Glycation of Human IgG Induces Structural Alterations Leading to Changes in its Interaction with Anti-IgG

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ABSTRACT

Background: Glycation of proteins is a non-enzymatic spontaneous process that occurs in diabetes mellitus and aging, altering the structure and function of proteins. IgG undergoes glycation leading to changes in its reactivity to antigen and fixation of complement. **Objective:** This study aimed at revealing the effect of glycation on the interaction of IgG with anti-IgG using electroimmunoassay. **Methods:** Purified human IgG was glycated with different concentrations of glucose and different periods of treatment. Glycation was measured using thiobarbituric acid reaction. Glycated and non-glycated IgG were subjected to electroimmunoassay, and the height of the precipitated rings were measured and compared. **Results:** The results showed that IgG was glycated in vitro and the level of glycation was dependent on the glucose concentration and duration of treatment with glucose. The height of glycated IgG peaks formed in the electroimmunoassay was significantly lower than those of non-glycated IgG ($p < 0.01$). **Conclusion:** The results indicated that in vitro glycation of IgG leads to structural changes altering its mobility in the electroimmunoassay. Moreover, it suggests that this alteration may cause the weakness of its interaction with anti-IgG. This phenomenon may play a role in the susceptibility of diabetic patients to infection.

Keywords: Diabetes, Electroimmunoassay, Glycation, IgG

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INTRODUCTION

Non-enzymatic glycosylation is the covalent binding of single sugars to alpha- or epsilon- amino groups on proteins (1,2). It is quite different from enzyme catalyzed glycosylation, where short oligosaccharide chains become attached to asparagines or serine side chains through glycosidic bonding (3). This reaction is controlled by glycosyl transferases, whereas in nonenzymatic glycosylation or glycation, sugars attack in a time and concentration dependent manner, glycating N-terminal amino acids or side chains of lysine residues (4).

Many biologically important proteins are known to become increasingly glycated in diabetics, and it is thought to play a major role in many of the long-term complications of diabetes mellitus (5). Several evidences show that glycation changes the structure of proteins and subsequently induces the functional alteration (6). Although there are evidences that chronic hyperglycemia is the major cause of the complications of diabetes, the mechanisms thereof are not well known (7).

Dolhofer et al. were the first who propose that glycation significantly impairs the functional ability of IgG when they found glycated IgG fixed less complement than unglycated IgG (8). There is also another report showing that incubation of human IgG with glucose in vitro leads to covalent incorporation of the sugar concomitant with marked changes in the molecular structure and subsequently impairment of complement fixation (9). Also, it was shown that in vitro glycation of IgG changes the functional properties of the Fc fragment (10). Formation of a 500-kDa protein has been reported for glycated IgG (11). The comparison of functional properties of the 500-kDa protein with non-glycated monomeric IgG has indicated a marked reduction in binding to protein A and fixation of the compound (11). Kennedy et al. in an in vitro study also showed that glycation can significantly lower the affinity of an antibody for its antigen (12). These findings were in contrast to those reported by Morin et al. As reported by them, nonenzymatic glycation of human immunoglobulins dose not impair the antigen-antibody binding (13) and their immunoreactivity (14).

The aim of this study was to show more evidence in glycation of IgG and also to demonstrate the changes in the interaction of glycated IgG with anti-IgG. The other question that was proposed to be answered is whether the structural alteration induced by glycation leads to an alteratrion in the mobility of IgG in electroimmunoassay.

MATERIALS AND METHODS

IgG was extratcted from human healthy serum and its purity was checked and confirmed using SDS-PAGE. Anti-human IgG developed in sheep was purchased from Biogen (Iran). All other chemicals were obtained from Sigma and Merck in high pure grade.

Preparation of Glycated IgG. IgG solution (1 mg/ml) was glycated by treatment with 50 and 100 mM glucose in 0.2 M phosphate buffer, pH 7.4 containing 0.04% sodium azide at 27ºC & 37ºC for 10 and 20 days (15). The controls were treated under the same conditions but without glucose. On days 10, and 20 from the beginning of incubation, microbiological testing of the samples was carried out to confirm the absence

of microbiological contamination. To avoid the interference of glucose, the samples were dialyzed against the phosphate buffer at 4º C overnight.

Glycation Assay. Glycation was measured using the thiobarbituric acid colorimetric reaction (16). The colorimetric method with 2-thiobarbituric acid is based on the hydrolysis of the glycated proteins using oxalic acid at 100º C yielding 5-hydroxymethyl furfural (5-HMF) which reacts with thiobarbituric acid. The absorbance was measured at 443 nm. 5-HMF was used as a standard and glycation of IgG was calculated and expressed as µmol HMF per g protein.

Electroimmunoassay. The interaction of glycated IgG with sheep-anti-human IgG was studied using electroimmunoassay (Rocket electrophoresis) (17). Agarose gel (1%) in 25 mM barbitone buffer (pH 8.6) containing 2µl/ml of sheep-antihuman- IgG was prepared on a gel-bond (Pharmacia) between two glass plates. The wells were cut (5 mm diameter) in gel and 10 µl of different concentrations of glycated IgG or native IgG (control) was loaded into the wells in triplicates. Electrophoresis was carried out using an LKB flatbed electrophoresis system at 25A for 3h. After electrophoresis, the plate was dried and stained with Coomasie Briliant blue. The height of immunoprecipitation peaks was measured.

Statistical Analysis. The data were analyzed using non-parametric Man-Whitney U test.

RESULTS

Tables 1 and 2 show the results of glycation measurement for incubated IgG aliquots with two different concentrations of glucose for 10 and 20 days at 27° C & 37° C. As

Table 1. Glycation of IgG (µmol HMF/g protein) for different incubation period and different concentrations of glucose at 27º C

Glucose concentration			
100 mM	50 mM	0 mM (Control)	Time (day)
20.00 ± 1.2*	10.18 ± 0.06*	1.45 ± 0.25	10
28.91 ± 1.5*	12.70 ± 1.10*	2.54 ± 0.35	20

The data are Mean ± SD of two separate triplicate experiments.
* $p < 0.01$ compared to the relevant control.

Table 2. Glycation of IgG (µmol HMF/g protein) for different incubation period and different concentrations of glucose at 37º C

Glucose concentration			
100 mM	50 mM	0 mM (Control)	Time (days)
23.04 ± 0.72*	13.03 ± 1.1*	1.62 ± 0.13	10
29.51 ± 1.4*	16.8 ± 1.65*	2.23 ± 0.68	20

The data are Mean ± SD of two separate triplicate experiments.
* $p < 0.01$ compared to the relevant control.

Table 3. Interaction of glycated- and non-glycated IgG with anti IgG. IgG was glycated by treatment with 50 mM glucose at 27º C for 10 & 20 days. Data are heights of precipitated peaks in rocket electrophoresis

Mean of deccrease (%)	Percent of deccrease	Height of peaks (mm) Non-glycated IgG	Glycated IgG	IgG concentration g/l	Time (day)
	19.0	21.0 ± 0.75	17.0 ± 0.45	2.86	
	27.4	19.3 ± 0.59	14.0 ± 0.47	1.43	
23.9	25.4	16.5 ± 0.63	12.3 ± 0.88	0.71	**10**
	29.2	19.5 ± 0.88	13.8 ± 0.75	2.86	
	28.0	17.1 ± 0.78	12.3 ± 0.49	1.43	
26.2	21.3	15.0 ± 0.49	11.8 ± 0.84	0.71	**20**

$P < 0.05$ compared to non-glycated IgG

Table 4. Interaction of glycated- and non-glycated IgG with anti IgG. IgG was glycated by treatment with 100 mM glucose at 27° C for 10 & 20 days. Data are heights of precipitated peaks in rocket electrophoresis

Mean of deccrease (%)	Percent of deccrease	Height of peaks (mm) Non-glycated IgG	Glycated IgG	IgG concentration g/l	Time (day)
	25.0	18.0 ± 0.71	13.5 ± 0.65	2.86	
	27.6	16.3 ± 0.49	11.8 ± 0.84	1.43	
27.5	31.0	14.5 ± 0.74	10.0 ± 0.46	0.71	**10**
	30.8	17.2 ± 0.71	11.9 ± 0.52	2.86	
	38.0	16.8 ± 0.52	10.4 ± 0.75	1.43	
32.4	28.4	13.7 ± 0.65	9.8 ± 0.48	0.71	**20**

$P < 0.05$ compared to non-glycated IgG

shown in these Tables, in vitro incubation of IgG with glucose led to its glycation and this process depended on the duration of incubation and concentration of glucose. In all treated IgGs, the level of glycation was significantly higher than those for untreated IgG (control) ($p < 0.01$).

Fig. 1 shows a typical pattern of rocket electrophoresis of glycated and non-glycated IgG. This fig. indicates the changes in the mobility of glycated IgG that shows glycation altered the interaction of IgG with anti-IgG. The results of height measurement of immuno-precipitated peaks are summarized in Tables 3-6.

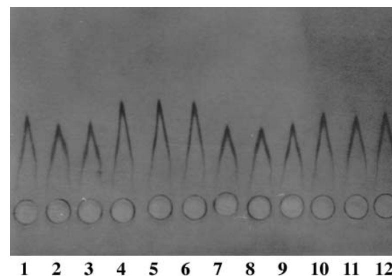

Figure 1. A typical plate of rocket-electrophoresis
Peaks 1-3 and 7-9 contained glycated IgG. Peaks 4-6 and 10-12 contained non-glycated IgG (control)

Table 5. Interaction of glycated- and non-glycated IgG with anti IgG. IgG was glycated by treatment with 50 mM glucose at 37° C for 10 & 20 days. Data are heights of precipitated peaks in rocket electrophoresis

Mean of deccrease (%)	Percent of deccrease	Height of peaks (mm) Non-glycated IgG	Glycated IgG	IgG concentration g/l	Time (day)
	32.0	19.7 ± 0.54	13.8 ± 0.56	2.86	
	22.4	16.5 ± 0.66	12.8 ± 0.82	1.43	
26.0	23.8	14.7 ± 0.45	11.2 ± 0.65	0.71	**10**
	40.9	20.5 ± 0.47	12.1 ± 0.49	2.86	
	37.5	16.0 ± 0.65	10.0 ± 0.54	1.43	
37.8	35.1	14.8 ± 0.45	9.6 ± 0.78	0.71	**20**

$P < 0.05$ compared to non-glycated IgG

Table 6. Interaction of glycated- and non-glycated IgG with anti IgG. IgG was glycated by treatment with 100mM glucose at 37° C for 10 & 20 days. Data are heights of precipitated peaks in rocket electrophoresis

Mean of deccrease (%)	Percent of deccrease	Height of peaks (mm) Non-glycated IgG	Glycated IgG	IgG concentration g/l	Time (day)
	34.7	19.7 ± 0.54	12.2 ± 0.65	2.86	
	25.5	16.5 ± 0.66	10.5 ± 0.68	1.43	
28.8	26.2	14.7 ± 0.45	9.0 ± 0.58	0.71	**10**
	32.2	18.0 ± 0.39	12.4 ± 0.43	2.86	
	37.5	16.5 ± 0.79	9.5 ± 0.68	1.43	
38.7	35.1	13.9 ± 0.54	8.9 ± 0.73	0.71	**20**

$P < 0.05$ compared to non-glycated IgG

As these data show, in all cases the height of glycated IgG peaks was lower than those for non-glycated IgG. Also, these alterations in the height of peaks were higher in upper levels of glycation.
Statistical analysis of these data shows that difference in the height of the peaks between glycated- and non-glyacted IgG was significant ($p < 0.05$). Data also indicates that changes in the height of the peaks depend on the level of glycation.

DISCUSSION

Non-enzymatic glycation of proteins in serum and tissues is a pathophysioloical consequence of hyperglycemia in diabetes mellitus, and also correlates with aging (18). The products of glycation lead to irreversible physicochemical and biological changes of proteins (19). Modification of oxygen affinity in glycated hemoglobin (20), alterations in the binding properties for bilirubin and fatty acids for serum albumin (21), and modification of solubility of the lens crystalline and collagen have been reported (22,23).

In this study, serum IgG was used to evaluate the functional alteration of glycated proteins. We also showed further evidence in the non-enzymatic incorporation of D-glucose under in vitro physiological conditions into human IgG. Our observation confirmed the previous studies reporting that the glycation of IgG is time and concentration dependent (4). A report published recently also revealed more details in the kinetics of in vitro glycation of IgG (24). In this study, IgG was incubated with normal and diabetic glucose concentrations but in a longer time period; 90 and 270 days at temperatures of 4° C to 60° C indicating that glycation of IgG resembles the first order kinetics.

To show the effect of glycation on the interaction of IgG with anti-IgG, we examined the mobility of IgG in an electroimmunoassay system. Our data indicated that glycated IgG had lower mobility than non-glycated IgG. Higher degrees of glycation caused lower mobility in the rocket electrophoresis. What can be concluded from these results is a structural change induced by glycation of IgG. This observation can be due to the binding of glucose that changed the net charge of IgG molecule, leading to a lower mobility. As in this electro-immunoassay IgG molecules moved to cathode, it is possible that glycation induced a conformational change to the IgG with less positive charge. Another possibility is that these conformational changes altered its reactivity with anti-IgG. Since we obtained similar results using single radial immunodifusion method (Manuscript Submitted), which is independent of electric charge, the second possibility is more plausible.

The glycation of IgG seems interesting for several reasons. The most important one is that diabetics have impaired resistance to infection. It has been suggested that glycosylation may impair the IgG function and thereby contribute to increased susceptibility to infection. IgG molecule contains polysaccharide chains which play an important role in the Fc fragment functions, such as the clearance of IgG from plasma by the liver asialoprotein receptors, complement activation and IgG binding to Fc receptors (25). It is quite possible that glycation can modify these activities. According to the data obtained from our study, it is likely that formation of glycated IgG alters the IgG ability to bind to anti-IgG antibody.

Dolhofer-Bliesener et al. reported that functional properties of the Fab region were unaffected upon glycation (10). They also showed that functional changes of the Fc fragment were observed at glycation levels comparable to those found in diabetes (8). In contrast to these findings, Lapolla et al., in an in vivo study found that the immunodeficiency observed in diabetic patients may be due to the inhibition of molecular recognition between antibody and antigen as a result of a change in functionality of the modified Fab fragment of IgG (26).

These data and the above-mentioned observations lead us to speculate that changes in the biological activity of the glycated IgG molecule is due to the alteration of some of its free amino groups by the non-enzymatic glycosylation. Our findings open an interesting view on the possible alterations of another biological function in glycated IgG which may contribute to complications of diabetes. Further studies are needed to clarify the role of this process in the pathophysiology of diabetes mellitus.

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