera of cancer patients proven with histopathological diagnosis were obtained from J.N. Medical College Hospital A.M.U., Aligarh and from All India Institute of Medical Sciences, New Delhi.

Blood was collected from voluntary donors with history of type 1 diabetes mellitus (n = 31, male = 19, female = 12) under treatment in J.N. Medical College Hospital A.M.U., Aligarh. The control blood samples (n = 10, male = 6, female = 4) were collected from normal healthy subjects. The post prandial blood sugar level for diabetic patients was > 200 mg/dl while it was < 140 mg/dl for healthy subjects (without family history of diabetes). The glycated hemoglobin (HbA1C) was considered to be normal at < 6% and that of diabetes patients of poor glycemic control was > 6%.

All sera were decomplemented by heating at 56°C for 30 min and stored in aliquots at -20°C with sodium azide as preservative.

Protein Estimation

Protein was estimated by the methods of Lowry et al. (1951), Bradford (1976) or HSA concentration was measured spectrophotometrically using $E_{\text{1cm}}^{1}%$ of 5.30 at 280 nm (Wallevik, 1973).

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 alkali 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. One 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 observed when Coomassie Brilliant Blue G–250, an acidic dye, binds hydrophobically to protein having positively charged groups (Bradford, 1976). In the environment of these positively charged groups, protonation is suppressed and a blue color is observed ($\lambda_{max} = 595$ nm).

(a) **Dye Preparation**

One hundred 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 liter and filtered through a Whatman No.1 filter paper to remove undissolved particles.
(b) Protein Assay
To 1ml solutions, containing 10–100 μg protein, 5 ml of dye solution was added and the contents were mixed by vortexing. The absorbance was read at 595 nm after 5 min, against a reagent blank.

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

(a) Crystallization of Diphenylamine
Diphenylamine (2 gm) was dissolved in 200 ml boiling hexane. After adding 0.5 gm 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 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.

Purification of Calf Thymus DNA
Commercially available 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 isoamylalcohol (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.

**Polyacrylamide Gel Electrophoresis**

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 18.2 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) **Electrode Buffer**

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

(iv) **Procedure**

Glass plates, separated by 1.5 mm thick spacer were sealed with 1% agarose. The 7.5% 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 2.5% stacking gel. After the polymerization of separating gel, 2.5% stacking gel mixture was poured and allowed to solidify. Protein samples containing 10% (w/v) glycerol, or 2% (w/v) SDS, or 5% (v/v) mercaptoethanol and 0.002% bromophenol blue were applied. The electrophoresis was carried out at 50V to 80V for 3 to 8 h and protein were visualized using coomassie brilliant blue R-250 or by silver nitrate staining.
Recipe for Native PAGE

<table>
<thead>
<tr>
<th>Solution</th>
<th>7.5% Resolving gel</th>
<th>2.5% Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide</td>
<td>7.5 ml</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>3.75 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>16.95 ml</td>
<td>5.65 ml</td>
</tr>
<tr>
<td>1.5% Ammonium persulphate</td>
<td>1.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>15 (\mu L)</td>
<td>0.75 (\mu L)</td>
</tr>
</tbody>
</table>

Staining Procedures

Two staining methods were employed

(a) Coomassie Brilliant Blue Staining

Protein bands were detected by staining with 0.1% Coomassie Brilliant Blue R-250 in 40% isopropanol and 10% glacial acetic acid. Destaining was carried out in a mixture of 10% glacial acetic acid and 20% methanol.

(b) Silver Nitrate Staining

The procedure described by Merril et al. (1982) was followed with some modifications. After electrophoresis, the protein bands were fixed by rapidly immersing in a mixture of 40% methanol and 13.5% formaldehyde for 15 min with instant shaking. The gel was washed with distilled water twice, transferred to 0.02% \(Na_2S_2O_3\) solution for 2 min. The gel was again rinsed twice with distilled water. This was followed by incubation with 0.1% AgNO\(_3\) solution for 20 min. The gel was rinsed with distilled water briefly, immersed in developer solution (3% sodium carbonate solution containing 0.5% formaldehyde and 0.02% \(Na_2S_2O_3\)) for 15 min or until properly stained. The reaction was stopped by transferring the gel to stopper solution (25% isopropanol solution containing 10% acetic acid glacial) for 5 min. The gel was washed twice with distilled water and finally stored in distilled water.
**Agarose Gel Electrophoresis**

Agarose (0.8% or 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. DNA samples 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 was carried out for 1.5 - 2.5 h at 30 mA in TAE (40 mM Tris-acetate 9 mM EDTA, pH 8.0) buffer. The gels were stained with ethidium bromide (0.5 µg/ml), viewed by illumination under UV light.

**Modification of Human Serum Albumin**

HSA was modified in PBS (10 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.4) by a published procedure with slight modifications (Waris and Alam, 1998; Mansoor et al., 2005). An aqueous solution of native HSA (3.75 µM) was modified by hydroxyl radical, generated by the UV irradiation (30 minutes) of hydrogen peroxide (31 mM) at 254 nm. Excess hydrogen peroxide was removed by extensive dialysis against PBS.

**Spectroscopic Analysis**

The ultraviolet spectra of native and ROS-HSA samples were recorded in the wavelength range of 200-400 nm on a Shimadzu UV-240 spectrophotometer. The modifications incurred on native HSA were also analyzed by UV-difference spectroscopy.

**Circular Dichroism Measurements**

C.D. measurements were carried out with Jasco spectropolarimeter, model J-720 equipped with a microcomputer. The instrument was calibrated with D-10-camphorsulfonic acid. The CD measurements were made at 25°C with a thermostatically controlled cell holder attached to Neslab’s RTE 110 water bath with an temperature accuracy of ± 0.1°C. Spectra were taken with a scan speed of 20 nm/min at a response time of 1 s. Each spectrum was the average of four scans. Far-UV CD spectra were taken at protein concentration of 3.0 µM with a cell of 1 mm
path length and the near-UV CD spectra were taken at 20.0 μM protein concentration with a 10 mm path length. The MRE (mean residue ellipticity) was expressed in deg.cm².mol⁻¹, MRE = CD / (10×n×l×Cp) where CD in milli-degree, n is the number of amino acid residues (585), l is the path length of the cell, and Cp is the mole fraction. Helical content was calculated from the MRE values at 222 nm using the following equation (Chen et al., 1972).

\[
\% \text{Alpha helix} = \left( \frac{\text{MRE}_{222 \text{ nm}} - 2340}{30300} \right) \times 100
\]

Fluorescence Measurements

Fluorescence measurements were performed on spectrofluorimeter (Shimadzu RF-1501, Japan). The fluorescence spectra were measured at 25 ± 0.1°C with a cell of 1 cm path length. The excitation and emission slits were set at 5 and 10 nm, respectively. Intrinsic fluorescence was measured by exciting the protein solution at 280 or 295 nm and emission spectra were recorded in the range of 300-400 nm. Loss of fluorescence intensity (F.I.) was calculated using the following equation

\[
\% \text{Loss of F.I.} = \left( \frac{\text{F.I. native HSA} - \text{F.I. modified HSA}}{\text{F.I. native HSA}} \right) \times 100
\]

SDS-PAGE Under Reducing and Non-Reducing Conditions

Native and ROS modified HSA were characterized by SDS-PAGE under reducing and non-reducing conditions, using 12% and 7.5% (w/v) acrylamide gels, respectively. For reducing SDS-PAGE, samples were heated for 5 minutes at 95°C in a samples buffer that contained 50 mM Tris-HCl, pH 6.3, 2% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) mercaptoethanol, 0.002% (w/v) bromophenol blue. For non-reducing SDS-PAGE, samples were not heated and samples buffer contained all above chemicals except mercaptoethanol. Gels were electrophoresed at 50 V for 3-4 h and proteins were visualized using silver nitrate staining and coomassie brilliant blues R-250.

Absorption-Temperature Scan

Thermal denaturation profile of protein was performed on a Shimadzu UV-240 spectrophotometer equipped with a temperature programmer and controller assembly (Hasan and Ali, 1990). Protein samples were in PBS, pH 7.4 and
absorbance was recorded at a fixed wavelength of 280 nm. Percent denaturation was calculated using the equation:

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

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.

**HPLC Analysis**

HPLC analysis was carried out with Amershan Biosciences akta purifier column resource Q anion exchanger, equipped with a microcomputer. Native or modified HSA at a concentration of \(5.11 \times 10^{-5}\) M in 0.01 M Tris, pH 7.4 was allowed to bind the exchanger and elution was done by 0 to 0.5 M NaCl gradient.

**Assay of Carbonyl Formation**

Carbonyls contents of native and ROS-modified HSA were analyzed according to Levine et al. (1990) with slight modifications. The reaction mixture containing 15 μM native HSA or ROS-HSA, 0.5 ml of 10 mM 2, 4-dinitrophenylhydrazine (DNPH) / 2.5 M HCl was added and thoroughly mixed. After addition of 250 μM TCA (20%) and centrifugation, the pellet was collected and washed three times with 1 ml ethanol : ethylacetate (1:1) mixture. The pellet was then dissolved in 1 ml of 6 M guanidine solution and incubated at 30°C for 15 min. After centrifugation, the supernatant was collected and carbonyl contents were estimated from the absorbance at 370 nm using a molar absorption coefficient of 22,000 M⁻¹cm⁻¹. Samples were spectrophotometrically analyzed against a blank of 1 ml of guanidine solution (6 M). Protein concentration was determined in the samples by the method of Lowry et al. (1951). Carbonyl contents were expressed as nmol/ mg protein. Similar procedure was used for the estimation of protein carbonyl contents in patient serum protein and in isolated HSA from patients of SLE, cancer and diabetes.
**Immunization Schedule**

Native and ROS-modified HSA (100 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 600 μg of antigen in the course of 6 injections. Blood was collected from marginal vein of the ear. Serum was separated and decomplexed by heating at 56 °C for 30 min. 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 Human Serum Albumin**

Human serum albumin (HSA) was prepared according to the method described earlier (Tayyab and Qasim., 1990). Briefly plasma as obtained from normal human blood and patients of SLE, bladder cancer and diabetes was brought to 2.26 M in ammonium sulphate by adding requisite volume of 4M ammonium sulphate solution, pH 7.0. The mixture was kept for 12 hours at room temperature and then carefully diluted with water and with frequent addition of 0.5 N H₂SO₄ such that the final concentration of ammonium sulphate was reduced to 1.9 M and the pH to 4.2. After incubating it for 12 hours at room temperature, the precipitate was collected by centrifugation at 3000g for 30 mins. It was washed 3 times with 2.2 M ammonium sulphate solution, pH 4.2 and then dissolved in 0.06 M sodium phosphate buffer, pH 7.4 containing 0.08 M NaCl. The protein preparation thus obtained was serum albumin, extensively dialyzed against 6 liters of the 0.06 M sodium phosphate buffer pH 7.4 and stored at 4°C.

**Gel Chromatography**

Column of Sephacryl S-200 HR (40×2.0 cm) was packed according to the published procedure (Tayyab et al., 1991) and equilibrated with 0.06 M sodium phosphate buffer, pH 7.0. The void volume of the column was determined by passing a band of blue dextran (10 mg/2ml) and was found to be 40 ml. Nearly 10 mg of each dialyzed samples in 1 ml of 0.06 M sodium phosphate buffer, pH 7.0 were loaded on to the column and eluted with the same buffer at a flow rate of 15-20 ml/h in 3-5 ml
fractions. Protein in each fraction was monitored by the method of Lowry et al. (1951).

Isolation of Plasmid DNA

Plasmid DNA was isolated from *E. coli* strain DH<sub>5</sub>α “KS” by using Qiagen plasmid Mega kit (QIAGEN GmbH-Germany or USA).

(a) Material Used

Cell Strains and Media - *Escherichia coli* strain DH<sub>5</sub>α “KS” was used throughout the study. Luria Broth was used and ampicillin was added at a concentration of 100 μg/ml. RNase A solution was added to buffer P1 to give a final concentration of 100 μg/ml supplied in the kit.
### (b) Composition of buffers in the kit

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
<th>Storage</th>
</tr>
</thead>
</table>
| Buffer P1 (resuspension buffer) | 50 mM Tris-HCl, pH 8.0  
10 mM EDTA;  
100 μg/ml RNase A | 4 °C, after addition of RNase A |
| Buffer P2 (lysis buffer)       | 200 mM NaOH, 1% SDS                               | room temp.                     |
| Buffer P3 (neutralization buffer) | 3.0 M potassium acetate, pH 5.5                  | room temp. or 4 °C             |
| Buffer FWB (QIAfilter wash buffer) | 750 mM NaCl;  
50 mM MOPS, pH 7.0  
15% isopropanol | room temp.                     |
| Buffer QBT (equilibration buffer) | 750 mM NaCl;  
50 mM MOPS, pH 7.0  
15% isopropanol;  
0.15% Triton X-100 | room temp.                     |
| Buffer QC (wash buffer)         | 1.0 M NaCl;  
50 mM MOPS, pH 7.0;  
15% isopropanol | room temp.                     |
| Buffer QF (elution buffer)      | 1.25 M NaCl;  
50 mM Tris-HCl, pH 8.5  
15% isopropanol | room temp.                     |
| Buffer QN (elution buffer)      | 1.6 M NaCl;  
50 mM MOPS, pH 7.0  
15% isopropanol | room temp.                     |
| TE                            | 10 mM Tris-HCl, pH 8.0  
1 mM EDTA | room temp.                     |
| STE                           | 100 mM NaCl;  
10 mM Tris-HCl, pH 8.0;  
1 mM EDTA | room temp.                     |


**Preparation of Plasmid DNA**

Plasmid containing *E. coli* cells were grown in 10 ml L-broth containing 100 µg/ml ampicillin. After ~8 hrs. incubation at 37 °C, with vigorous shaking (~300 rpm) the culture was transferred to 500 ml L-broth containing 100 µg/ml ampicillin and further incubated for 12-16 hrs. at 37 °C. Cells were harvested by centrifugation at 6000 x g (6000 rpm) for 15 min. at 4 °C. The supernatant was discarded off. The open centrifuge tube was left in an inverted position to allow all of the supernatant to drain away. The bacterial cell pellet was resuspended in 50 ml of buffer P1 containing RNase A. The cells were resuspended completely by vortexing or pipetting up and down until no cell clumps remain. Added 50 ml of buffer P2, mixed gently but thoroughly by inverting 4-6 times, and incubated at room temperature for 5 min. Then added 50 ml of chilled buffer P3 mixed immediately but gently by inverting 4-6 times and incubated on ice for 30 min. Mixed the sample several times during the incubation on ice. The sample was centrifuge at ≥20,000 x g for 30 min. at 4 °C. Re-centrifuged the supernatant again at ≥20,000 x g for 15 min. at 4 °C. Removed promptly supernatant containing plasmid DNA. Equilibrated a QIAGEN-tip 2500 by applying 35 ml buffer QBT and allowed the column to empty by gravity flow. The supernatant was applied to the QIAGEN-tip and allowed it to enter the resin by gravity flow. Washed the Qiagen-tip with 2 x 100 ml buffer QC. DNA was eluted with 35 ml buffer QF. Precipitated DNA by adding 24.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mixed and centrifuged immediately at ≥15,000 x g for 30 min at 4 °C. The supernatant was carefully decanted off. DNA pellet was washed with 7 ml of room-temperature 70% ethanol and centrifuged again at ≥15,000 x g for 10 min. Carefully decanted the supernatant without disturbing the pellet. Air-dried the pellet for 10-20 min and redissolved the DNA in a suitable volume of TE, pH 8.0. DNA concentration was determined by UV absorbance and subjected to agarose gel electrophoresis.

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

Serum IgG was isolated by affinity chromatography on Protein A-Sepharose CL-4B column. Serum (0.3 ml) diluted with equal volume of PBS, pH 7.4 was applied to column (12 mm 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 with 1 ml of 1M Tris-HCl, pH 8.5. Three ml fractions were collected and read at 251 and 278 nm. IgG concentration was determined considering 1.40 OD$_{280}$ = 1.0 mg/ml. The isolated IgG was then dialyzed against PBS, pH 7.4 and stored at -20°C with 0.1% sodium azide.

**Immunological Detected of Antibodies**

Sera were tested for antibodies by enzyme linked immunosorbent assay, immunoduffsion, immunoprecipitin titration 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 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**

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

(ii) **Procedure**

An enzyme linked immunosorbent assay (ELISA) was carried out on polystyrene plates with slight modification (Ali and Alam, 2002; Dixit and Ali, 2004; Dixit *et al.*, 2005; Habib *et al.*, 2005; Khan *et al.*, 2005; Mansoor *et al.*, 2005; Khan
Polystryrene polysorp immunoplates were coated with 100 µL of native or modified HSA (10 µg/ml). Polystryrene maxisorp immunoplates were coated with 100 µL of plasmid DNA or native calf thymus DNA (2.5 µg/ml). The plates were coated for 2 h at 37°C and overnight at 4°C. Each sample was coated in duplicate and half of the plates served as control devoid of only antigen coating. Unbound antigen was washed thrice with TBS-T (20 mM Tris, 150 mM NaCl, pH 7.4 containing 0.05% Tween-20) and unoccupied sites were blocked with 2% fat free milk in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) for 4-6 h at 37°C. After incubation the plates were washed four times with TBS-T. The test serum serially diluted in TBS-T or affinity purified IgG in TBS (100 µL/well) was adsorbed for 2 h at 37°C and overnight at 4°C. Bound antibodies were assayed with anti-rabbit/anti-human IgG alkaline phosphatase conjugate using p-nitrophenyl phosphate as substrate. The absorbance of each well was monitored 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 $A_{\text{test}} - A_{\text{control}}$.

(b) Competition ELISA

The antigenic specificity of the antibodies was determined by competition ELISA (Dixit and Ali, 2001; Hasan et al., 1990; Dixit and Ali, 2004; Dixit et al., 2005; Habib et al., 2005; Khan et al., 2005; Mansoor et al., 2005; Khan and Ali, 2006). Varying amounts of inhibitors (0-20 µg/ml) were mixed with a constant amount of antisera or IgG. The mixture was incubated at room temperature for 2 hrs. and overnight at 4°C. The immune complex thus formed was coated in the wells instead of the serum/IgG. 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) Immunodiffusion

Immunodiffusion (ID) was carried out by Ouchterlony Double Diffusion System with slight modification (Ouchterlony, 1949). Six ml of 1% molten agarose in PBS, containing 0.1% sodium azide, was poured onto a glass petridish and
allowed to solidify at room temperature. Wells of 5 mm diameter were cut into the hardened gel and an appropriate concentration of antigen and antibody were placed in the wells. The petridish was allowed to stand in a moist chamber at room temperature for 48-72 hrs. The gel was washed with 5% sodium citrate to remove non-specific precipitin lines and the result was analyzed visually.

(d) Formation and Quantification of Immune Complexes
The assay was carried out in eppendorf microfuge tubes as described earlier with some modifications (Dixit and Ali, 2004). Increasing amount of antigen (0-100 μg) was added to a constant amount of immune or SLE IgG (120 μg) in an assay volume of 200 μL. Preimmune or normal human IgG was used as control. The mixture was incubated for 2 h at 37 °C and overnight at 4°C. The immune complexes were pelleted and supernatant was carefully taken out to fresh assay tube. To supernatant which contains unbound antigen and unbound IgG was added Protein A-Sepharose beads and the mixture incubated for 2 h at room temperature. The beads were pelleted and the unbound antigen in the supernatant was estimated spectrophotometrically. IgG bound to Sepharose beads was eluted with 0.58% glacial acetic acid containing 0.85% sodium chloride. After five minutes of incubation, the pH of supernatant was adjusted by 1.0 M Tris, pH 7.5. The eluted IgG was estimated by spectrophotometry and represent unbound IgG. Apparent affinity constant was determined by Langmuir plot (Langmuir, 1981; Benefield et al., 1982).

The Langmuir equation is commonly written as follows (Langmuir, 1918):

\[
x/m = \frac{a b C}{1 + a C}
\]

where,

- \(x\) = amount of material adsorbed or bound antigen.
- \(m\) = weight of adsorbent or bound IgG.
- \(C\) = concentration of material remaining in the solution after adsorption is complete or unbound antigen.
Taking the reciprocal of both sides of the above defined Langmuir equation

\[ \frac{1}{(x/m)} = \frac{1}{b} + \frac{1}{abC} \]

If adsorption follows the Langmuir analysis, a linear trace should result when the quantity \(1/(x/m)\) is plotted against \(1/C\) as shown in the below figure. Values of the constants \(a\) and \(b\) can be determined from the slope and intercept of the plot.
(e) Band shift assay

For the visual detection of antigen antibody binding and immune complex formation, gel retardation assays were performed (Sandford et al., 1988; Dixit and Ali, 2001; Dixit and Ali, 2004; Dixit et al., 2005; Habib et al., 2005; Khan et al., 2005; Mansoor et al., 2005; Khan and Ali, 2006). A constant amount of antigen (native and modified HSA) was incubated with varying amounts of IgG in PBS, pH 7.4 for 2 hrs. at 37 °C and overnight at 4 °C. One-forth volume of ‘sample’ dye was added to the mixture and electrophoresed on 7.5% native PAGE with 2.5% stacking gel for 3-8 h with 80V at 4°C. The gels were visualized using silver nitrate and/or coomassie brilliant blue (R-250) staining. Agarose gel electrophoresis was performed in case of plasmid DNA, native ctDNA and ROS-DNA. Immune complexes were prepared by incubating constant amount of DNA with varying amounts of IgG in PBS for 2 h at 37°C and overnight at 4°C or overnight at 37°C. The 1% or 0.8% agarose gel was run with TAE buffer (40 mM Tris acetate-9 mM EDTA, pH 8.0) at 30 mA for 1.5 – 2.5 h. The gels were stained with ethidium bromide (0.5 µg/ml), viewed by illumination under UV light.

Statistical Analysis

Data are presented as mean ± SD or mean ± SE. Significance of differences from control values were determined with the Student’s t-test (Statgraphics, Origin 6.1; MS-Excel; Sigma 2000). A value of $p < 0.05$ was considered to indicate statistical significance.