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CLINICAL STUDY

The oxidative by-product, hydroxyl radical, damaged immunoglobulin-G in patients with non-insulin dependent diabetes mellitus

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Abstract: *Background and objective:* Free radical-mediated oxidative damage and consequent protein modifications are important mediators of cell toxicity and disease pathogenesis. Earlier investigations from our laboratory revealed an increased oxidative damage in diabetes patients and hydroxyl radicals ($^{\cdot}$OH) caused an extensive damage to immunoglobulin G (IgG) and oxidatively modified IgG was found to be highly immunogenic in rabbits. Here, for the first time, we evaluated the role of hydroxyl radical-damaged IgG ($^{\cdot}$OH-IgG) in non insulin dependent (Type 2) diabetes patients.
Methods: IgG was isolated from normal human serum by affinity chromatography and modified by hydroxyl radicals, generated by UV irradiation of hydrogen peroxide. Type 2 diabetes patients (n=38) were accessed by direct binding and competitive inhibition ELISAs and the results were compared to healthy age and sex matched human subjects (n=22).
Results: A high degree of specific binding by 31.6 % of diabetes sera antibodies towards $^{\cdot}$OH-IgG was observed, in comparison to its native analog ($p<0.01$). Antibodies from normal human subjects showed negligible binding with either of antigens ($p>0.05$).
Conclusion: Our results suggest that hydroxyl radicals' modification of IgG causes structural perturbations, resulting in the generations of neo-epitopes, and making it a potential immunogen. The IgG modified with hydroxyl radicals may be one of the factors for the induction of circulating type 2 diabetes autoantibodies. The $^{\cdot}$OH-IgG may proves one of the best markers to determine the oxidative stress in type 2 diabetes patients *(Tab. 1, Fig. 4, Ref. 44)*. Full Text in free PDF *www.bmj.sk.*
Key words: ROS, Immunoglobulin-G, ROS-IgG, Non-insulin dependent diabetes mellitus.

Worldwide, there were approximately 246 millions of adults aged 20-79 years with diagnosed diabetes mellitus (DM) in 2007 (with type 2 diabetes accounting for 90-95 % of all diagnosed cases), and that number is expected to increase to 333 million over the next 20 years (1). Diabetes is associated with increased incidence of coronary artery, cerebrovascular and peripheral vascular disease, with up to 80 % of deaths in people with diabetes caused by cardiovascular disease. Diabetes is also largely responsible for blindness, amputations and end stage renal disease in the developed world (1).

A currently favoured hypothesis is the oxidative stress, which is the common pathogenic factor leading to insulin resistance, â-cell dysfunction, impaired glucose tolerance (IGT) and ultimately to type 2 DM (2). To optimize the treatment in these patients, it is necessary to understand the root cause of oxidative stress. Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide and hydroxyl radicals are products of oxygen metabolism in all aerobic organisms. Environmental agents such as ultraviolet light, ionizing radiation, redox chemicals and cigarette smoke also readily generate ROS. Oxidative stress occurs when the production of ROS exceeds the body's natural antioxidant defense mechanisms, causing damage to biomolecules such as lipids, proteins and DNA. Indeed, oxidative DNA damage has been implicated as the cause of some forms of cancer (3). ROS such as $^{\cdot}$OH and H_2O_2, produced by inflammatory cells have been proposed to contribute multistage carcinogenesis by inducing DNA or tissue damage in inflamed tissues (3). Oxidative stress has been implicated in a wide range of diseases including atherosclerosis, hypertension, diabetes, ischemia reperfusion injury and a range of neurodegenerative disorders (4). It is well documented that oxidative damage of protein and DNA presents unique neoepitopes, which is one of the factors in antigen-driven autoimmune response in various diseases (5, 6) including type 2 diabetes (7). More recent stidies also reported autoantibodies in type 2 diabetes patients (8).

Studies from our laboratory have demonstrated that after modification with ROS, proteins became highly immunogenic

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and oxidative by-products could lead to neoantigens which would initiate autoimmunity in various diseases (9–13). In view of these, it was hypothesized that oxidative by-products like hydroxyl radicals, would react with IgG and help to initiate autoimmunity in patients with type 2 diabetes mellitus. To test this hypothesis, IgG was isolated from normal human serum and modified by hydroxyl radicals, generated by UV irradiation of hydrogen peroxide. The binding characteristic of circulating antibodies in type 2 diabetes sera directed against native and ROS-modified IgG was assessed.

Materials and methods

SDS-molecular weight markers, anti-human IgG-alkaline phosphatase conjugate, p-nitrophenyl phosphate, Folin-ciocalteu reagent, Coomassie Brilliant Blue G-250 and Tween-20 were purchased from Sigma Chemical Company, U.S.A. Protein A-Sepharose CL-4B was purchased from Genei, India. Sephacryl™ S-200 was purchased from Pharmacia, Sweden. Polysorp microtitre flate bottom ELISA plates with 96 wells were purchased from NUNC, Denmark. Acrylamide, ammonium persulphate, bisacrylamide, N,N,N,N-tetramethyl-ethylene diamine (TEMED) were purchased from Bio-Rad Laboratory U.S.A. All other reagents/chemicals were of the highest analytical grade. Protein was estimated by the methods of Lowry et al (14).

Human subjects

Blood was collected from voluntary donors with a history of type 2 diabetes mellitus (n=38, male=18, female=20, aged 54.22±11.62) under treatment in J.N. Medical College & Hospital, A.M.U., Aligarh. The control samples (n=22, male=14, female=8, aged 52.95± 8.96) were collected from healthy subjects. The average (±SD) of postprandial blood sugar level for 63 diabetic patients was 354±84 mg.dL^{-1}, while it was 108±6 mg.dL^{-1} for healthy subjects (without family history of diabetes). 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 -20 °C with 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 as previously described (9). Briefly, serum (0.3 ml) diluted with equal volume of PBS, pH 7.4 was applied to column 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 and neutralized with 1 ml of 1 M Tris-HCl, pH 8.5. Three ml fractions were collected and read at 251 and 278 nm. The IgG concentration was determined considering 1.40 OD_{280} = 1.0 mg IgG/ml. The isolated IgG was then dialyzed against PBS, pH 7.4 and stored at -20 °C with 0.1 % sodium azide.

Modification of Immunoglobulin-G

Affinity purified IgG was modified by hydroxyl radicals generated by UV irradiation of hydrogen peroxide as previously described (9). Briefly, solution of IgG (1.6 mM) in PBS, pH 7.4 was irradiated under 254 nm UV light for 30 min at room temperature in the presence of hydrogen peroxide (32 mM). Hydrogen peroxide excess was removed by an extensive dialysis against PBS (pH 7.4).

Enzyme Linked Immunosorbent Assay

Enzyme Linked Immunosorbent Assay (ELISA) was carried out on polystryrene plates with slight modification (11). Polystyrene polysorp microtitre plates were coated with 100 ěL of native or modified IgG (10 mg.ml^{-1}) in carbonate-bicarbonate buffer (0.05 M, pH 9.6). The plates were coated for 2 hr 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 hr at 37 °C. After incubation the plates were washed four times with TBS-T. The test serum serially diluted in TBS-T (100 ěL well^{-1}) was adsorbed for 2 h at 37 °C and overnight at 4 °C. Bound antibodies were assayed with anti-human IgG alkaline phosphatase conjugate using p-nitrophenyl phosphate as substrate. The absorbance (A) of each well monitored at 410 nm on an automatic microplate reader. Each sample ran in duplicate. The control wells were treated similarly but were devoid of antigen. Results were expressed as the mean of $A_{test} - A_{control}$.

Competitive Inhibition ELISA

The antigenic specificity of the antibodies was determined by competition ELISA (12). Varying amount of inhibitor (0–20 mg.ml^{-1}) was mixed with a constant amount of antiserum. The mixture was incubated at room temperature for 2 h 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. The percent inhibition was calculated using the following formula:

Percent inhibition = $[1 - (A_{inhibitor/} A_{uninhibitor})]$ ´ 100

Statistical analysis

Data are presented as the mean±SD. Significance of differences from control values were determined using the Student's t-test (Statgraphics, Origin 6.1). The value of $p<0.05$ was considered statistically significant.

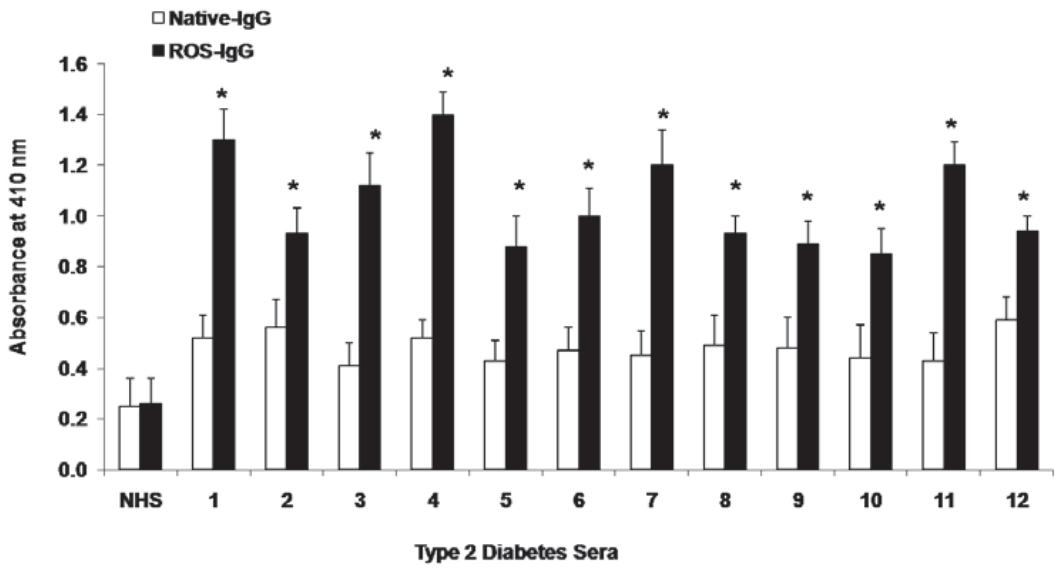

Fig. 1. Direct binding ELISA of 1:100 diluted Type 2 diabetes serum samples. The microtitre plates were individually coated with IgG and ROS–IgG (10 µg/ml). The number of serum samples was 12 (IgG, ROS-IgG). Normal human serum samples were 22 (IgG, ROS-IgG). Each bar represents a mean ± SEM of three independent assays. *p<0.01 vs native IgG.

Results

Immunoglobulin-G was isolated from normal human serum by affinity chromatography on Protein A–Sepharose CL-4B column and affinity purified IgG was modified by hydroxyl radicals generated by UV irradiation of hydrogen peroxide. We previously showed that the purified IgG was found to elute as a single symmetrical peak and gave a single band on SDS-PAGE (9). We also showed that hydroxyl radicals caused an extensive damage to IgG as evident by significant structural changes in modified IgG (9). The UV absorption spectra of hydroxyl radical modified IgG (ROS-IgG) revealed a marked hypochromicity at 280, the oxidation of tryptophan residues on damaged IgG was evident by the 61.2 % fluorescence intensity at 340 nm us-

Tab. 1. Biochemical and immunological parameters of Type 2 diabetes subjects.

DM patients	Age (years)	Sex (M/F)	Blood glucose (mg/dl)	HbA_{1C}	Detection of anti-ROS-IgG antibody		Detection of anti-IgG antibody	
					A_{410}	MPI	A_{410}	MPI
					(ROS-IgG)		(Native-IgG)	
1.	76	M	400	9.1	1.30	57.3	0.52	12.4
2.	68	M	440	8.3	0.93	62.1	0.56	19.5
3.	53	F	438	9.3	1.12	35.7	0.41	10.1
4.	54	M	400	8.6	1.41	62.4	0.52	33.3
5.	46	M	390	7.8	0.88	49.8	0.43	20.4
6.	57	M	377	8.6	1.00	52.7	0.47	14.1
7.	41	M	325	8.7	1.20	53.6	0.45	20.7
8.	48	F	330	7.4	0.98	44.3	0.49	11.2
9.	59	M	354	8.8	0.89	46.4	0.48	19.4
10.	52	M	373	7.3	0.85	63.8	0.44	31.9
11.	66	M	380	7.7	1.20	58.1	0.43	26.1
12.	63	F	398	8.9	0.94	66.8	0.59	17.2
Mean±S.D.	56.91±10.01	9M/3F	383.75±36.03	8.38±0.67	0.99±0.28*	54.42±9.18**	0.46±0.08	19.92±0.79
Control Mean±S.D.	52.95±8.96 (n = 22)	16M/6F (n = 22)	108.5±9.79 (n = 22)	4.78±0.83 (n = 22)	0.26±0.04 (n = 22)	16.98±1.49 (n = 22)	0.25±0.05 (n = 22)	15.41±1.85 (n = 22)

DM – diabetes mellitus; M – male; F – female; n – number of samples tested; A_{410} – absorbance at 410 nm calculated by direct binding ELISA; MPI – maximum percent inhibition at 100 µg/ml of inhibitor concentration calculated by competitive inhibition ELISA. *p<0.05 vs A_{410} (native IgG); **p<0.001 vs MPI (native IgG)

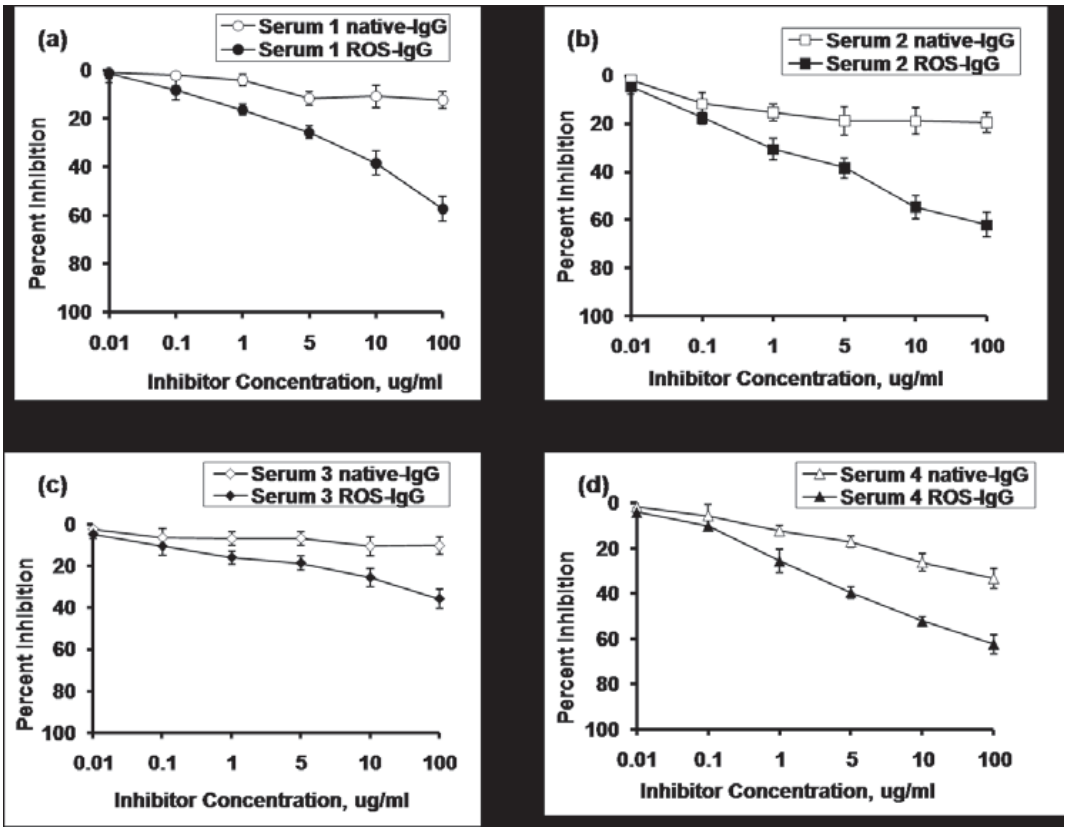

Fig. 2. Competitive inhibition ELISA of Type 2 diabetes serum antibodies from four patients (1, 2, 3, 4). The inhibitors used were native IgG and ROS-IgG. The microtitre plates were coated with the ROS-IgG (10 µg/ml).

ing an excitation wavelength of 295 nm. A damage of tryptophan residues was confirmed by the loss of 64.2 % fluorescence intensity at 340 nm using an excitation wavelength of 280 nm. The far-UV circular dichroism measurements of ROS-IgG showed an increased ellipticity values in the range 210 to 230 nm as compared to the circular dichroism measurements of unmodified IgG, indicating a significant loss of secondary structural of protein caused by hydroxyl radicals (9).

The present study comprised of serum samples from patients suffering from type 2 diabetes mellitus (DM). Each sample was obtained after a careful clinical examination of patients with proven diagnosis at J.N. Medical College Hospital, A.M.U., Aligarh. Sera from sixty two type 2 DM and twenty two normal human subjects were tested for binding to native and ROS-modified IgG by direct binding ELISA. Approximately 32 % of DM sera (12/38) showed a strong binding to ROS-IgG over native IgG at 1:100 serum dilution ($p<0.05$) (Fig. 1). No appreciable binding was observed in the normal subjects. The average absorbance at 410 nm (±SD) of 12 DM sera binding to native and ROS-damaged IgG was 0.46±0.08 and 0.99±0.28, respectively (Table 1). The absorbance at 410 nm (±SD) of twenty two normal human sera binding to both protein antigens was 0.25±0.05 and 0.26±0.04, respectively. The binding specificity of antibodies from 12 selected sera of DM patients was evaluated by a competition ELISA using native and ROS-IgG as inhibitors. Microtitre plates were coated with ROS-IgG. Results pointed to a higher reactivity of autoantibodies in patient's sera towards ROS damaged IgG over native IgG. The sera showed high percent inhibitions with modified IgG over native IgG as evaluated by an inhibition ELISA (Figs 2-4). The average percent inhibition (±SD) in the binding of 12 type 2 DM sera to native and ROS-IgG were 19.92±0.79 and 54.42±9.18, respectively (Tab. 1). The data revealed striking differences in the recognition of native and ROS-IgG by DM autoantibodies ($p<0.001$).

Discussion

Reactive oxygen species are continuously formed in living cells as the consequence of cellular metabolism and exogenous agents but increase in their steady states is attributed to the variety of human degenerative diseases (15). An excess generation of ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) have potential to induce significant biomolecular damage to cell macromolecules including proteins (5, 16-18) and DNA (4, 12, 19). Proteins are the major target for free radical attack, the most damaging species being

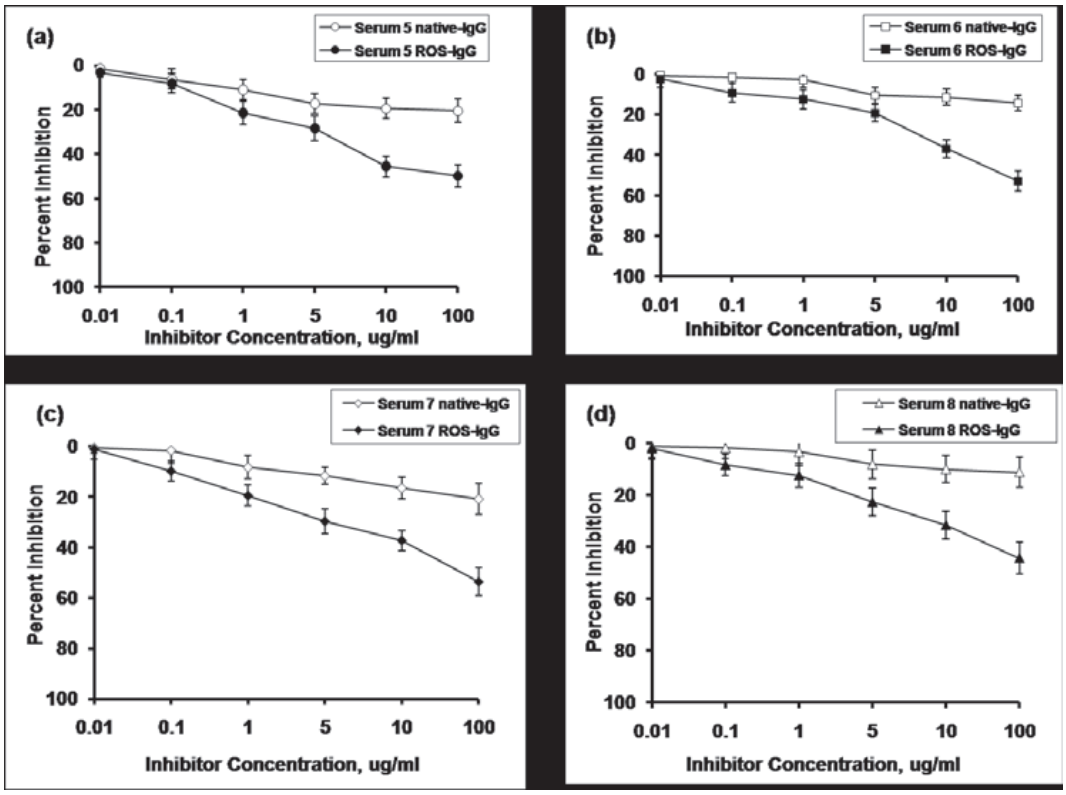

Fig. 3. Competitive inhibition ELISA of Type 2 diabetes serum antibodies from four patients (5, 6, 7, 8). The inhibitors used were native IgG and ROS-IgG. The microtitre plates were coated with the ROS-IgG (10 μg/ml).

·OH, which react with most biomolecules with a rate constant close to the diffusion control limit (20). Protein oxidation results in cellular dysfunctions, functional disruption and structural changes (21) and contributes to the etiology of many human diseases (22). These oxidative modifications on protein may lead to the formation of neoantigens which could in turn initiate autoimmunity (5, 9, 11, 12). In fact, oxidative stress plays a central role in the onset of diabetes mellitus as well as in diabetes-associated complications (23). Various studies have shown that diabetes mellitus is associated with an increased formation of free radicals and decrease in antioxidant potential. This leads to the oxidative damage of cell components such as proteins, lipids and nucleic acids. An increased oxidative stress has been reported in both type-1 and type 2 diabetes patients (24).

Hyperglycemia initiates a reactive oxygen species (ROS) chain reaction (25) and increases the production of ROS and decreases their scavenging (antioxidants) by various mechanisms, resulting in lower than normal levels of antioxidant activity (26). Impaired antioxidant activity might allow free radicals produced from other sources to go unchecked. Such increase of ROS may disturb or modify various cellular functions and alter gene expression due to oxidative stress. This increased oxidative load in NIDDM is associated with an increased oxidative damage to DNA (27). The high glucose induced mitochondrial overproduction of ROS (28) and abnormal activation of NAD(P)H oxidize (29, 30) are thought to be the major sources of ROS associated with hyperglycemia, while sustained hyperglycemia may also decrease radical scavenging by manganese superoxide dismutase (31) and the glutathione redox cycle (32).

Thus, chronic oxidative stress is thought to be the major cause of diabetic complications, and clinical evaluation of the accumulation of oxidative stress induced by sustained enhancement of ROS production could be useful to assess the risk of diabetes complications. Current clinical markers of ROS in diabetes, such as urinary 8-OH-2'-deoxyguanosine (27), urinary 8-iso $PGF_{2\alpha}$ (33), plasma lipid peroxide (34) and the oxidized form of human non mercaptalbumin (35, 36), all reflect short-term ROS formation and not cumulative oxidative stress.

In the present study, human immunoglobulin-G was isolated from normal human serum by affinity chromatography on Protein-A Sepharose CL-4B column. Affinity IgG was modified by hydroxyl radicals, generated by in vitro reaction of IgG and H_2O_2 in presence of UV-B light. It is well documented that IgG is quite vulnerable to ROS (9). Previously, we reported that ROS-modified IgG was observed to be potent antigenic stimulus inducing high titre antibodies in experimented animals as compared to native IgG (9), suggesting that neo-epitopes were generated due to the structural perturbation caused by hydroxyl radicals. However, it was established that oxidative damage of protein or DNA leads to various autoimmune diseases (5). This is

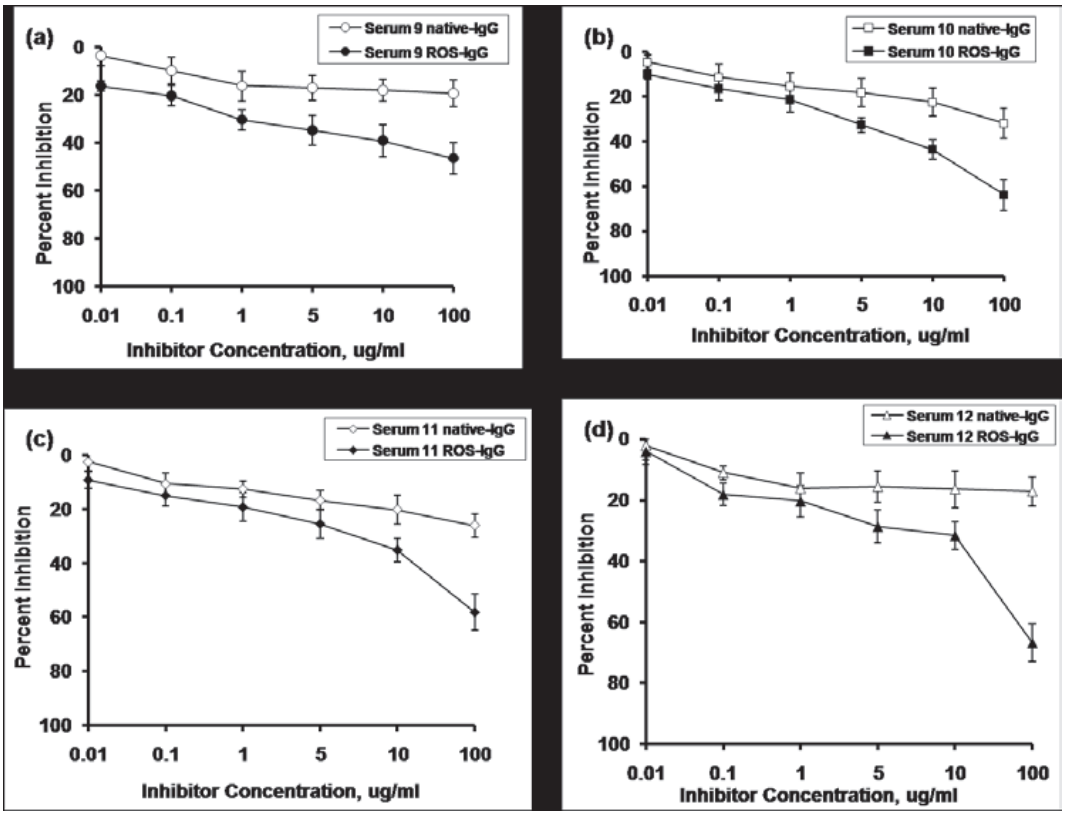

Fig. 4. Competitive inhibition ELISA of Type 2 diabetes serum antibodies from four patients (9, 10, 11, 12). The inhibitors used were native IgG and ROS-IgG. The microtitre plates were coated with the ROS-IgG (10 μg/ml).

due to the oxidative modification of biomolecules that might result in formation of chemical adducts, which could serve as neoantigens that the immune system has probably not been exposed to. There are other examples of an immune response against oxidatively modified self-antigens. Malondiadehyde (MDA)-cross linked proteins are actually present in vivo and their production is stimulated by oxidative stress. Antigens modified by oxidative by-products have been shown to induce immune responses in alcoholic liver diseases (37). Oxidative stress occurs when antioxidant mechanisms are not overwhelmed in some ways, possible due to pathology in the disease. Hydrogen peroxide is generated by number of ways (38); these peroxides are normally eliminated by glutathione which acts as a reducing agent. Oxidative stress plays a central role in the onset of Diabetes Mellitus (DM) as well as in diabetic associated complications (39). The role of oxidative protein damage in the pathogenesis of the diabetic state is being studied extensively (40). In type 2 DM, autoantibodies are present in patient's sera that bind to oxidized low-density lipoprotein (7).

Immunoglobulin-G (IgG) is a major class of serum glycoprotein with 82-96 % of protein and 4-18 % of carbohydrate, and it functions as a major effector molecule of the humoral responses in human. Earlier studies demonstrate that the structural alterations in IgG can take place by free radicals (9, 41, 42) and this altered IgG can induce antibodies that react selectively with the immunogen (43). Autoantibodies directed to normal IgG are frequently observed in rheumatoid arthritis (9). It is also reported that these autoantibodies directed against the Fc fragment of immunoglobulin G are present in the sera of more than 75% patients of rheumatoid arthritis (44).

In this study, antibodies from sixty two different type 2 DM sera were screened by direct binding and competitive inhibition ELISA. Out of these, ~31 % of sera showed a preferentially high binding towards ROS-IgG as compared to native-IgG ($p<0.001$). No detectable antibody activity was found in normal human sera.

Our results demonstrated the recognition of ROS-damaged IgG by diabetes sera due to the possible participation of damaged immunoglobulin-G in type 2 diabetes and complications associated with the disease pathogenesis. Thus, the present study clearly demonstrated structural perturbation in IgG by reactive oxygen species, rendering it immunogenic. The modification of IgG unique epitopes might play a role in the induction of circulating autoantibodies in type 2 diabetes mellitus. Thus, it is clear that oxidative processes are the important factors to induce autoimmunity in the clinical scenario; it may be possible to prevent the development of type 2 diabetes and associated complications in genetically susceptible individuals by administration of antioxidant or other dietary manipulations to decrease formation of oxidative byproduct. There is indeed an urgent need to redouble efforts both in vitro and in vivo study on ROS modi-

fied proteins that are potentially harmful to generate autoimmune diseases and to search for antioxidant role to pursue novel strategies to cope with diagnosis and treatment of autoimmune diseases.

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