Materials and Methods
Materials

Chemicals

Human IgG, guanidinium hydrochloride, thiobarbituric acid (TBA), bovine serum albumin (BSA), molecular weight marker, anti-human and anti-rabbit-IgG-alkaline phosphatase conjugate, p-nitrophenyl phosphate, Tween-20, Coomassie Brilliant Blue G-250, sodium dodecyl sulphate (SDS), protein A-agarose, Freund’s complete and incomplete adjuvants, agarose, sodium azide, human serum albumin (HSA), H2A histone, haemoglobin, lysine, arginine, tyrosine, tryptophan, phenylalanine, human DNA, 5,5’-Dithio-bis(2-nitrobenzoic acid) and dialysis tubing were purchased from Sigma Chemical Company, U.S.A. Acrylamide, bisacrylamide, ammonium persulphate and N,N,N’,N’-tetramethylethylenediamine (TEMED) were from Bio-Rad Laboratories, U.S.A. Nε-(carboxymethyl)lysine (CML) was from PolyPeptide Laboratories, France, SAS. D-Glucose, Nitroblue Tetrazolium (NBT), 2,4-dinitrophenyl hydrazine (DNPH) and silver nitrate were obtained from SRL (India). Flat bottom polysorp ELISA modules were purchased from NUNC, Denmark. Ethylenediaminetetraacetic acid (EDTA) (disodium salt), chloroform, methanol, glacial acetic acid, iso-propanol, sodium chloride, sodium carbonate, sodium nitrite, sodium hydroxide, sodium bicarbonate, sodium acetate, magnesium chloride, hydrochloric acid, oxalic acid, copper sulphate, trichloro acetic acid, trifluoro acetic acid and potassium chloride were from Qualigens, India. All other reagents/chemicals were of the highest analytical grade available.

Equipments

ELISA microplate reader (Labsystem Multiskan EX, Finland), Shimadzu UV-1700 Spectrophotometer equipped with a thermo-programmer and controller unit, Spectrofluorimeter (Shimadzu, RF-5301), Spectropolarimeter (Jasco J-815), FT-IR Spectrometer (Shimadzu, 8201-PC 4000 cm\(^{-1}\)- 400 cm\(^{-1}\), U.S.A.), ELICO pH meter (L1-120), polyacrylamide gel electrophoresis assembly (Genei, India), Avanti 30 table top high speed refrigerated centrifuge (Beckman, U.S.A.), Gel-doc (Bio-Rad laboratories, U.S.A) and HPLC column (2622 SC, 4.6 x 60 mm; Hitachi) were the major equipments used in this study.
Collection of sera and blood samples

26 normal human sera were obtained from healthy subjects. 81 rheumatoid factor positive sera (Tested by Rhelax RF- slide test for rheumatoid factor) of RA patients were collected from the Department of Microbiology, J.N.Medical College, A.M.U. Blood samples from RA patients were obtained from outdoor and indoor patients of the department of Orthopaedics, J.N.M.C., A.M.U. Verbal consent of patients as well as healthy subjects was obtained before taking blood samples. The patients were chosen for the study after having a preliminary evaluation consisting of a brief medical history, smoking, alcohol consumption and physical examination. Patients with any history of chronic diseases such as liver diseases, diabetes mellitus, respiratory disorders, cardiovascular diseases and alcohol usage and smoking were not included in this study. Those RA patients who were pregnant, not ambulant or taking oral corticosteroids and under treatment with disease modifying anti-rheumatic drugs were excluded. None of the RA patients had other autoimmune disorders. Blood was taken from the subjects and the erythrocyte sedimentation rate (ESR) was determined by Westergren method (Gambino et al., 1965). Serum level of C-reactive protein (CRP) in RA patients was measured by nephelometric assay (Pearle et al., 2007). Samples were collected in a glass test tube and left to clot for 30 min at 37 °C. Serum was separated by centrifugation at 3000 rpm for 10 min. Serum samples were heated at 56 °C for 30 min to inactivate complement proteins and stored in aliquots at −20 °C with 0.1% sodium azide as preservative.

Methods

Determination of protein concentration

Protein estimation was carried out 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 solutions at high pH).
(a) *Folin-Ciocalteau reagent*

The reagent was 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 tartrate.

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 test 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 the change in absorption maxima of an acidic solution of Coomassie Brilliant Blue G-250 from 465 nm to 595 nm when protein binds to the dye. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change.

**Dye preparation**

100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol and 100 ml of 85% (v/v) orthophosphoric acid was added to it. The resulting solution was diluted to a final volume of 1.0 litre and filtered through a Whatman filter paper (No. 1) to remove undissolved particles.
Protein assay

To 1.0 ml of solutions, containing 10-100 µg protein, 5.0 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.

IgG glycation

Commercially available human IgG (0.825 µM) was glycated by incubating with 5 mM D-glucose in PBS (10 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.4). The reaction mixture was incubated under sterile conditions at 37 ºC for different time intervals ranging from 5 - 25 days. After incubation, the solutions were extensively dialyzed against PBS to remove the excess glucose. The solution of IgG without glucose served as control.

Absorbance spectroscopy

The absorption profile of native and glycated samples incubated for 5, 10, 15, 20 and 25 days were recorded on Shimadzu UV-1700 spectrophotometer in the wavelength range of 200-400 nm in quartz cuvette of 1 cm path length.

SDS- polyacrylamide gel electrophoresis

The electrophoretic behaviour of protein samples was analysed by SDS-PAGE on 10% polyacrylamide gel in non-reducing conditions according to the procedure described by Laemilli (1970) with slight modifications. The following stock solutions were prepared.

Acrylamide-bisacrylamide (30:0:8)

A concentrated stock solution of 30% acrylamide (30 gm) containing 0.8% bis-acrylamide (0.8 gm) was prepared in 100 ml of distilled water. After being prepared, this solution was filtered and stored at 4 ºC in a dark bottle.
**Resolving gel buffer**

A stock solution was prepared by dissolving 9.08 gm tris (1.5 M) in 40 ml of distilled water. The contents were mixed, pH adjusted to 8.8 by 6 N HCl and the final volume brought to 50 ml with distilled water.

**Electrophoretic buffer**

3.0 gm tris (0.025 M), 14.4 gm glycine and 1 gm SDS (0.1%) were dissolved in distilled water and volume made up to one litre.

**Procedure**

Cleaned glass plates, separated by 1.5 mm thick spacer, were sealed from the bottom and sides with 1% agarose. The 10% non-reducing resolving gel was prepared by mixing the components, poured into the space between the glass plates and allowed to solidify at room temperature. Protein samples mixed with one fourth volume of sample dye (50% glycerol, 10% SDS, 1 M tris pH 6.8 and 1% w/v bromophenol blue) were applied into the wells and electrophoresis was carried out at 80 V for 3-4 hr. The electrophoresed proteins were visualized by staining with silver nitrate.

**Composition of 10% resolving gel**

The following solutions were mixed and instantly poured between the glass plates.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>Acrylamide – bisacrylamide</td>
<td>3.3 ml</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>10% ammonium persulphate</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>4.0 µl</td>
</tr>
</tbody>
</table>

**Native polyacrylamide gel electrophoresis**

Native PAGE of proteins samples was performed as described by Laemilli (1970). Reagents used and the procedure followed was same as for SDS-PAGE. The only difference was that the electrophoresis carried out in the absence of SDS.
Silver nitrate staining of gel

Silver staining of the gel was performed as described by Merril et al (1981). After electrophoresis, the protein bands were fixed by rapidly immersing the gel in a mixture (40% methanol and 13.5 % formaldehyde) for 10 min with intermittent shaking. The gel was then washed with distilled water twice at an interval of 5 min and transferred to 0.02% sodium thiosulphate (Na$_2$S$_2$O$_3$) solution and incubated for 1 min. The gel was again rinsed twice with distilled water at an interval of 20 sec. This was followed by treatment with 0.1% AgNO$_3$ solution for 10 min. The gel was washed with distilled water briefly and then immersed in a developer solution (3% sodium carbonate solution containing 0.05% formaldehyde and 0.02% Na$_2$S$_2$O$_3$) for 15 min or until the gel was properly stained. The reaction was stopped by transferring the gel to a stopper solution (25% isopropanol solution containing 10% glacial acetic acid) and left for 5 min. The gel was washed twice with distilled water and finally stored in distilled water.

Fluorescence studies

Fluorescence spectra were recorded on Shimadzu (RF-5301-PC) spectrofluorophotometer (Japan) at 25±0.1 °C in a 1 cm path length cell at 10 nm slit width. Intrinsic fluorescence was measured by exciting the protein samples at 280 nm and 295 nm and emission spectra were recorded in 300-400 nm range (Rasheed et al., 2009). Loss in the fluorescence intensity (F.I.) was calculated using the following equation:

\[
\% \text{ loss of F.I.} = \left[ \frac{(\text{F.I. native sample} - \text{F.I. glycated sample})}{\text{F.I. native sample}} \right] \times 100
\]

Possible presence of AGEs in the glycated sample was verified with AGE-specific fluorescence at 440 nm after excitation at 370 nm (Lapolla et al., 1992). Increase in fluorescence intensity (F.I.) was computed by the following equation:

\[
\% \text{ increase of F.I.} = \left[ \frac{(\text{F.I. glycated sample} - \text{F.I. native sample})}{\text{F.I. glycated sample}} \right] \times 100
\]
Circular dichroism measurements

Far-UV CD measurements were carried out with Jasco spectropolarimeter, model J-815 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 a temperature accuracy of ± 0.1 °C (Chen et al., 1972). Native and glycated IgG samples (2.5 µM) were placed in 1 mm path length cuvette and spectra were recorded in the wavelength range of 200-250 nm. Each spectrum was the average of 2 scans and all the scans were recorded at an interval of 1 nm wavelength. A scan speed of 20 nm/min and response time of 1 sec was chosen to record CD spectra.

FT-IR spectroscopy

The native and glycated IgG samples were then subjected to spectral recording on a Shimadzu FT-IR spectrophotometer (8201-PC). Samples to be analysed on FT-IR spectrophotometer was first lyophilized and prepared as KBr pellets (Jovanovic et al., 2008).

Thermal denaturation studies

Thermal denaturation studies of IgG were performed to ascertain its thermal stability upon glycation. Mid point melting temperature (Tm), of native and glycated IgG samples was determined by subjecting them to heat denaturation on Shimadzu UV-1700 Spectrophotometer equipped with a thermo-programmer and controller unit (Khan et al., 2006). All the samples were melted from 30°C to 90°C at a rate of 1.0 °C/min. The change in absorbance at 280 nm was recorded with increasing temperature and percent denaturation was computed using the following equation:

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 (90 °C).
- \( A_{30} \) = Initial absorbance at 30 °C.
Detection of an AGE, N^ε-(carboxymethyl) lysine in glycated IgG sample by high performance liquid chromatography (HPLC)

Samples of native and AGE-IgG were subjected to HPLC analysis for CML after acid hydrolysis. The retention time of commercially available CML was taken as reference for comparison. Brief descriptions of sample preparation are as follows: The native and glycated IgG samples were first hydrolyzed with 6 N HCl for 24 hr at 110°C. The hydrolyzed samples were then subjected to ultrafiltration through 0.42 µM Millex filter. The filtered samples were analyzed for CML using an ion-exchange HPLC column (2622 SC, 4.6 x 60 mm; Hitachi) as described previously (Ikeda et al., 1996; Nagai et al., 2000).

Determination of protein-bound carbonyl groups

Carbonyl contents of native and glycated IgG samples were analyzed by previously published procedure (Levine et al., 1994) with slight modifications. Quantitative estimation of carbonyl groups in proteins is carried out by DNPH reagent and analyzed spectrophotometrically. In the reaction mixture containing 6.6 µM native IgG/ glycated IgG, 0.5 ml of 10 mM DNPH (dissolved in 2N HCl) was added and thoroughly mixed. After incubation for 1 hr at room temperature, the DNP-hydrazones were precipitated by adding 0.5 ml of trichloroacetic acid (20% v/v) and centrifuged for 3 min at 11,000 x g. The pellet was washed with 1 ml of ethanol-ethyl acetic acid mixture (1:1; v/v) to get rid of any extra DNPH reagent. Samples were incubated for 10 min at room temperature followed by centrifugation at 11,000 x g for 5 min. The supernatant was discarded and pellet was washed twice with ethanol-ethyl acetic acid mixture. The protein pellet was finally suspended in 1 ml of 6 M guanidinium chloride dissolved in 20 mM phosphate buffer pH 2.3 (adjusted with trifluoroacetic acid). Samples were incubated at 37 °C for 15-30 min for complete solubility of proteins. All samples were then centrifuged to remove any insoluble material. The concentration of DNPH was determined by absorbance measurement at 360 nm against guanidinium chloride (as blank) using the molar extinction coefficient of 22,000 M^-1.cm^-1. IgG concentration in samples was determined by recording absorbance at 278 nm and protein carbonyl content was expressed as nmole/mg of protein.
**Determination of free sulfhydryl groups**

The estimation of free sulfhydryl groups in native and glycated IgG samples was performed according to Ellman’s method (Sedlak and Lindsay., 1968). First, the DTNB (5, 5'-Dithio-bis[2-nitrobenzoic acid]) stock and the tris dilution buffer were prepared as follows: DTNB solution- 50 mM sodium acetate (NaAc), 2 mM DTNB in distilled water; Tris solution- 1 M tris/ pH 8.0. DTNB stock was diluted in order to prepare the working solution by adding 100 µl tris solution and 840 µl water in 50 µl of DTNB stock solution. 10 µl of protein samples was added to 990 µl of DTNB reagent. It was thoroughly mixed and incubated for 5 min at 37 ºC. Absorbance was taken at 412 nm. The free sulfhydryl group content was determined using an extinction coefficient of 13600 M⁻¹.cm⁻¹.

**NBT reduction assay**

The ketoamine moieties formed by the glycation of IgG was determined by NBT reduction assay (Ansari et al., 2011) with slight modifications. IgG (0.825 µM) was incubated with 5 mM glucose for 20 days at 37 ºC in 10 mM PBS and absorbance was taken each day to determine the content of ketoamine moieties. 0.1 ml of native and glycated IgG samples were mixed with 1 ml of 100 mM carbonate buffer (pH 10.8) containing 0.25 mM NBT and incubated for 45 minutes at 37 ºC. The color was read by taking the absorbance at 525 nm. The content of ketoamine moieties (nmol/ml) was determined using an extinction coefficient of 12640 M⁻¹.cm⁻¹ for monoformazan (Mironova et al., 2005).

**Thiobarbituric acid assay**

Gottschalk first discovered that treating fructosamines with acids yields 5-Hydroxymethylfurfural (HMF) (Gottschalk., 1952). However, the typical approach, first reported by Keeney and Bassette, has been to react HMF with thiobarbituric acid (TBA) to form a derivative that has an absorbance maximum at 443 nm (Keeney and Bassette., 1959). Absorbance of protein samples was taken each day for 20 days, in this assay also, in order to find out the HMF content. Briefly, 1 ml each of the native and glycated samples were mixed with 1M oxalic acid and incubated at 100 ºC for 2 hr (Fluckiger and Winterhalter., 1976). The protein from the assay mixture was
removed by precipitation with 40% trichloroacetic acid. TBA (0.05 mol/l) was added to protein-free filtrate and incubated at 37 ºC for 40 min. The color was developed and the amount of HMF (nmol/ml) was calculated using molar extinction coefficient value of $4 \times 10^4$ M$^{-1}$.cm$^{-1}$ at 443 nm.

**Immunization schedule**

The immunization of random bred, female New Zealand white rabbits was performed as described previously (Dixit *et al.*, 2005). Fresh rabbits weighing 1-1.5 kg were selected for immunization and it was making sure that the rabbits were infection free. Briefly, rabbits were immunized in duplicates (n=4; two each for native and glycated antigens) intramuscularly at multiple sites with 100 µg of antigen, emulsified with an equal volume of Freund's complete adjuvant. The animals were boosted in Freund's incomplete adjuvant at weekly intervals for 6 weeks with the same amount of antigen. Test bleeds were performed 7 days post boost and antibody titre was determined. Marginal ear vein of the rabbit was punctured and blood was collected in the serum tube. This should be done carefully in order to prevent hemolysis. Serum was separated from the blood and heated at 56 ºC for 30 min to inactivate complement proteins. Preimmune sera were obtained from the blood collected prior to immunization. The sera were stored at -20 ºC in small aliquots with 0.1% sodium azide as preservative. The institutional ethic committee has approved the study protocol.

**Isolation of IgG by protein A-agarose column**

Serum IgG was isolated by affinity chromatography on protein A-agarose affinity column (Dixit *et al.*, 2011). Serum (0.3 ml) diluted with equal volume of PBS (pH 7.4) was applied on top of the column pre-equilibrated with the same buffer. The wash through was recycled 2–3 times and unbound material 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., 1978) and collected in a tube containing 1.0 ml of 1.0 M Tris–HCl (pH 8.5). Three ml fractions were collected and read at 278 nm. The IgG concentration was determined considering $1.4 \text{OD}_{278} = 1.0 \text{ mg IgG} / \text{ml}$. The isolated IgG was dialyzed against PBS, pH 7.4 and stored at -20 ºC with 0.1% sodium azide.
**Immunological detection of antibodies**

Preimmune and immune sera were tested for antibodies against native and glycated IgG by enzyme linked immunosorbent assay and band shift assay.

**Enzyme linked immunosorbent assay**

Following reagents were prepared and used in ELISA.

**Antigen coating buffer:** 15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6.

**Tris-buffered saline (TBS):** 10 mM tris, 150 mM NaCl, pH 7.4.

**Tris-buffered saline containing Tween 20 (TBS-T):** 20 mM tris, 144 mM NaCl, 2.68 mM KCl, pH 7.4 containing 500 µl Tween-20 per litre.

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

**Substrate:** 500 µg p-nitrophenyl phosphate (p-NPP) per ml of carbonate-bicarbonate buffer.

**Procedure**

ELISA was performed on flat bottom polystyrene plates as described earlier (Ali and Alam., 2002). 96-wells polystyrene polysorp immunoplates were coated with 100 µl of the native or glycated IgG (10 µg/ml) in antigen coating buffer. The plates were incubated for 2 hr at 37 °C and overnight at 4 °C. Each sample was coated in duplicate and half of the plate, devoid of antigen coating, served as control. The wells of the test plates were washed three times with TBS-T and unoccupied sites were blocked with 150 µl of 2.5% fat-free skimmed milk in TBS for 4-5 hr at 37 °C. After incubation, the plates were washed 5-6 times with TBS-T. Test sera serially diluted in TBS-T was added to each well (100 µl/well) and reincubated for 2 hr at 37 °C and overnight at 4 °C respectively. After incubation, the plates were washed thrice with TBS-T and bound antibodies were assayed with anti-Immunoglobulin G alkaline phosphatase conjugate in TBS. After incubation for 2 hr, the plates were again
washed three times with TBS-T and twice with distilled water using p-nitrophenyl phosphate as substrate. Absorbance (A) of each well was monitored at 410 nm on an automatic microplate reader and the mean of duplicate readings for each sample was recorded. Results have been expressed as a mean of A_{test} - A_{control}.

**Competition ELISA**

The antigenic specificity of the antibodies was determined by competition ELISA (Habib et al., 2005). Varying amounts of inhibitors (0–20 µg/ml) were mixed with a constant amount of antiserum or affinity purified 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 (Ansari et al., 2009). Immune complexes were prepared by incubating constant amount of native or glycated IgG with varying amounts of affinity purified IgG in PBS for 2 hr at 37 °C and overnight at 4 °C. One-fourth volume of sample dye was added to the mixture and electrophoresed on 10% SDS-polyacrylamide gel for 3 hr at 80 V. The gels were visualized by staining with silver nitrate.

**Statistical analysis**

Data are presented as mean ± SD. Statistical significance of the data was determined by Student’s t test, and a value of \( p < 0.05 \) was considered as statistically significant.