Advanced Glycation End Products (AGEs) Damaged IgG, a Target for Circulating Autoantibodies in Patients with Type 1 Diabetes Mellitus

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Abstract: The role of advanced glycation end products (AGEs)–damaged immunoglobulin G (AGE-IgG) in type 1 diabetes has been investigated in the present study. IgG was isolated from the normal humans and was subjected to *in vitro* glycation with glucose. The AGEs caused extensive damaged to IgG. The AGE-IgG was found to be highly immunogenic in rabbits as compared to native IgG. The binding characteristics of circulating autoantibodies in type 1 diabetes mellitus (DM) patients against native and AGE-IgG were assessed. Type 1 DM patients (n=31) were examined by ELISA and their results were compared with healthy age-matched human controls (n=22). High degree of specific binding by 61.3 % of DM sera autoantibodies towards AGE-IgG was observed, in comparison to its native analog ($p< 0.05$). Sera from those type 1 DM patients having smoking history, high aging with high degree of disease showed substantially stronger binding to AGE-IgG over native IgG in particular. IgG from type 1 DM patients (DM-IgG) contained higher levels of carbonyls as compared to normal human subjects (normal-IgG) ($p<0.001$). Collectively, the AGEs modification of IgG causes perturbations, resulting in the generation of neo-epitopes, and making it a potential immunogen. The IgG modified with AGEs may be one of the factors for the induction of circulating type 1 diabetes autoantibodies.

Keywords: AGEs, IgG, AGE-IgG, Type 1 diabetes mellitus.

INTRODUCTION

Hyperglycemia and poor glycemic control are considered to be the key basal factors in the development of diabetes and its associated secondary complications. Acute or chronic hyperglycemia is known to enhance the formation of advanced glycation products (AGEs) and is a primary factor that initiates and promotes diabetes and diabetic associated complications [1]. Non-enzymatic glycation of proteins in serum and tissues is a pathophysiological consequence of hyperglycemia in diabetes mellitus, that also correlates with aging [2]. Patients with diabetes having higher serum glucose concentration, are thought to be more prone to glycation than healthy persons. It is well documented that AGEs alter unique three-dimensional integrity of various plasma proteins, such as albumin, hemoglobin, collagen, etc, which could induce the functional abnormalities and thereby lead to several pathophysiological conditions including diabetes, rheumatic arthritis and their associated secondary complications [3,4]. Glycation of IgG is of special interest due to its influence on the functionality of immunoglobulin's and overall immunocompetence. Several studies showed the extent of glycation of IgG and its influence on the biological functionality of IgG [5-13], but the immunological role of AGEs damage IgG (AGE-IgG) in patients with type 1 DM is still not fully understood. Proper extensive clinical investigation of this aspect with a high level of confidence are needed.To meet this need, we hypothesized that AGE-IgG helps to initiate autoimmunity in type 1 diabetes mellitus patients. To test this hypothesis, we immunized rabbits with AGE-IgG or native IgG and found that autoimmunity was established faster and more vigorously in the animals that were immunized with the damaged IgG. We studied the presence of circulating autoantibodies in type 1 DM sera directed against native and AGE-IgG. The evaluation of the effects of oxidative stress on the biological properties of isolated IgG from diabetes patients was also attempted.

MATERIALS AND METHODOLOGY

Materials

Anti-rabbit-IgG-alkaline phosphatase conjugate, Anti-human-IgG-alkaline phosphatase conjugate, p-nitrophenyl phosphate, and Tween-20 were purchased from Sigma Chemical Company, U.S.A. Protein A-Sepharose CL-4B was purchased from Genei, India. Polystyrene microtiter flat bottom ELISA plates and modules were purchased from NUNC, Denmark. All other chemicals were of analytical grade. Protein was estimated by the methods of Lowry *et al.* [14] or Bradford [15].

Human Subjects

Blood was collected from voluntary donors with the history of type 1 diabetes mellitus (n=31, male=19, female=12, aged 48±15) under treatment in J.N. Medical College Hospital A.M.U., Aligarh and Jolly Grand Hospital, Dehradun, UK, India. The control samples (n=22, male=17, female=5,

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aged 43±17) were collected from normal healthy subjects. The average (±SD) post prandial blood sugar level for 31 diabetic patients was 354±84 mg/dl, while it was 110±8 mg/dl for 22 healthy subjects (without family history of diabetes). The glycated hemoglobin (HbA_{1C}) was 8.0±1.5 for patients, whereas 4.7±0.4 for normal subjects. Insulin antibodies (IAA), and anti-tyrosine phosphatase antibodies (IA-2A) were found in 74% and 39% of the cases. Immunopositivity for at least one of these autoantibodies was found in all patients. Forty eight percent male patients have smoking history. All other information on demographics and cumulative clinical and laboratory manifestations over the course of disease was obtained by both chart review and discussion with the patient and his/her family members. The informed consent was obtained from each patient or from patients' family members. All sera were decomplemented by heating at 56 °C for 30 min and stored in aliquots at −80 °C with sodium azide as preservative.

Purification of Immunoglobulin G

IgG from normal human sera was isolated by affinity chromatography on Protein A-Sepharose CL-4Bs column by previously published procedure [16]. Briefly, serum (0.3ml) diluted with equal volume of PBS, pH 7.4 was applied to the column (12 mm×45 mm) equilibrated with the same buffer. The flow was reloaded onto the column 2–3 times. Unbound proteins were removed by extensive washing with PBS, pH 7.4.The bound IgG was eluted with 0.58% acetic acid in 0.85% sodium chloride and neutralized with 1.0 ml of 1.0 mol/l Tris– HCl, pH 8.5, 3 ml fractions were collected and read at 251 and 278 nm. The IgG concentration was determined considering 1.38 OD_{278}=1.0 mg IgG/ml. The isolated IgG was dialyzed against PBS, pH 7.4 and stored at −20 °C. The homogeneity of isolated IgG was checked by polyacrylamide gel electrophoresis.

AGEs Preparation

For the preparation of AGE-IgG, affinity purified IgG at a concentration of 33.3 mM in 20 mM PBS, pH 7.4 was filtered through a 0.45 μm Millipore filter and incubated with 50 mM D-glucose under sterile conditions in capped vials for 0 - 60 days at 37^0C. Solution of IgG without glucose served as control. After incubation, the solutions were extensively dialyzed against PBS and stored at -80^0C.

Assay of AGE-IgG

The AGEs on IgG were quantified by a published colorimetric procedure using nitroblue tetrazolium (NBT) [17] with slight modification. BSA (10 mg/ml) was incubated with 0.5 M glucose for 15 days at 37°C in 20 mM PBS, which resulted in complete modification of protein with subsequent formation of ketoamines [18]. Native and AGE-IgG samples (50 μl) were added to 96-well microtiter plates in duplicate. 100 μl NBT reagent (250 μM in 0.1 M carbonate buffer, pH 10.35) was added to each well and incubated at 37°C for 2 h. The plate was read in a microplate reader at 550 nm. The amount of AGE-IgG in the sample was calculated using the standard curve constructed with glycated BSA.

Fluorescence Studies

Fluorescence measurements were performed on Hitachi F-200 spectrofluorimeter (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 [19]. Increase of fluorescence intensity (F.I.) was calculated by using the following equation:

$$\% \text{ Increase of F.I.} = [(F.I._{\text{Modified sample}} - F.I._{\text{Unmodified sample}}) / F.I._{\text{Modified sample}}] \times 100$$

Assay of Carbonyl Formation

Carbonyl contents of native and AGE-IgG were analyzed by previously published procedure [20] with slight modifications. The reaction mixture containing 6.7 μM native IgG or AGE-IgG, and 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 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^{-1} cm^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.* [14]. Carbonyl contents were expressed as nmol/mg protein.

Immunization Schedule

The immunization of random bred, New Zealand white rabbits was performed as described previously [21]. Briefly, rabbits (n=4; two each for native and AGE-IgG antigens) were immunized 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, which gave appropriate titer of the antibody. The animals were bled and the serum separated from the blood was heated at 56 °C for 30 min to inactivate complement proteins and stored at −20°C.

Enzyme Linked Immunosorbent Assay

Enzyme linked immunosorbent assay (ELISA) was performed on flat bottom 96-wells polystyrene polysorp immunoplates as described previously [22,23]. Briefly, plates were coated with 100 μL of respective antigen (10 μg/ml) for 2 h at 37 °C and overnight at 4 °C. The plates were washed 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. The test serum serially diluted in TBS-T or affinity purified anti- IgG antibodies 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 (A) 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 $A_{\text{test}} - A_{\text{control}}$.

Competition ELISA

The antigenic specificity of the antibodies was determined by competition ELISA [24]. Varying amount of inhibitors (0–20 μg/ml) were allowed to interact with a constant amount of antiserum or anti-IgG antibodies for 2 h and overnight at 4 °C, the mixture was added to antigen-coated plates and the residual antibody level was detected by ELISA. Percent inhibition was calculated by using the formula:

Percent inhibition = [1- ($A_{inhibited}$ / $A_{uninhibited}$)] X 100

Statistical Analysis

All statistical analyses were performed using Origin 6.1 software package (one paired two tailed *t*-test with one way ANOVA) and $P < 0.05$ was considered significant. Values shown are mean ± SD unless stated otherwise.

RESULTS

Isolation, AGEs-Modification, and Characterization of IgG

IgG was purified from normal human sera (NHS) by Protein-A Sepharose CL-4B affinity column. The purified IgG was found to elute as a single symmetrical peak and gave a single band on SDS-PAGE (Fig. **1**). Affinity purified IgG was then modified by 50 mM glucose and changes induced in IgG were analyzed by fluorescence spectroscopy. The oxidation of tryptophan residues on damaged IgG was evident by the loss of 53.6 % fluorescence intensity (FI) at 340 nm using an excitation wavelength of 295 nm (Fig. **2a**). The damage on tryptophan residues was confirmed by the loss of 51.3 % FI at 340 nm using an excitation wavelength of 280 nm (Fig. **2b**). The ketoamine moieties formed by the glycation of IgG were measured colorimetrically by using NBT (Table **1**). The average ketoamines (±SD) of five independent assays of AGE-IgG and native IgG were 9.4±1.3 and 2.3±1.1 nmol/mg protein, respectively (Table **1**). Oxidation of protein typically results in an increase in protein carbonyl contents, a known biomarker of oxidative stress. The average carbonyl contents (±SD) of five independent assays of AGE-IgG and native IgG were 18.1±1.2 and 2.8±1.4 nmol/mg protein, respectively (Table **1**), and a *p* value of < 0.001 indicates significant difference in the carbonyl contents of native and AGE-IgG. Characterization of native and AGE-IgG has been summarized in Table **1**.

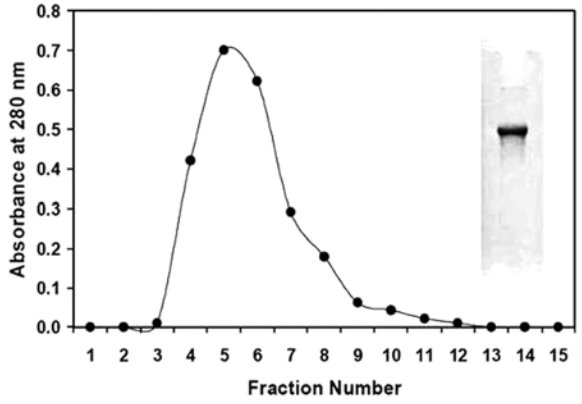

Fig. (1). Elution profile of normal human IgG on Protein-A Sepharose CL-4B affinity column. Inset: SDS-PAGE of purified IgG.

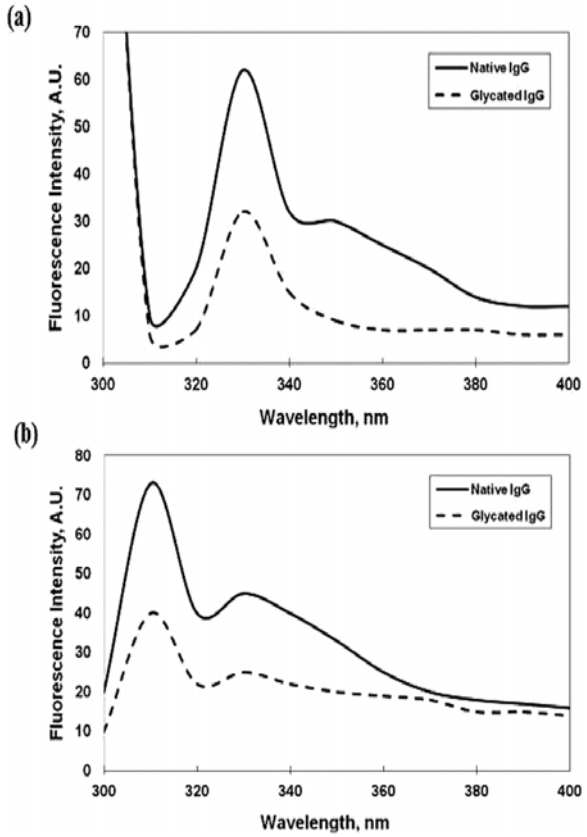

Fig. (2). Fluorescence emission spectra of native (—) and AGE-modified **(---)** IgG. Protein was in PBS, pH 7.4 at a concentration of 1.5 μM. The excitation wavelength was 295 nm (**a**) and 280 nm (**b**). The spectra are the average of three determinations.

Antigenicity of AGE-IgG

Direct binding ELISA showed AGEs-modified IgG as a potent immunogen in rabbits induces high titer antibodies as compared with native IgG titer under identical experimental conditions. Preimmune serum served as negative control, and did not show appreciable binding with the respective immunogens (Fig. **3**). Protein A-Sepharose isolated anti-AGE-IgG antibodies showed strong binding with their respective immunogens as evident by competitive inhibition assays. Preimmune affinity purified antibodies showed negligible binding with their respective immunogens under identical experimental conditions (Fig. **4**). The average percent inhibition (±SD) of five independent assays of affinity purified anti-AGE-IgG antibodies showed 92.8±3.2 at 20 μg/ml of inhibitor (AGE-IgG) concentration (Fig. **4a**). The concentration of competitor (AGE-IgG) required for 50% inhibition was only 0.81 μg/ml. Whereas, anti-IgG antibodies showed average percent inhibition (±SD) of 85±2.1 at 20 μg/ml of inhibitor (IgG) concentration under identical conditions (Fig. **4b**). The concentration of competitor (native IgG) required for 50% inhibition was 9.6 μg/ml. Preimmune IgG with AGE-IgG and IgG showed negligible inhibition under identical experimental conditions (Fig. **4**). Results point that autoimmunity was established faster and more vigorously in the animals that were immunized with AGE-modified IgG.

Table 1. Characterization of Native and AGE-modified IgG under Identical Experimental Conditions

Parameters	Native IgG	AGE-IgG	Modification
Florescence intensity (ext. 295 and emm. 340 nm)	656.3	304.1	53.6 % loss
Florescence intensity (ext. 280 and emm. 340 nm)	768.8	374.4	51.3 % loss
Ketoamine estimation (nmole/mg protein)	2.3±1.1	9.4±1.3*	75.5 % Increased
Carbonyl contents (nmol/mg protein)	2.8±1.4	18.1±1.2*	84.5% Increased

*p < 0.001 vs native IgG.

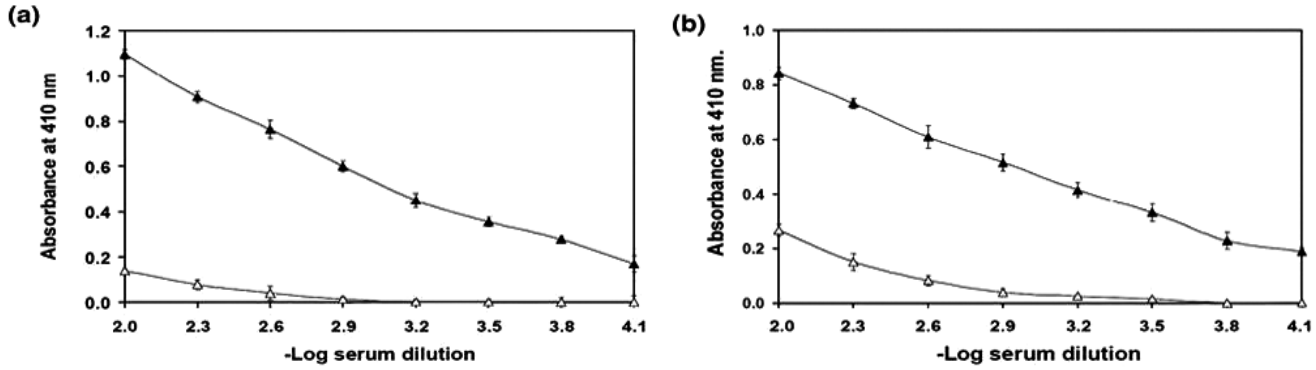

Fig. (3). Level of induced antibodies against native and AGEs-modified IgG. Direct binding ELISA of (**a**) AGEs-IgG and (**b**) IgG with immune (▲) and preimmune (△) sera. Microtiter plates were coated with the respective antigen (10 μg/ml). Each point represents a mean±S.D. of five independent assays.

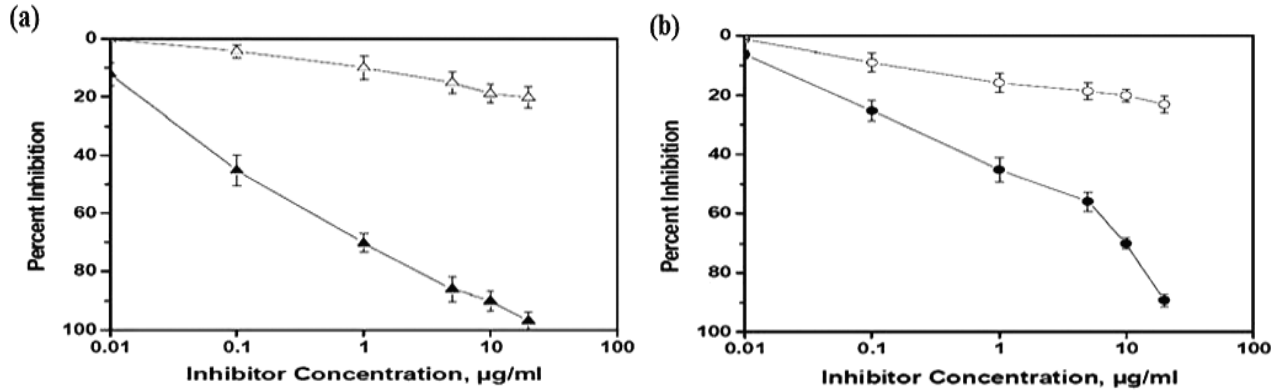

Fig. (4). (**a**) Inhibition of anti-AGE-IgG antibodies (▲) and preimmune antibodies (Δ) binding to AGEs-modified IgG. The inhibitor was AGE-IgG. The microtiter plates were coated with the AGE-IgG (10 μg/ml). (**b**) Inhibition of anti-native IgG antibodies (●) and preimmune antibodies (○) binding to native IgG. The inhibitor was native IgG. The microtiter plates were coated with the native IgG (10 μg/ml). Each point represents a mean ± S.D. of five independent assays.

Detection of Autoantibodies Against Native IgG and AGE-IgG in Type 1 DM Patients

Our study comprised serum samples from patients suffering from type 1 DM. Each sample was obtained after careful clinical examination of patients with proven diagnosis at J.N. Medical College Hospital, A.M.U., Aligarh and Jolly Grand Hospital, Dehradun, UK, India. Sera from thirty one type 1 DM and twenty two normal human subjects were tested for binding to native and AGE-IgG by direct binding ELISA (Fig. **5**). Majority of DM sera (19/31) showed strong binding to AGE-IgG over native IgG at 1:100 serum dilution ($p < 0.05$). No appreciable binding was observed with the normal human subjects. The average absorbance (±SD) at 410 nm of 19 DM sera binding to native and AGE-IgG was 0.51±0.07 and 0.85±0.06, respectively. Where as, the absorbance at 410 nm (±SD) of 22 normal human sera binding to both protein antigens was 0.24±0.04. The binding specificity of antibodies from 19 selected sera of DM patients was evaluated by competition inhibition ELISA using native and AGE-IgG as inhibitors. Microtiter plates were coated with AGE-IgG. The average percent inhibition (±SD) in the binding of 19 type 1 DM sera to native and AGE-IgG was 28.3±5.3 and 50.8±9.5, respectively (Table **2**). The data reveals striking differences in the recognition of native and modified IgG by DM autoantibodies ($p < 0.001$). Results point to a higher reactivity of autoantibodies in patient's sera towards AGE-IgG over native IgG. Table **2** summarizes complete demographics, clinical, biochemical and immunological characteristics of all 19 selected DM sera with their controls. Among 19 DM sera, 11

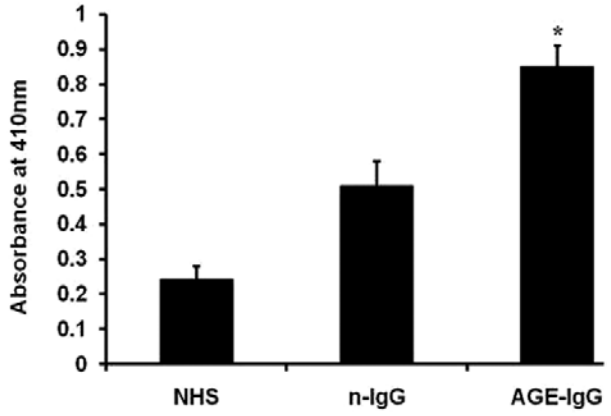

Fig. (5). Direct binding ELISA of 1:100 diluted type 1 diabetes serum samples. The microtiter plates were individually coated with IgG and AGE-IgG (10 μg/ml). Binding of type 1 diabetes sera to native IgG (n-IgG), and AGEs-IgG-modified IgG (AGE-IgG). Normal human serum (NHS) with either of the antigens. The numbers of diabetes serum samples were 17 (IgG, AGE-IgG) and normal human serum samples were 22 (IgG, AGE-IgG). Standard deviation has been indicated with each bar. $^{*}P<0.001$ vs. n-IgG.

sera were from male patients having smoking history of 25.5±10.1 years. Direct binding and competition ELISA results point to a strong reactivity of all DM smokers towards AGE-IgG over native IgG. Sera from aging patients with high degree of diabetes showed substantially higher binding to AGE-IgG as compared to native IgG (Table **2**). Protein carbonyl groups as biomarker of oxidative stress and AGEs formation, lead to modification of protein that typically results in an increase in carbonyl contents. The data showed that serum protein carbonyl contents were significantly ($p<0.05$) increased in eleven DM patients, as compared with eleven normal human subjects. The average carbonyl contents (±SD) of eleven independent assays of DM serum proteins and normal human serum proteins were 3.15± 0.35 and 2.28±0.25 nmol/mg protein, respectively (Table **2**). To investigate the extent of alterations in the biological properties of IgG in DM patients, IgG was isolated from same eleven diabetes patients (DM-IgG) and also from the same eleven normal human subjects (NH-IgG), and their carbonyl contents were compared. The purified IgG was found to elute in a single symmetrical peak on Protein A sepherose CL-4b affinity column. Polyacrylamide gel of purified IgG showed a single homogenous band (data not shown). The average carbonyl contents (±SD) of eight independent assays of DM-IgG and NH-IgG were 1.1±0.15 and 0.66±0.18 nmol/mg protein, respectively (Table **2**), and a *p*-value of <0.05 indicates significant difference in the carbonyl contents of DM-IgG and NH-IgG. The results were similar to those obtained with serum. High carbonyl contents were observed in those DM patients who are having high aging, high degree of diabetes and having smoking history. Low carbonyl contents were observed in those DM patients who are slightly younger in age and do not have any smoking record. Table **2** summarizes biochemical and immunological characteristics of DM sera. Direct binding and competition ELISA results point towards a strong reactivity of type 1 DM autoantibodies towards AGE-damaged IgG over native IgG. Our results are in full agreement to the view that glyco-oxidative damage of protein presents unique epitopes, which help to initiate autoimmunity.

DISCUSSION

Non-enzymatic glycosylation of proteins in serum and tissues is a pathophysiological consequence of hyperglycemia in diabetes mellitus that also correlates with aging [1,2] There is an increasing evidence that chronic hyperglycemia is the major cause of diabetes and its associated secondary complications [25,26]. Discovery of glucose dependent chemical modification of various proteins suggested that they could induce functional abnormalities, and thereby lead to the pathophysiology of diabetes [27]. Glycation of albumin changes its binding to drugs and its kidney transport [28]. Lens crystalline in diabetic patients undergo glycation reaction leading to the formation of high molecular weight mass that causes turbidity in lenses [29]. For collagen, it is shown that *in vitro* glycation changes its physicochemical properties including decrease solubility and elasticity [30]. Although there are evidences for *in vitro* and *in vivo* glycation of IgG [31,32], some discrepancies in this field still continues [33] and the immunologinicity of AGE-IgG in patients with type 1 diabetes is still unclear, however, AGE-IgG was found to be a target for circulating autoantibodies of RA patients [34]. In view of these, we studied the presence of circulating autoantibodies in type 1 DM sera directed against native and AGE-IgG.

In the present study, IgG was purified from normal human sera by Protein-A Sepharose CL-4B affinity column, and homogeneity of purified IgG was checked on SDS-PAGE. The affinity purified IgG was then modified by AGEs, generated by gylcation of IgG with glucose, resulting in extensive damage to IgG as evident by various phsicochemical techniques. Oxidation of tryptophan residues of IgG upon AGEs modifications was confirmed by the decrease in the fluorescence intensity when the protein was excited at 295 nm, these modifications on tryptophan residues were further confirmed by exciting the protein at 280 nm. Protein carbonyl contents are actually the most general indicator and by far the most commonly used biomarkers of protein oxidation and AGEs formation [20]. The oxidation of AGEs-IgG was further evident by the significant increase ($p<0.05$) in carbonyl contents. Modified IgG was found to be a potent antigenic stimulus inducing high titer antibodies in rabbits as compared to the titer induced by native IgG. The antigenic specificity of affinity purified anti-AGE-IgG antibodies and anti-IgG antibodies reiterated that induced antibodies that are immunogen specific. The substantially enhanced immunogenicity of AGE-IgG in comparison to native IgG could possibly due to the generation of potential neo-epitopes against which antibodies are raised.

Type 1 DM is an organ-specific autoimmune disease, in which the insulin-producing beta cells in the pancreatic islets are selectively eliminated. The autoimmune attack destroys the beta cells resulting in decreased production of insulin and consequently increased levels of blood glucose. B-lymphocytes play a major pathogenic role by the generation of autoantibodies. Autoantibodies to beta cells may also contribute to cell destruction by facilitating either complement-mediated lysis or antibody-dependent cell-mediated cytotoxicity [35].

Table 2. Biochemical and Immunological Details of Study Subjects

DM Patients	Age (Year)	Sex (M/F)	Blood Glucose (mg/dl)	HbA_{1C}	Smoking Duration	Detection of Anti-AGE-IgG Antibodies		Detection of Anti-IgG Antibodies		Cabonyl Contents (nmol /mgprotein)	
S. No.						A_{410} AGE-IgG	MP1	A_{410} IgG	MP1	DM serum	DM-IgG
1	60	M	440	8.9	32	0.92	65.1	0.49	25.5	3.5	1.3
2	61	M	439	8.8	34	0.93	64.3	0.48	27.2	3.6	1.1
3	61	M	435	9.2	36	0.94	64.4	0.53	27.2	3.5	1.2
4	60	M	415	8.2	34	0.93	62.2	0.54	21.1	3.3	1.1
5	57	M	404	8.8	33	0.89	57.4	0.46	32.3	3.1	1.0
6	59	M	415	8.5	30	0.81	54.2	0.43	29.4	3.5	1.2
7	59	M	405	7.8	29	0.90	53.3	0.37	30.2	3.0	1.1
8	41	M	395	7.4	16	0.89	51.5	0.36	29.0	-	-
9	40	M	373	8.2	13	0.89	52.6	0.37	35.8	-	-
10	40	M	372	7.8	15	0.85	49.0	0.35	38.4	-	-
11	39	M	365	8.1	9	0.84	43.8	0.31	33.0	-	-
12	34	M	324	7.1	-	0.81	39.4	0.31	30.2	-	-
13	45	F	329	7.3	-	0.71	41.4	0.37	31.3	2.6	0.9
14	25	F	372	8.5	-	0.79	47.8	0.42	20.1	-	-
15	24	F	364	8.0	-	0.77	37.1	0.41	31.8	2.9	1.0
16	58	F	390	8.6	-	0.92	60.2	0.51	27.2	3.0	1.2
17	43	F	352	8.8	-	0.90	43.1	0.37	20.1	2.7	0.8
18	35	F	342	7.2	-	0.88	42.2	0.38	28.1	-	-
19	30	F	340	7.3	-	0.86	37.1	0.39	19.4	-	-
Mean±SD	45.8±13.0	12M/7F	383±36.6	8.13±0.65	25.5±10.1	0.85±0.06*	50.8±9.5**	0.51±0.07	28.3± 5.3	3.15±0.35#	1.1±0.15##
Control Mean±SD	42.0±15.0 (n=22)	14M/8F (n=22)	115±7.5 (n=22)	4.6±0.5 (n=22)	22.1±6.4 (n=22)	0.26±0.04 (n=22)	14.2±6.3 (n=22)	0.23±0.04 (n=22)	16.2±4.3 (n=22)	NH serum 2.28±0.25 (n=11)	NH-IgG 0.60±0.18 (n=11)

DM: type 1 diabetes mellitus; M: male; F: female; n: number of samples tested; DM-serum: serum from DM patients; DM-IgG: IgG from DM patients; NH-serum: serum from normal human; NH: normal human; NH-IgG: IgG from normal humans; A_{410}: absorbance at 410 nm calculated by direct binding ELISA; MPI : maximum percent inhibition at 20 μg/ml of inhibitor concentration calculated by competitive inhibition ELISA. *p<0.05 vs. A_{410} (native IgG); **p<0.001 vs. MPI (IgG); #p<0.05 vs. NH-serum; ##p<0.05 vs. NH-IgG.

In view of these, type 1 DM sera were screened for the presence of autoantibodies reactive to native and AGE-IgG. The binding of circulating autoantibodies from sera of thirty one type 1 DM patients and twenty two normal subjects to native and AGE-IgG was studied by direct binding ELISA. Of these, 61.3% diabetes sera showed preferentially high binding to AGE-IgG as compared to its native analog. No appreciable binding was observed with the normal human subjects. Competition ELISA reiterated the direct binding ELISA results. The data showed that circulating autoantibodies from sera of DM patients having history of smoking, aging and high degree of disease (high blood sugar and HbA_{1C}) showed better recognition of AGE-IgG as compared to native IgG. The oxidation of a protein typically results in an increase in carbonyl contents. This increase is due to the oxidation of lys, arg, pro or other amino acid residues, which lead to the formation of AGEs. In short, protein carbonyl groups are the biomarkers of oxidative stress [36]. Present data showed that total serum protein carbonyl contents were significantly (p<0.01) increased in type 1 DM patients as compared with normal subjects. Further, to investigate the extent of alterations in the biological properties of IgG, in diabetic patients IgG which were isolated from eleven type 1 DM patients (DM-IgG) and also from eleven normal subjects (NH-IgG), their carbonyl contents were compared. A p-value of <0.05 indicates significant difference in the carbonyl contents of DM-IgG and NH-IgG. Isolated IgG from smokers, and aged patients with high degree of disease showed great alteration in their carbonyl formation. The above mentioned results suggest that in diabetic patients with increased AGEs,

the oxidative modification of plasma proteins are greatly enhanced. Since the most common protein of serum is IgG, it is likely to be extensively damaged and might be responsible for the pathological conditions associated with type 1 DM. These results suggest that IgG is continuously exposed to AGEs, so much, that alterations in its biological properties could result in the conformational changes of IgG. The results indicate that the conformation of DM-IgG, in particular, involves an increase in accessible hydrophobic regions, as compared with normal-IgG.

Our results demonstrate the presence of AGEs-induced IgG damage in type 1 DM patients, which might play an active part in the progression of disease. We conclude that IgG after modification with AGEs presents unique antigenic determinants for the production of type 1 diabetes autoantibodies. The present study also provides evidence to suggest that the modification of isolated IgG from DM patients leads to alterations in the biological properties of IgG and neo-epitopes thus generated might play a role in the induction of circulating autoantibodies in type 1 diabetes.

AUTHORS' CONTRIBUTIONS

ZR conceived of the study, its design, coordination, data interpretation and drafting the manuscript. LK carried out the experimental work, collection and interpreted the data. S, IP, NAA carried out some experimental work. RA participated in literature search and drafting the manuscript.

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