Immunological functions of oxidized human immunoglobulin G in type 1 diabetes mellitus: Its potential role in diabetic smokers as a biomarker of elevated oxidative stress

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Abstract. The role of oxidized immunoglobulin G in type 1 diabetic smokers has been investigated in the present study. Human immunoglobulin G (IgG) was modified by reactive oxygen species (ROS). The binding characteristics of circulating autoantibodies in type 1 diabetes patients against native and modified IgG were assessed by direct binding ELISA. High degree of specific binding by 68.5% of patients sera towards ROS-modified IgG was observed in comparison to its native analogue ($p < 0.05$). In addition, diabetic smokers ($n = 28$) were examined and the results were compared with diabetic non-smokers ($n = 26$). Circulating antibodies of diabetic smokers showed substantially stronger binding to modified IgG as compared with the antibodies present in diabetic non-smokers ($p < 0.05$). Normal human sera ($n = 53$) showed negligible binding with either antigen. Competitive inhibition ELISA reiterates the direct binding results. The increase in total serum protein carbonyl levels in the diabetic smokers was largely due to an increase in oxidized IgG. Diabetic smokers showed substantially higher carbonyl contents in sera as well as in purified IgG as compared with sera and IgG of diabetic non-smokers. Collectively, the oxidation of plasma proteins, especially IgG, might enhance oxidative stress in type 1 diabetes smokers.

Keywords: Type 1 diabetic smokers, oxidized IgG, autoantibodies, reactive oxygen species

1. Introduction

Diabetes mellitus (DM) and its associated secondary complications are the leading cause of deaths worldwide [1]. The number of diabetes patients has been increased by exposure to environmental risk factors including cigarette smoking [2]. The prevention of diabetes is a serious worldwide challenge, but it is clear that the successful prevention of diabetes will depend on the reduction of risk factors along with better methods of screening and early detection. Type 1 diabetes is an autoimmune disease resulting from the selective destruction of pancreatic β cells. Its incidence in different ethnic groups is extremely variable, suggesting that the involvement of genetic as well as environmental elements [3]. In type 1 diabetes, multiple autoantibodies against pancreatic β cell antigens may arise and persist even after the disease becomes clinically overt. In humans, glutamic acid decarboxylase (GAD), IA-2 and insulin would be the most important targets against which autoantibodies as well as autoreactive T cells

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are directed [3]. The precise mechanism of β-cells destruction leading to diabetes remains unclear [4].

Oxidative stress plays a central role in the onset of diabetes as well as in diabetes associated complications [1,2,5]. Various studies have shown that diabetes is associated with increased formation of free radicals and decrease in antioxidant potential. This leads to oxidative damage of cell components such as proteins, lipids and nucleic acid [5]. Increased oxidative stress in diabetic patients leads to protein oxidation [5]. The conversion of protein to protein carbonyl (PCO) derivatives may occurs via direct oxidation by ROS, with the eventual formation of oxidized amino acids [6].

Immunoglobulin G is a major protein of serum whose function lies in the specific interactions with and clearance of antigens. In diabetes and rheumatoid arthritis patients, IgG dysfunctions have been reported; it behaves not only as an antibody, but also as a putative antigen for diabetes and rheumatoid arthritis autoantibodies [7,8]. It has been well documented that IgG is quite vulnerable to ROS [7–10]. Therefore, IgG is continuously exposed to oxidative stress, so that alterations of the conformation and function of IgG could occur, resulting in modification of its biological properties.

It is well documented that oxidative damage of protein and DNA presents unique neo-epitopes, which may be one of the factors in antigen-driven autoimmune response in autoimmune diseases [11,12]. Recently, we have demonstrated that after modification with ROS, proteins became highly immunogenic and oxidative by-products with proteins could lead to neoantigens which would initiate autoimmunity in various diseases including diabetes [10,13–19]. In view of this, the present study was based upon a hypothesis that oxidative by-products, hydroxyl radical damage IgG, help to initiate autoimmunity in type 1 diabetes patients. To test this hypothesis, IgG was purified from normal human sera by affinity chromatography. Affinity purified IgG was modified with hydroxyl radicals (·OH). Type 1 diabetes patients having smoking or non-smoking history were evaluated for presence of circulating autoantibodies directed against native and ·OH-modified IgG.

2. Materials and methods

2.1. Study subjects

Blood was collected from human voluntary donors of type 1 diabetes mellitus ($n = 54$, male $= 43$, female $= 11$, aged 44 ± 15). Fifty two percent diabetic male

having smoking history, whereas twenty seven percent were diabetic non-smokers. The control samples were collected from healthy subjects ($n = 53$, male $= 38$, female $= 15$, aged 45 ± 10.2). Forty seven percent of normal human were cigarette smokers, whereas twenty four percent were non-smokers. All studied females whether diabetic or normal were non-smokers. The average ($\pm$ SD) of post prandial blood sugar level for 54 diabetic patients was 378 ± 26 mg/dl, while it was 108 ± 7.5 mg/dl for 53 healthy subjects (without family history of diabetes). The glycated hemoglobin (HbA1C) was 8.1 ± 0.8 for patients, whereas 4.75 ± 0.4 for normal subjects. The complete demographic details of study subjects have been summarized in Table 1. Glutamic acid decarboxylase antibodies (GADA), insulin antibodies (IAA), anti-tyrosine phosphatase antibodies (IA-2A) were found in 42%, 71% and 36% of the cases, respectively. Immunopositivity for at least one of these autoantibodies was found in all patients. 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.

2.2. Immunoglobulin G purification

IgG from normal human sera was isolated by affinity chromatography on Protein A-Sepharose CL-4B column as previously described [11]. Briefly, serum (0.3 ml) diluted with equal volume of PBS, pH 7.4 was applied to the column (12 mm $\times$ 45 mm) equilibrated with the same buffer. The flow through 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 M Tris-HCl, pH 8.5, 3ml 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.

2.3. Modification of human IgG

Human IgG was modified in PBS (10 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.4) by

Table 1
Characteristics of the study subjects

	Type 1 diabetes patients		Normal human subjects	
	Smokers	Non-smokers	Smokers	Non-smokers
Number of cases	28	26	25	28
Age (years)	47 ± 15.8	41 ± 14.2	42 ± 9.3	45 ± 10.2
Sex (M/F)	28M	15M / 11F	25M	13M / 15F
Blood Glucose (mg/dL)	385.2 ± 31.2	371.1 ± 21.3	111.3 ± 6.7	105.4 ± 8.3
HbA$_{1C}$	8.2 ± 0.71	7.9 ± 0.91	4.8 ± 0.32	4.7 ± 0.39
Smoking duration (years)	22 ± 5.5	–	24 ± 6.7	–
Smoking intensity (no. of cigarettes/day)	24 ± 10.2	–	29.2 ± 8.8	–

Data represents mean $\pm$ S.D.. M, male; F, female.

our published procedure [11]. An aqueous solution of IgG (1.2 μM) was modified by hydroxyl radical, generated by the irradiation (30 minutes) of hydrogen peroxide (15.1 μM) at 254 nm. Excess hydrogen peroxide was removed by extensive dialysis against PBS.

2.4. Carbonyl estimation

Carbonyl contents in smoking DM sera were analyzed according to Levine et al. [20] with slight modifications. The reaction mixture containing 10 μL of sera, 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^{-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. [21]. Carbonyl contents were expressed as nmol/ mg protein.

2.5. Direct binding ELISA

Presence of antibodies against native and ROS modified IgG in sera of diabetes patients having smoking or non-smoking history was evaluated by enzyme immunoassays. Enzyme linked immunosorbent assay (ELISA) was carried out on polystyrene polysorp plates as described previously [14–17]. Polystryrene polysorp microtitre plates were coated with 100 μL of native or modified IgG (5 μg 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 μL 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. Absorbance (A) was monitored at 410 nm on an automatic microplate reader and mean of duplicate readings for each sample was recorded. Results were expressed as a mean of A$_{test}$ − A$_{control}$.

2.6. Competitive inhibition ELISA

The antigen binding specificity of the antibodies was evaluated by competition ELISA [18,19]. Varying concentrations of inhibitors (0–20 μg ml^{-1}) were allowed to interact with a constant amount of antiserum for 2h at 37°C and overnight at 4°C. The immune complexes were incubated in the wells (instead of the serum taken in direct binding ELISA) and the bound antibody levels were detected as in direct binding ELISA. Percent inhibition was calculated using the formula:

Percent Inhibition =
$$[1 - (A_{\text{inhibited}}/A_{\text{uninhibited}})] \times 100$$

2.7. Statistical analysis

All measurements were performed in duplicates and repeated at least 3 times. All statistical analyses were performed using Origin 6.1 software package (one paired two tailed t-test with one way ANOVA analysis) and $p < 0.05$ was considered significant. Values have been shown as mean $\pm$ SEM unless stated otherwise.

Table 2
Competitive inhibition of antibodies from type 1 diabetes smoking and non-smoking patients by native and ROS-modified immunoglobulin G

Type of sera	Number of patients	Maximum inhibition (%)[#]	
		Native IgG	ROS-IgG
DM smokers	20	22.1 ± 1.9^{a}	54.2 ± 1.2^{b}
DM non-smokers	17	21.1 ± 1.1^{a}	40.3 ± 3.3^{c}
NH smokers	25	20.4 ± 1.2^{a}	22.2 ± 7.5^{a}
NH non-smokers	28	19.2 ± 2.1^{a}	20.7 ± 6.5^{a}

Results are representative (mean $\pm$ SEM) of triplicate experiments and differ without a common letter $P < 0.05$. Microtitre plates were coated with ROS-IgG (5 μg/ml). [#]Maximum percent inhibition at 20 μg/ml of inhibitor concentration. DM: type 1 diabetes mellitus; NH: normal human; IgG: immunoglobulin G.

3. Results

3.1. Characterization and immunization of native and ROS-modified human IgG

Our earlier reports showed alterations in IgG following exposure to the hydroxyl radical, generated by the UV-irradiation of hydrogen peroxide [11]. Loss of secondary structures, hypochromicity at 280 nm, loss of tryptophan fluorescence intensity, increase in protein carbonyl contents were observed in human IgG following hydroxyl treatment.

Previously, we also demonstrated that ROS-IgG was a potent immunogen in rabbits that induced high-titre (> 1:12,800) antibodies, whereas native human IgG showed a titre < 1:6400 [11]. Preimmune serum serving as a negative control did not show appreciable binding with the respective immunogens under identical experimental conditions.

3.2. Recognition of ROS-modified IgG by diabetic smokers autoantibodies

Our study comprised of 54 serum samples from type 1 diabetes patients having smoking or non smoking history. The majority of diabetic smokers (20/28) and non-smokers (17/26) showed strong binding to ROS-IgG over native IgG ($p < 0.01$). No appreciable binding was observed in normal healthy smokers and non-smokers ($p > 0.05$) (Fig. 1). The average OD. at 410 nm ($\pm$ SEM) of 20 diabetic smokers antibodies binding to native and ROS-damaged IgG was 0.53 ± 0.03 and 0.88 ± 0.04, respectively. Whereas average OD. at 410 nm ($\pm$ SEM) of 17 diabetic non-smokers binding to native and ROS- IgG was 0.29 ± 0.03 and 0.56 ± 0.04, respectively. Average OD. at 410 nm ($\pm$SEM) of 25 normal human (NH) smokers antibodies to native and ROS-

damaged IgG was 0.23 ± 0.06 and 0.29 ± 0.04, respectively, whereas the results for 28 NH non-smokers antibodies binding to native and ROS-damaged IgG were 0.22 ± 0.04 and 0.21 ± 0.03, respectively (Fig. 1). The binding specificity of antibodies from diabetic smokers and non-smokers was further evaluated by competitive inhibition ELISA using native and ROS-IgG as inhibitors. Microtitre plates were coated with ROS-IgG. Figure 2 illustrates inhibition of type 1 diabetes autoantibodies (in sera 1, 2 and 3) by native and modified IgG. Results point to a higher reactivity of autoantibodies in patient's sera towards ROS-modified IgG over native IgG. Circulating antibodies from diabetic smokers showed preferentially strong binding to ROS-IgG as compared with the diabetic non-smokers antibodies ($p < 0.05$). Similarly, the rest of the sera showed high percent inhibitions with modified IgG over native IgG (data summarized in Table 2). Average percentage inhibition ($\pm$ SEM) at 20 μg/ml of inhibitor concentration in the binding of 20 diabetic smokers antibodies to native and ROS-IgG was 22.1 ± 1.9 and 54.2 ± 1.2, respectively. Whereas percent inhibition of serum antibodies from 17 diabetic non-smokers binding to native and ROS-IgG was 21.1 ± 1.1 and 40.3 ± 3.3, respectively (Table 2). Circulating antibodies from the negative controls did not show significant inhibition to either inhibitor. The data revealed striking differences in the recognition of ROS-IgG by antibodies from diabetic patients ($p < 0.01$).

3.3. Oxidation in serum protein and purified IgG

Protein carbonyl content is a general indicator and the most commonly used biomarker for protein oxidation. Our data showed significant increase in total serum protein carbonyl content in diabetic smokers ($n = 20$) and diabetic non-smokers ($n = 17$) compared with their respective controls ($p < 0.05$). The average carbonyl content ($\pm$ SEM) of 20 independent assays in serum samples of diabetic smokers and normal healthy smokers were 3.92 ± 0.08 and 2.11 ± 0.05 nmol/mg protein, respectively. Whereas in non-smoking DM serum proteins and non-smoking NH serum proteins the average results were 2.81 ± 0.05 and 2.10 ± 0.07 nmol/mg protein, respectively (Table 3).

To investigate the extent of alterations in the biological properties of IgG in DM patients, IgG was isolated from same diabetic smokers (S-DM-IgG), same diabetic non-smokers (NS-DM-IgG), normal healthy smokers (S-NH-IgG) and normal healthy non-smokers (NS-NH-IgG) by affinity chromatography using Pro-

Table 3
Carbonyl contents in serum proteins and in purified immunoglobulin G from smoking and non-smoking type 1 diabetes patients

Study subjects	Number of samples	Carbonyl contents (nmol/mg protein)	
		Serum proteins	Purified IgG
Smokers DMS	20	3.92 ± 0.08^a	2.84 ± 0.04^b
Non-smokers DMS	17	2.81 ± 0.07^b	1.99 ± 0.06^b
Smokers NHS	25	2.11 ± 0.05^b	1.62 ± 0.04^c
Non-smokers NHS	28	2.10 ± 0.07^b	1.51 ± 0.06^c

Results are representative (mean $\pm$ SEM) of triplicate experiments with type 1 diabetes mellitus serum (DMS) samples obtained from age and sex matched normal human serum (NHS) and differ without a common letter $p < 0.05$.
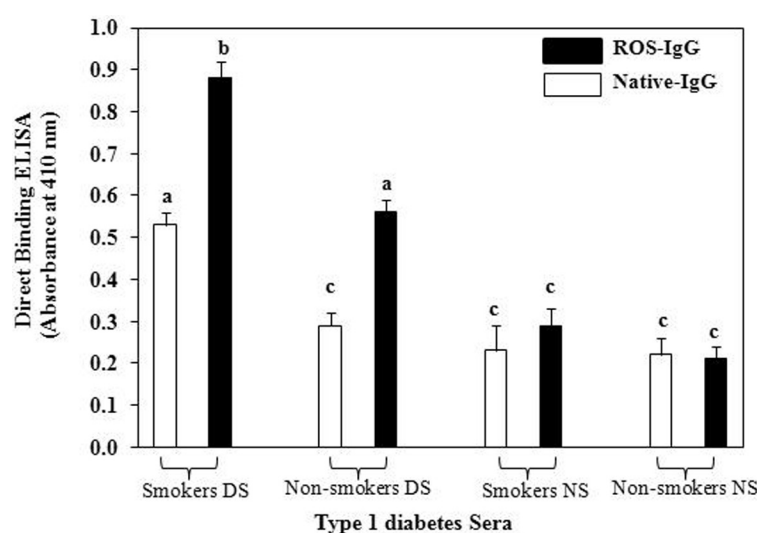

Fig. 1. Direct binding ELISA of serum samples (1:100 diluted) from smoking type 1 diabetes patients (smokers DM), non-smoking DM patients (non-smokers DM), healthy smoking subjects (smokers NS) and healthy non-smoking subjects (non-smokers NS) to native IgG and ROS-modified IgG. The microtitre plates were individually coated with native IgG and ROS-IgG (5 μg/ml). The number of smoking DM, non-smoking DM, smoking NS and non-smoking NS samples were 20, 17, 25 and 28, respectively. Results are representative (mean $\pm$ SEM) of triplicate experiments and differ without a common letter $P < 0.05$.

tein A-sepharose CL-4B column. Polyacrylamide gel electrophoresis of affinity purified IgG showed a single homogenous band (data not shown). Average carbonyl contents ($\pm$ SEM) of their respective independent assays of S-DM-IgG and S-NH-IgG were 2.84 ± 0.04 and 1.62 ± 0.04 nmol/mg protein, respectively ($P < 0.05$), whereas in NS-DM-IgG and NS-NH-IgG the results were 1.99 ± 0.06 and 1.51 ± 0.06 nmol/mg protein, respectively ($p < 0.05$). Importantly, $p < 0.05$ indicated a significant difference in carbonyl content in the S-DM-IgG and NS-DM-IgG groups (Table 3).

4. Discussion

Despite the power of modern molecular approaches and persisting investigative efforts, diabetes mellitus

remains an enigmatic disorder or the agent (or agents) triggering this disease remains to be identified [22]. Oxidative stress plays a central role in the onset of diabetes as well as in diabetes associated complications [1]. Various studies have shown that diabetes is associated with increased formation of free radicals and decrease in antioxidant potential. This leads to oxidative damage of cell components such as proteins, lipids and nucleic acid in both type 1 and type 2 diabetes [2]. The role of oxidative protein damage in the pathogenesis of the diabetic state is being investigated extensively [5,7,16]. Considerable evidence indicates that the maintenance of protein redox status is of fundamental importance for cell function, whereas structural changes in protein are considered to be among the molecular mechanisms leading to diabetes and its associated complications [3].
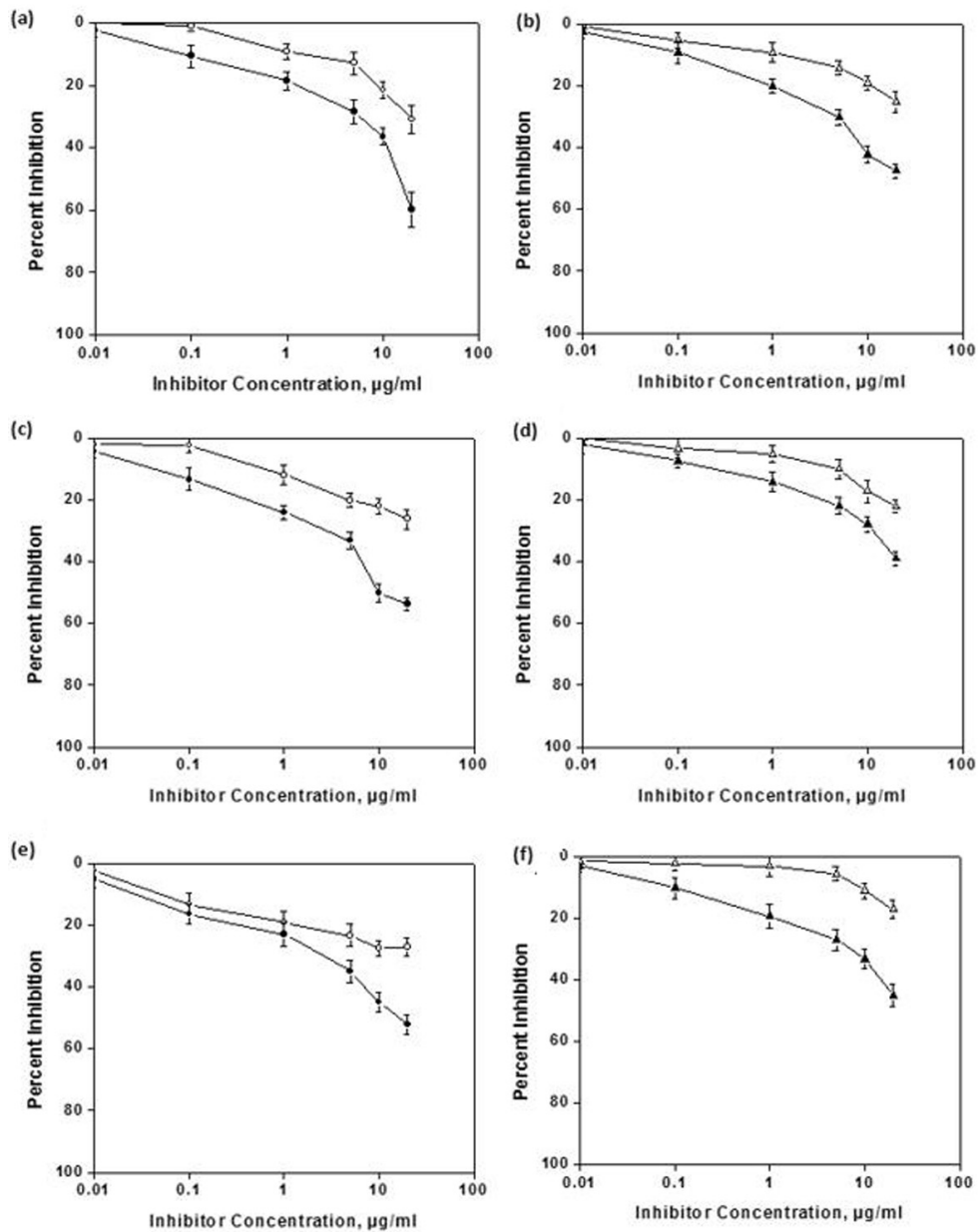
Fig. 2. Competitive inhibition ELISA of serum antibodies from three smoking (a,c,e) and three non-smoking (b,d,f) type 1 diabetes patients. The inhibitors used were native IgG ($\bigcirc$, $\square$, $\triangle$) and ROS-IgG ($\bullet$, $\blacksquare$, $\blacktriangle$). The microtitre plates were coated with ROS-IgG (5 μg/ml). Varying amounts of inhibitors (0–20 μg/ml) were allowed to interact with a constant amount of antiserum 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.

Immunoglobulin G is an abundant protein in the circulatory system, whose redox modification modulates its physiologic function, may serves as a biomarker of oxidative stress [7–11]. It is well documented that IgG is quite vulnerable to reactive oxygen species [10]. Therefore, IgG is continuously exposed to oxidative stress, so that alterations of the conformation and func-

tion of IgG could occur, resulting in modification of its biological properties. Many reported studies show the presence of elevated levels of oxidized IgG, in patients with inflammatory diseases such as rheumatoid arthritis [9–11] and very recently in non-insulin dependent diabetes mellitus [7]. But still it is unclear whether oxidized IgG has a pathologic role in type 1

diabetes patients. In agreement with previous studies the present work also found oxidatively altered IgG in patients with type 1 diabetes mellitus. It may reflect a physiological response directed towards the cleaning of oxidative proteins from the circulation, rather than a true autoimmune response with clinical consequences.

We and others have reported the presence of elevated levels of oxidized protein products (advanced oxidation protein products) such as oxidized plasma protein in patients with various diseases [9–26]. Furthermore, ROS modified IgG is highly immunogenic in rabbits, and the antigenic specificity of affinity purified anti-ROS-IgG antibodies and anti-IgG antibodies suggest that induced antibodies are immunogen-specific. The substantially enhanced immunogenicity of ROS-IgG in comparison to native IgG may be due to the generation of potential neo-epitopes against which antibodies are raised [11]. In the present study, type 1 diabetic smokers and non-smokers were screened for the presence of autoantibodies reactive to native and ROS-modified IgG using a direct-binding ELISA. Results revealed that circulating antibodies in diabetes serum samples showed preferentially high binding to ROS-IgG, compared with its native analogue ($p < 0.05$), whereas control samples did not show appreciable binding ($p > 0.05$). Circulating diabetes antibodies also showed some binding to native IgG, this may be due to the generation of cryptic epitopes, which might be generated during isolation. Competitive inhibition ELISA reiterates the direct binding results indicated that the ROS-modified IgG was an effective inhibitor showing substantial difference in the recognition of damaged IgG over native IgG ($p < 0.05$). Importantly, our notable novel data showed that circulating antibodies from smokers diabetes patients showed better recognition of ROS-IgG as compared with the circulating antibodies from diabetic non-smokers ($p < 0.05$). As the abundant plasma protein, IgG is known to play a major role as an antigen clearance, but under oxidative stress IgG dysfunctions have been reported [7,9–11]. In this context, the characterization of the oxidation status of IgG would provide not only useful information about the redox state of the human body but also alterations in the conformation and function of IgG that may result in modification of its biological properties. The oxidation of a protein typically results in an increase in carbonyl content [20,27]. This increase is due to the oxidation of lys, arg, pro and other amino acid residues [20,27]. In short, protein carbonyl groups are the biomarkers of oxidative stress [27,28]. Our data show that carbonyl content in serum of diabetic smokers and diabetic non-smokers was high as compared with their respective smokers or non-smokers controls. Importantly, serum carbonyl content of diabetic smokers was significantly higher as compared to diabetic non-smokers ($p < 0.05$). In addition, the purified IgGs from diabetic smokers showed significantly higher carbonyl content as compared with the IgGs from normal healthy smokers ($p < 0.05$). Similar results were obtained in diabetic non-smokers and their respective controls. Our data point out that higher carbonyl content was found in diabetic smokers compared with diabetic non-smokers ($p < 0.05$). These results indicated that IgG is continually exposed to oxidative stress, which results in alterations in its biological properties, could results in conformational changes, these changes may be one of the factors for the presentation of unique epitopes for the induction of autoantibodies in type 1 diabetes patients. Our data supported the view that cigarette smoking increases the oxidative stress in diabetes patients. This suggests that diabetes patients with increased oxidative stress showed enhanced oxidative modification of plasma proteins and IgG is likely to be extensively damaged. The present report demonstrates the structural perturbation in IgG due to oxidative stress, rendering it immunogenic. The neo-epitopes generated, which may be one of the responsible factors for the induction of autoantibodies in patients with type 1 diabetes. This might play a significant role in the etiology of diabetes and might represents a new biomarkers especially for cigarette smoking type 1 diabetes patients.

5. Conclusions

The present article is the first report that demonstrates the presence of ROS-induced IgG damage in type 1 diabetes patients. We conclude that IgG after modification with ROS presents unique epitopes for the induction of autoantibodies in type 1 diabetes with special preference to those patients having cigarette smoking history. Thus, ROS-modified IgG might represents a novel therapeutic targets for diabetic smokers.

Competing interests

The authors declare that they have no competing interests.

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