In vitro Glycation of Human IgG and Its Effect on Interaction with Anti-IgG

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ABSTRACT

Non-enzymatic glycosylation of proteins is one of the key mechanisms in the pathogenesis of diabetic complications. Glycation of IgG is of special interest due to its possible influence on the functionality of immunoglobulins and overall immuno-competence. The aim of this study was to clarify more details of *in vitro* glycation of IgG and to study the effect of this modification on its interation with anti-IgG.

Purified human IgG was glycated in the presence of 50 and 100 mM glucose. Glycation was measured using spectrophotometric thiobarbituric acid method. To study the effect of glycation on interaction with anti IgG the Single Radial Immunodiffusion (SRID) was used and the diameters of precipitation rings of glycated IgG and non-glycated IgG were measured and compared.

The results showed that IgG was glycated in presence of 50 and 100 mM glucose at 27° and 37° C and the extent of glycation was dependent on glucose concentration and time of incubation. In higher concentration of glucose and longer period of incubation glycation was higher at 27° C ($p<0.01$). Similar results were obtained at 37° C.

The results of SRID indicated that glycated IgG showed reduced interaction with anti-IgG. The diameters of precipitated rings for glycated IgG were significantly lower than those of non-glycated IgG ($p < 0.01$).

It can be concluded that modification that occurred in IgG structure due to glycation can be the reason of the reduction of its interaction with anti-IgG.

Keywords: Anti-IgG; Diabetes Mellitus; Glycosylation; Immunoglobulin G

INTRODCUTION

Non-enzymatic glycosylation of proteins in serum and tissues is a pathophysiological consequence of hyperglycemia in diabetes mellitus, and also correlates with ageing.[1,2] There is an increasing evidence that chronic hyperglycemia is the major cause of secondary complications of diabetes such as microangiopathy, retinopathy, neuropathy, and nephropathy.[3-5]

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Discovery of glucose dependent chemical modification of various proteins suggested that they could induce functional abnormalities in these proteins, and thereby lead to the pathophysiology of diabetes.[6]

Glycation of albumin changes its binding to drugs and its kidney transport.[7] Lens crystalline in diabetic patients undergoes glycation reaction leading to formation of high molecular weight mass that causes turbidity in lenses.[8] For collagen it is shown that *in vitro* glycation changes its physicochemical properties include decrease solubility and elasticity.[9] There is evidence for *in vitro* and in vivo glycation of IgG.[10,11] Treatment of purified human IgG with glucose

induced its glycation and this process was time and pH dependent.[10] This study also indicated that this modification changed the complement fixation activity of IgG.[10] Kenedy et al. showed that the level of glycated IgG, IgM, and IgA increased in diabetic patients.[12] Although reduction of the ability of glycated IgG for complement fixation has been reported,[10] another group of investigators believe that glycated IgG can stimulate complement activity higher than non-glycated.[13]

Although there are several reports on the effect of IgG glycation on some functions of IgG, some discrepancies between the results and research in this field still continues.[14] Furthermore there are few reports about the effect of the modification on interaction of glycated IgG with anti-IgG. Newkrik et al. showed that advanced glycation end products on IgG was a target for circulating antibodies in rheumatoid arthritis patients.[14] The aim of this study was to obtain further details about the *in vitro* glycation of IgG and also to show that glycation of IgG alters its interaction with anti-IgG.

MATERIALS AND METHODS

IgG was a gift from Dr. A. Mostafaei, its purity was checked and confirmed using SDS-PAGE. Anti human IgG developed in sheep was purchased from Biogen (Iran). All other chemicals were from Sigma and Merk in high pure grades.

Glycation of IgG

Prepared human IgG was glycated by treatment with different concentrations of glucose at different intervals.[16] Aliquots of IgG (1 mg/ml) in dialysis bag were incubated with 50 and 100 mM glucose in 0.2 M phosphate buffer, pH 7.4 containing 0.04% sodium azide, at 27ºC and 37º C for 10 and 20 days. Controls were treated under the same conditions but without glucose. On days 10, and 20 from the beginning of incubation, microbiological testing of samples was carried out to confirm the absence of microbiological contamination. To avoid the interference by glucose, we dialyzed the samples overnight against physiological saline at 4ºC with 3 times buffer exchange.

Measurement of Glycation

Glycation was measured using the thiobarbituric acid colorimetric reaction.[17] The colorimetric method with 2-thiobarbituric acid is based on the hydrolysis of the glycated proteins using oxalic acid at 100ºC 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.

Interaction of Glycated IgG with Anti IgG

The interaction of glycated IgG with sheep-anti human IgG was studied using single radial immuno-diffusion (SRID). Agarose gel (1%) was prepared according to the method of Bialey.[18] This gel contained 2µl/ml of sheep-antihuman- IgG. Wells were cut (5 mm diameter) in gel and 10 µl of different concentrations of glycated IgG or native IgG (control) were placed in each well. The gel was incubated in a humid covered box at room temperature for 48 hours. After this period of time the diameter of precipitated rings were measured.

Statistical Analysis

The results were analyzed using non-parametric Man-Whitney U test. P value less than 0.05 was considered significant.

RESULTS

Results of the measurements of IgG glycation in different conditions are summarized in Table 1.

As this table shows treatment of IgG with 50 and 100 mM glucose in 27°C leading to glycation of IgG and the levels of glycation in both concentrations of glucose being higher compared to that of control. Glycation of IgG also was significantly higher when it was treated with 100mM glucose compared to 50 mM glucose ($p<0.01$). Similar results were obtained when glycation was carried out at 37°C (Table 1). Glycation of IgG by treatment with 50 and 100 mM glucose at 37°C was significantly higher than that of control ($p<0.01$).

Comparison of the effect of two different concentrations of glucose at 37° C also indicated that the level of glycation was elevated in higher

Table 1. Glycation of IgG (µmol HMF/g protein) in different incubation times and different concentrations of glucose at 27ºC and 37ºC.

Temperature	Incubation Time (days)	Glucose Concentration		
		0 mM (Control)	50 mM	100 mM
27º C	10	1.45 ± 0.25	10.18 ±0.06*	20.00 ± 1.2*
	20	2.54 ± 0.35	12.70 ±1.10*	28.91 ± 1.5*
37º C	10	1.62 ± 0.13	13.03 ± 1.1*	23.04 ± 0.72*
	20	2.23 ± 0.68	16.8 ± 1.65*	29.51 ± 1.4*

The data are mean ± SD of two separate triplicate experiments.

* p <0.01 compared to the relevant control.

concentration of glucose (p<0.01). The interaction of different concentrations of glycated IgG with anti IgG was studied using SRID (Figure 1), and the diameters of precipitated rings were compared to that of non-glycated protein (Tables 2-6).

As Table 2 shows there were statistically significant differences between the diameters of precipitated rings in glycated and non-glycated states of IgG (p<0.05). For all studied concentrations of IgG the observed diameter of precipitated rings were significantly lower than those of non-glycated IgG (p<0.05).

Table 3 shows similar results for glycated IgG formed by treatment with 100 mM glucose at 27° C. In this condition also there was a significant decrease in diameters of precipitated rings of glycated IgG compared to non-glycated (p<0.05).

The mean of decrease in diameters of precipitated rings was 36.6% and 37.4% for 10 and 20 days treatment, respectively.

Glycated IgG formed at 37° C also showed similar trends (Tables 4-5). The results in Table 5 are for IgG which was glycated by treatment with 50 mM glucose at 37° C for 10 and 20 days. These results showed that glycated IgG formed smaller precipitated rings compared to non-glycated (p<0.05).

Glycated IgG which was produced by treatment with 100 mM glucose at 37° C for 10 and 20 days also formed lower diameters ring compared to non-glycated IgG (p<0.01). Comparing the results obtained at temperature 27° C and 37° C, it can be seen that the increase of 10 degrees in temperature could significantly reduce the binding affinity between glycated IgG and its anti-component at all concentrations(p<0.05).

Table 2. Interaction of Glycated and non-glycated IgG with anti IgG.
IgG was glycated by treatment with 50 mM Glucose at 27ºC for 10 and 20 days. Data are diameter (X ± SD) of precipitated rings in SRID.

Incubation Time (Days)	IgG Concentrations µg/µl	Diameters of Precipitated Rings (mm x 10)		Percent of Decrease	Mean of Decrease (%)
		Glycated IgG	Non-glycated IgG		
10	1.83	130 ± 9.1	175 ± 6.5	25.7	22.7
	0.91	125 ± 6.8	157 ± 4.5	20.3	
	0.45	105 ± 8.4	130 ± 5.1	19.2	
	0.22	86 ± 0.1	108 ± 6.5	20.3	
	0.11	46 ± 4.6	64 ± 4.5	28.1	
20	1.83	120 ± 9.1	172 ± 7.5	30.2	28.18
	0.91	114 ± 6.1	150 ± 6.3	24.0	
	0.45	97 ± 7.3	132 ± 9.2	26.5	
	0.22	65 ± 6.1	101 ± 5.3	35.6	
	0.11	49 ± 6.2	65 ± 3.4	24.6	

Table 3. Interaction of Glycated and non-glycated IgG with anti IgG.
IgG was glycated by treatment with 100 mM Glucose at 27°C for 10 and 20 days. Data are diameters (X ± SD) of precipitated rings in SRID.

Incubation Time (Days)	IgG Concentrations µg/µl	Diameters of Precipitated Rings (mm x 10)		Percent of Decrease	Mean of Decrease (%)
		Glycated IgG	Non-glycated IgG		
10	1.83	109 ± 9.3	160 ± 9.1	32.0	36.6
	0.91	87 ± 8.5	140 ± 9.5	37.8	
	0.45	72 ± 6.4	113 ± 8.3	36.2	
	0.22	57 ± 4.3	94 ± 8.2	40.0	
	0.11	31 ± 2.5	49 ± 5.2	36.7	
20	1.83	111 ± 8.2	171 ± 7.5	35.1	37.4
	0.91	96 ± 7.5	146 ± 7.3	34.2	
	0.45	79 ± 7.3	128 ± 9.2	38.3	
	0.22	62 ± 6.4	101 ± 5.9	38.6	
	0.11	51 ± 6.2	86 ± 3.4	40.7	

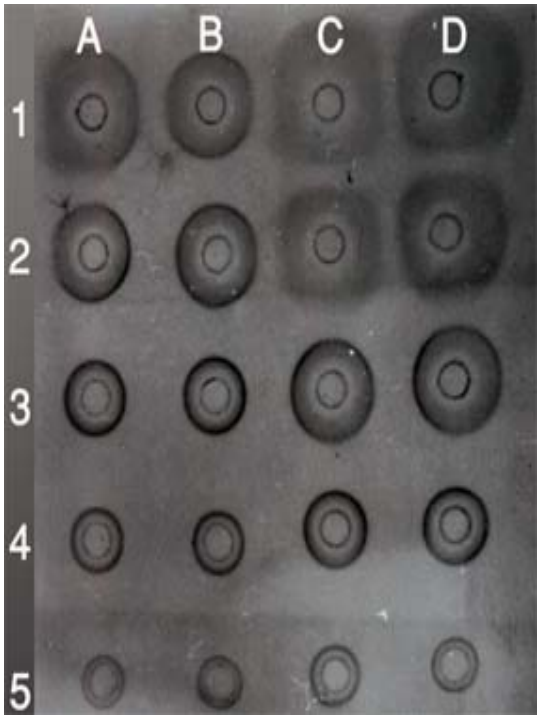

Figure 1. A typical plate of SRID showing precipitin rings due to the interaction of glycated and non-glycated IgG with anti IgG.
IgG was glycated by treatment with 50 mM glucose for 10 days at 27°C.
Lines 1, 2, 3, 4, and 5 in columns A and B contained 18.3, 9.15, 4.55, 2.27, and 1.13 µg glycated IgG respectively. Columns C and D contained similar amounts of non-glycated IgG.

DISCUSSION

In this study we demonstrated the *in vitro* non-enzymatic incorporation of D-glucose under physiological conditions into human IgG. Dolhoffer-Bliesener et al. also reported that IgG glycation might occur during *in vitro* incubation of IgG with glucose solutions; however the condition that they used was at pH 9 which was different from our study and far from the physiologic pH. [10] Our results are consistent with those reported by Davin et al.[13] They have confirmed a direct relationship between the percentages of glycated IgG, glucose concentration, and incubation time. A recently published article also indicated that IgG undergoes *in vitro* glycation and this reaction nearly resembles the first order reaction kinetics, which confirms our results.[19] The study of interaction of glycated IgG with anti IgG in agarose gel using SRID and comparing the data with non-glycated IgG indicated that covalent binding of glucose altered the structure of IgG and consequently affected its interaction with anti-IgG. The conformational changes induced by glycation may cover or alter the epitopes in IgG structure. The alteration in function of glycated IgG has been reported previously. Some reports showed that nonenzymatic glycosylation of human immunoglobulins does not impair their immuno-reactivity[20] and antigen-antibody binding.[21] Hennessey et al showed that glycation of IgG impairs its complement fixation.[22]

Table 4. Interaction of Glycated- and non-glycated IgG with anti IgG.
IgG was glycated by treatment with 50 mM Glucose at 37°C for 10 and 20 days. Data are diameter (X ± SD) of precipitated rings in SRID.

Incubation Time (Days)	IgG Concentrations µg/µl	Diameters of Precipitated Rings (mm x 10)		Percent of Decrease	Mean of Decrease (%)
		Glycated IgG	Non-glycated IgG		
10	1.83	127 ± 5.6	161 ± 7.1	21.2	24.0
	0.91	122 ± 6.1	145 ± 6.2	15.8	
	0.45	95 ± 8.6	122 ± 5.6	22.1	
	0.22	67 ± 5.8	97 ± 9.1	30.9	
	0.11	41 ± 3.1	59 ± 4.2	30.4	
20	1.83	115 ± 8.1	171 ± 7.3	32.7	29.52
	0.91	106 ± 6.9	139 ± 4.0	23.7	
	0.45	85 ± 7.1	123 ± 6.9	30.8	
	0.22	63 ± 3.4	88 ± 4.1	28.4	
	0.11	51 ± 4.3	75 ± 6.3	32.0	

They have demonstrated that a significant reduction in complement fixation by immunoglobulin occurs with elevated glucose concentration.[22] Formation of a 500-kDa protein has been reported for glycated IgG. Comparing the functional properties of the 500-kDa protein with nonglycated monomeric IgG, has indicated a marked reduction in binding to protein A and fixation of complement.[23] Our results confirming some previous reports indicated that glycation leads to changes in other functions of IgG.

The observed discrepancy between results is possibly due to different conditions of the experiments. While some investigators used short period of treatment with glucose, others carried out incubation at longer time.

It is important to note that in glycation, glucose reacts with free amino group of proteins to form labile Schiff bases, with subsequent Amadori rearrangement to stable ketoamines.

Formation of Amadori products is well established for a number of proteins and has been found to increase in diabetics.[24] The Amadori products very slowly undergo a series of still unknown rearrangement reactions to form yellow-brown, fluorescent and cross-linking substances, called advanced glycation end products (AGEs).[25]

Table 5. Interaction of Glycated- and non-glycated IgG with anti IgG.
IgG was glycated by treatment with 100 mM Glucose at 37°C for 10 and 20 days. Data are diameter (X ± SD) of precipitated rings in SRID.

Incubation Time (Days)	IgG Concentrations µg/µl	Diameters of Precipitated Rings (mm x 10)		Percent of Decrease	Mean of Decrease (%)
		Glycated IgG	Non-glycated IgG		
10	1.83	105 ± 5.9	155 ± 7.1	32.2	37.3
	0.91	89 ± 6.6	138 ± 6.2	35.5	
	0.45	69 ± 3.5	114 ± 4.6	39.9	
	0.22	59 ± 4.8	95 ± 5.5	37.8	
	0.11	32 ± 5.1	55 ± 4.4	41.8	
20	1.83	101 ± 6.2	160 ± 8.7	36.8	43.5
	0.91	81 ± 6.6	143 ± 5.8	43.3	
	0.45	68 ± 3.5	119 ± 7.1	42.8	
	0.22	51 ± 5.6	99 ± 6.1	48.4	
	0.11	30 ± 5.8	56 ± 3.9	46.4	

These products are thought to accumulate on proteins and play an important role in the development of late diabetic complications.[26]

Autoantibodies to IgG-AGE had previously been demonstrated in patients with sever longstanding rheumatoid arthritis.[27] In a recent study in patients with early synovitis, the presence of AGE-damaged IgG and IgM autoantibodies to IgG-AGE have been demonstrated.[28]

Both Fab and Fc regions of IgG may undergo glycation. Lapolla et al showed higher binding of glucose to Fab, possibly explained by its higher reactivity to glucose.[29]

Since binding of Fab-IgG to Fc-anti-IgG is very specific and precise, any modification such as glycation can affect this interaction.

This opens an interesting view on possible alterations of biological functions in glycated IgG, modification of antigen binding capacity or modifications of its ability for activation of the complement system.

Dolhofer-Bliesener et al studying *in vitro* glycation of IgG have reported that functional properties of the Fab region is unaffected upon glycation, whereas the functional changes of the Fc fragment were shown.[10]

Lapolla et al in a recently published article indicated that measurement of intermediate and late products of the glycation reaction is a precious method in verifying the relationship between glycation products and tissue modifications.[30] Controlling the glucose levels in diabetic patients can reduce the glycation of proteins and diminish the late complications of diabetes.

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