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Glycation of monoclonal antibodies impairs their ability to bind antigen

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SUMMARY

As elevated levels of glycated IgG have been detected in the plasma of patients with diabetes mellitus, a disease associated with increased susceptibility to infection, we have investigated whether glycation of MoAbs affects the kinetics and/or affinity of antigen binding. Three mouse MoAbs were incubated with 0·5 M glucose at pH 7·4 for 14–21 days at 37°C. Control MoAbs were incubated using identical conditions but with no added glucose. Using a surface plasmon resonance technique we found that glycation significantly increased the rate of dissociation (k_{diss}) of the antigen–antibody complex for all three MoAbs ($P < 0{\cdot}05$, $n = 4$), but had no significant effect on the rate of association (k_{ass}). For one of the MoAbs, against human IgG (Fab), we also measured k_{diss} by an alternative method utilizing radiolabelled antigen, which confirmed that glycation of the antibody significantly increases k_{diss} ($P < 0{\cdot}001$, $n = 8$). We also found using an ELISA-based method that glycation of the same MoAb significantly increased the equilibrium dissociation constant (K_d) ($P < 0{\cdot}05$, $n = 6$). A significant increase in k_d was observed after glycation using glucose concentrations consistent with those found in poorly controlled diabetics ($P < 0{\cdot}02$, $n = 5$). We conclude that *in vitro* glycation can significantly lower the affinity of an antibody for its antigen, and significantly increases the rate of dissociation of the antigen–antibody complex.

Keywords glycation monoclonal antibody affinity kinetics diabetes mellitus

INTRODUCTION

Many serious diabetic complications result from increased susceptibility to infection [1,2]. Since immunoglobulin is a major component of the humoral response and since increased glycation of IgG, IgA and IgM have been detected in diabetics, we have investigated whether glycation significantly affects the primary function of antibody, that of binding antigen.

Protein glycation is a spontaneous reaction whereby reducing sugars become covalently bound to proteins *in vivo* [3,4], often resulting in functional impairment [5]. It is a non-enzymatic process, occurring quite distinctly from glycosylation, which is controlled by specific glycosyl transferases. Many biologically important proteins are known to become increasingly glycated in diabetics, and it is thought to have a major role in many of the long-term complications of diabetes [6].

It is well established that diabetics have increased amounts of glycated IgG [7–10]; the mean percentage glycation of IgG in normal subjects ranges from 6·6% to 9·3% for non-diabetics and from 14·6% to 21·6% for diabetics. Other immunoglobulins such as IgA and IgM also show increased levels of glycation [11]. Dolhofer *et al.* were the first to propose that glycation significantly impairs the functional ability of IgG when they found that glycated IgG fixed less complement than unglycated IgG [7]. However, Morin *et al.* were unable to find any impairment using a variety of different functional assays assessing both complement fixation assay and antigen binding [8,12]. Further studies by Dolhofer & Gerbitz confirmed that complement fixation and protein A binding are markedly impaired by glycation, but found little evidence that antigen binding of a polyclonal antibody was affected [12,13]. Recently, however, Sasaki *et al.* showed that glycation of an anti-DNA MoAb impaired its relative antigen-binding capacity in an ELISA [14].

We have investigated the effect of glycation on antibody function by measuring the kinetics and affinity parameters of the antigen–antibody reaction which provide a more detailed picture of antigen–antibody interactions than can be obtained by assays which compare the relative antigen-binding capacity of glycated and unglycated antibody in an immunoassay such as an ELISA. Using this approach we have found that *in vitro* glycation can significantly increase the rate of dissociation (k_{diss}) of the antigen–antibody complex, and also significantly

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increases the equilibrium dissociation constant K_d. These findings considerably strengthen the argument that impairment of antibody function, as a result of glycation, involves the Fab portion of antibody as well as the Fc portion.

MATERIALS AND METHODS

MoAbs against human IgG (Fab) (I 6135), *Escherichia coli* β-galactosidase (G 8021) and avidin (A 5680) were obtained in the form of unpurified ascites from Sigma Chemical Co. (Poole, UK). The concentration of MoAb in each of the ascites was 3·2 mg/ml (I 6135), 1·8 mg/ml (G 8021), and 3·5 mg/ml (A 5680), respectively. Total protein concentration of all three MoAbs was approximately 30 mg/ml. Mouse albumin, whole mouse IgG, glucose and Tween 20 were also purchased from Sigma and all other reagents from BDH (Poole, UK). All chemicals were of reagent grade.

Surface plasmon resonance (SPR) measurements were carried out using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden). The CM5 sensor chips, P20 surfactant, amine coupling kit containing *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N′*-(3-diethylamino-propyl)-carbo-diimide (EDC) and 1 M ethanolamine-hydrochloride pH 8·5 were all provided as gifts from Pharmacia Biosensor AB. Affinity-purified rabbit anti-mouse IgG Fc (RamFc) and rabbit anti-mouse IgG1 (RamG1) were provided as gifts by Pharmacia Diagnostics (Uppsala, Sweden).

Protein radiolabelling was carried out using Iodogen from Pierce and Warriner (Chester, UK) with ^{125}I from ICN Biomedicals Ltd. (Irvine, UK). PD-10 gel filtration columns were purchased from Pharmacia (Milton Keynes, UK) and 'Removastrip' 12-well microtitre plates were obtained from Dynatech Labs. (Billinghurst, UK). Radioactivity was measured using an Intertechnique CG-4000 Gamma Counter (Kontron Instruments Ltd., Watford, UK).

ELISA was carried out using 96-well (Immunolon 4) microtitre plates from Dynatech Labs. For plate washing a 'Well Wash 4' plate washer (Denley Instruments Ltd., Billinghurst, UK) was used, and absorbance measurements were made using a Titertek Multiskan microtitre plate reader with a 405-nm filter. Pre-assay dilutions were carried out using an RSP 5032 robotic sampler (Tecan Ltd., Reading, UK).

Glycation

MoAbs in the form of unpurified ascites against human IgG (Fab) and β-galactosidase were diluted 1:4 in 0·1 M phosphate pH 7·4 and glycated for approximately 21 days at 37°C. A MoAb against avidin was glycated under similar conditions, but in PBS at pH 7·4. Control antibodies were incubated under identical conditions but with no added glucose. Any residual glucose present in the diluted ascites before incubation was assumed not to contribute significantly to the level of glycation. For both SPR measurements and dissociation measurements using radiolabelled antigen, 0·5 M glucose was used for glycation of the MoAbs. However, the anti-IgG (Fab) MoAb was glycated using both 0·5 M and 15 mM glucose for the determination of K_d.

All incubations were carried out under sterile conditions in sealed polypropylene tubes, and the solutions were sterilized by filtration with a 0·22-μm filter (Millipore, Watford, UK). In addition, all buffers contained 6 mM sodium azide to prevent microbial growth. Once glycated, the MoAbs were kept at 4°C for a maximum of 1 week while the assays were carried out.

Measuring IgG glycation

An approximation of the degree of glycation of the mouse MoAbs was determined using whole IgG glycated under similar conditions to those used for glycation of the MoAbs. Whole mouse IgG and albumin was diluted in 0·1 M phosphate buffer pH 7·4 so that IgG concentration was 0·75 mg/ml and the total protein concentration 7·5 mg/ml, i.e. similar concentrations to those of the diluted MoAbs. Mouse albumin was used since this is the major constituent of mouse ascities, comprising approximately 90% of total protein. The IgG–albumin mixture was incubated for 21 days at 37°C with 0, 15, 50 and 500 mM glucose. The IgG was then purified by protein G affinity chromatography with any free sugar being removed by dialysis, and the samples were then frozen until required. The extent of IgG glycation was estimated using the phenylboronate affinity (PBA) chromatography method of Gould *et al.* [15] and using a periodate-based colorimetric method developed by us [16].

Measuring association and dissociation kinetics by monitoring surface plasmon resonance

The BIAcore system was used to determine the kinetic rate constants k_{ass} and k_{diss} using a method described in detail by Karlsson *et al.* [17]. The principles of SPR measurements and their application to measuring antibody–antigen kinetics have also been described recently by Malmqvist [18].

RamFc or RamG1 capture antibody was immobilized to the sensor chip CM5 using amine coupling with NHS and EDC [19]. Each analytical cycle consisted of a 2-μl injection of MoAb ascites (diluted 1:40 in pH 7·4 HEPES-buffered saline (HBS)) followed by 20 μl of antigen (diluted to between 10 and 160 nM in HBS), followed by buffer minus antigen resulting in dissociation of the antigen with regeneration of the sensor surface using two washes with 0·1 M HCl. The surface concentration of captured MoAb was less than 2000 SPR response units (RU) so as to minimize mass transport effects. For all kinetic determinations a buffer flow rate of 2 μl/min was maintained, and SPR was monitored continuously throughout the association and dissociation phases of the interaction. The data thus obtained were analysed using the supplied BIAlogue kinetic evaluation software (pre-release version) to calculate the kinetic rate constants.

Measuring dissociation kinetics using radiolabelled antigen

Human IgG was labelled with ^{125}I using the Iodogen method of Fraker & Speck [20]. Glass screw-top vials containing 200 μl of Iodogen (100 μg/ml) in chloroform, were warmed under a continuous flow of nitrogen until dry. After rinsing with 0·1 M phosphate pH 7·4, 100-μl aliquots of IgG (1 mg/ml) were added to each vial, followed by 5 μl (0·5 mCi) of radiolabelled sodium iodide. After 5 min of occasional mixing, the IgG solution was removed from the glass vial and applied to a PD-10 gel filtration column to remove free iodine. Fractions were eluted with 0·1 M phosphate buffer pH 7·4 and analysed by non-SDS–PAGE electrophoresis. Autoradiography revealed that no significant damage had occurred to the IgG (data not shown).

Mouse MoAb against human IgG (Fab) (ascites) was diluted 1:1000 to a concentration of 1·6 μg/ml (10·7 nM) and

100 μl added per well of the removable (12 × 1 well) microtitre plate strips. The strips were then incubated overnight at 4°C. Any unbound antibody was removed by three washes with PBS, containing 0·05% Tween 20 (PBS-T). Radiolabelled human IgG was then diluted to 8 μg/ml (53 nM) in PBS-T and 100 μl added to each well. After a second overnight incubation at 4°C, the strips were washed a further three times with PBS-T, to remove unbound labelled antigen, and 100 μl of unlabelled human IgG, diluted to 0·5 mg/ml (3·3 μM) in PBS-T were added to each well. Dissociation of the antigen–antibody complex occurred with radiolabelled human IgG being displaced by unlabelled IgG, thereby decreasing the amount of bound radioactivity. At 1 min ($t = 0$) and then 1, 3, 4 and 6 h after addition of unlabelled antigen, the contents of the wells were removed, the wells washed three times and dried, and the amount of radioactivity bound to each well was then measured using a gamma counter.

Determination of the dissociation constant (K_d)

K_d was determined using a modification of the ELISA procedure of Friguet *et al.* [21]. Microtitre plates were prepared by coating wells with 100 μl of a solution comprising 1 μg/ml of human IgG diluted in carbonate buffer pH 9·6. MoAb against human IgG (Fab) was incubated under several different incubation conditions for 21 days at 37°C. The conditions were as follows: dilution in 0·1 M phosphate with 0, 50 and 500 mM glucose and dilution in 15 mM phosphate with 0·5 M glucose. A further control was incubation with 0·5 M α-methyl glucose, which is methylated at the C-1 position and therefore cannot glycate protein. This control was included to rule out the possibility that the increase in solute concentration of solutions containing glucose affects K_d.

After incubation, the MoAbs were diluted 1:333 in PBS-T, and 10 μl of varying concentrations of human IgG between 1×10^{-7} M to 2×10^{-10} M were added. Control wells contained no antigen. Dilutions were made in PBS at pH 7·4 containing 0·5% Tween 20 which was added to minimize non-specific binding. Because the MoAb, which contains 0·5 M glucose, is diluted to 1:17 000 overall, the concentration of glucose in the antigen–antibody mixture is negligible. The tubes were then sealed and incubated overnight at room temperature. During this incubation period in PBS, the labile Schiff base (SB) glycation products dissociated [22], leaving only Amadori product (AP) bound to the protein. After the overnight incubation, 100-μl aliquots of each antigen–antibody mixture was transferred to the wells of a microtitre plate previously coated with human IgG. After 30 min incubation at 20°C, MoAb not bound to the plate was then removed by washing three times with PBS-T. A polyclonal anti-mouse IgG antibody conjugated to alkaline phosphatase was then diluted 1:2000 in PBS-T and 100 μl added to each well. After 1·5 h at 37°C, the plate was then washed with PBS-T to remove unbound conjugate and 100 μl of substrate (3·7 mg/ml of pNPP, 1 M diethanolamine and 5 mM $MgCl_2$) were added to each well. After incubation for 1 h at 37°C, the absorbance of each well at 405 nm was measured using a microtitre plate reader. The resulting absorbances were used to calculate K_d using the method of Stevens [23].

For determining K_d, MoAb was also incubated with and without 15 mM glucose. The effect on K_d of the binding of both SB and AP glycation products was investigated by modifying the ELISA affinity assay to prevent net SB dissociation during the overnight incubation of the affinity assay. This was achieved by adding 15 mM glucose to the antigen–antibody mixtures before the overnight incubation so that a constant glucose concentration was maintained. After the overnight incubation, 100 μl of each antibody–antigen mixture (containing 15 mM glucose) were added to the antigen-coated plate and the K_d measured as before. Glucose (15 mM) was also added to the unglycated antibody immediately before transferring the antigen–antibody mixture to the IgG-coated wells, as a control.

RESULTS

Extent of IgG glycation

Purified whole mouse IgG, prepared as described in Materials and Methods, was glycated aseptically for 21 days at 37°C using glucose concentrations of 0, 15 mM, 50 mM and 0·5 M. Table 1 shows the amount of AP per mole of protein of the purified IgG, as determined by a periodate-based colorimetric method for determining glycation. The extent of glycation of mouse IgG was also determined using PBA chromatography; the percentage glycated IgG being 95·4 % after incubation with 0·5 M glucose and 20·9% after incubation without added glucose. The data in Table 1 give an approximation of the degree of glycation products of the MoAbs used in the kinetic and affinity determinations.

The effect of glycation on the rates of association and dissociation using SPR measurements

Each of the three mouse MoAbs (ascites) was glycated with 0·5 M glucose for approximately 21 days at 37°C. Control antibodies were incubated under identical conditions but with no added glucose. The rate of formation of the antigen–antibody complex (k_s) of both glycated and unglycated forms of each antibody was determined at antigen concentrations of 10, 20, 40, 80 and 160 nM, by continuous monitoring of SPR.

In order to rule out the possibility that the value of k_{diss} is significantly affected by dissociation of MoAb from the capture antibody, the following controls were carried out. RamFc or RamG1 capture antibody was immobilized on the sensor chip CM5 and MoAb, diluted 1:10 in HBS pH 7·4, was allowed to

Table 1. The degree of glycation of whole mouse IgG incubated in varying concentrations of glucose

Conc. glucose (mM) ($n = 3$)	Moles Amadori product per mole protein	Range
0	1·2	0·9–1·5
15	1·8	1·7–1·9
50	2·6	2·3–2·8
500	6·0	5·7–6·3

Whole mouse IgG was glycated under similar conditions to those used for glycation of MoAbs, in order to determine the approximate levels of MoAb glycation. Whole mouse IgG (0·75 mg/ml) was incubated for 21 days with mouse albumin (7·5 mg/ml) in 0·1 M phosphate pH 7·4 at 37°C. Glucose concentrations were 0, 15, 50 and 500 mM. The IgG was then affinity purified and free sugar removed. The mean and range of the number of moles of AP per mole of purified IgG is shown, as determined by colorimetry after periodate oxidation.

Table 2. The association and dissociation constants of glycated and unglycated MoAbs as determined by surface plasmon resonance (SPR) measurements

MoAb against	k_{ass} ($\times 10^4$/MS)	k_{diss} ($\times 10^5$/s)	K_d ($\times 10^{-9}$M)
Human IgG (Fab)			
Unglycated	2·3	6·6 (5·4–5·9)	2·9
Glycated	2·5	10·0 (10·0–11·0)	4·0
β-galactosidase			
Unglycated	1·4	2·1 (0·9–2·9)	1·5
Glycated	1·2	5·4 (5·0–5·7)	4·5
Avidin			
Unglycated	2·6	3·4 (1·9–5·8)	1·3
Glycated	2·3	4·7 (4·6–5·9)	2·0

The k_{ass} and k_{diss} of glycated and unglycated forms of three MoAbs are shown, as determined by surface plasmon resonance (SPR) measurements. The values for k_{ass} (column 1) show the regression line obtained from four to five determinations of k_s using antigen in the range 10–160 nM. The values for k_{diss} (column 2) are the mean of five determinations with the range shown in parentheses. The values for K_d (column 3) are calculated from the ratio k_{diss}/k_{ass}. These values will approximate the theoretical K_d, since the conditions used were close to equilibrium.

bind. Buffer flowed at 2 μl/min and any dissociation of the MoAb was monitored for 300 s. During this period, a fall in SPR response of 3–5 RU was observed for all three MoAbs. This corresponded to < 5% of the SPR response observed when antigen dissociated from the antigen–antibody complex during the kinetic determinations, and was therefore considered negligible.

The association rate constant (k_{ass}) was determined by linear regression of k_s against antigen concentration using the BIAlogue software, the value of k_{ass} being equal to the regression coefficient. Table 2 shows the values of k_{ass} for the glycated and unglycated forms of each of the three MoAbs. The k_{ass} for glycated antibody did not vary significantly from that of unglycated antibody for any of the three MoAbs ($P > 0.05$).

The rate of dissociation of the antibody–antigen complex (k_{diss}) for the glycated and unglycated forms of each of the three MoAbs was determined with continuous removal of free antigen by the buffer flow. The dissociation rate constant (k_{diss}) was determined using the BIAlogue software by linear regression of ln (B_n/B_0) against $t_n - t_0$; where B_0 = initial SPR response (at time t_0) and B_n = SPR response after t_n seconds; the value of k_{diss} being equal to the regression coefficient. Table 2 shows the values of k_{diss} for the glycated and unglycated forms of each of the three MoAbs. In each case the k_{diss} of glycated antibody was significantly higher than for unglycated antibody ($P < 0{\cdot}05$, $n = 4$). Therefore, for all three MoAbs the rate of association of the antigen–antibody complex was found to be unaffected by glycation, whilst the rate of dissociation increased significantly.

The effect of glycation on the rate of dissociation using radiolabelled antigen

In order to confirm the findings of the SPR measurements, that glycation significantly increases the rate of dissociation of the antigen–antibody complex, an alternative method of measuring k_{diss} using radiolabelled antigen was used.

Mouse MoAb (ascites) against human IgG (Fab) was incubated for approximately 21 days with 0·5 M glucose at 37°C. Control antibody was incubated under identical conditions but with no added glucose. Glycated or unglycated control antibody was bound to the wells of a microtitre plate and the rate of dissociation of radiolabelled antigen from each well measured, in the presence of an excess of unlabelled antigen. Because unlabelled antigen was in 50-fold excess with respect to radiolabelled antigen, dissociation could be measured in the absence of any significant rebinding of radiolabelled antigen. Under these conditions a good approximation of k_{diss} can be obtained.

The value of k_{diss} was obtained using the same mathematical model as used in the BIAlogue software for calculating k_{diss} from SPR data, i.e. k_{diss} is equal to the regression coefficient of ln (B_n/B_0) against $t_n - t_0$; where B_0 = radioactivity bound to control wells (at time t_0) and B_n = bound radioactivity after t_n seconds. The k_{diss} (mean ± 1 s.e.m., $n = 8$) of glycated MoAb incubated for 21 days with 0·5 M glucose was significantly higher at $9{\cdot}6 \times 10^{-5}$/s (± 0·5) compared with $4{\cdot}0 \times 10^{-5}$/s (± 0·5) for incubated control MoAb ($P < 0{\cdot}001$, $n = 8$). The k_{diss} of untreated MoAb was $3{\cdot}6 \times 10^{-5}$/s ($n = 4$), therefore no significant difference between incubated and untreated MoAb was observed. The results obtained using radiolabelled antigen confirmed the findings of the SPR measurements, that glycation significantly increases the rate of dissociation of the antigen–antibody complex.

The effect of glycation on the dissociation constant (K_d)

If glycation significantly increases k_{diss} but does not affect k_{ass}, as is suggested by the SPR data and the radiolabelled antigen experiments, a significant increase in the equilibrium dissociation constant (K_d) should be apparent after glycation, since K_d is equal to the ratio k_{diss}/k_{ass} at equilibrium. We therefore measured the K_d of the anti-human IgG (Fab) MoAb, glycated under identical conditions to those used for measuring the kinetic rate constants, using the ELISA-based method of Friguet *et al.* [21].

Figure 1 shows the effect of various incubation conditions on the measurement of K_d. The K_d of MoAb glycated with 0·5 M glucose in 0·1 M phosphate pH 7·4 was more than three-fold higher than unglycated control antibody incubated under identical conditions but with no added glucose ($P < 0{\cdot}001$, $n = 6$). The K_d of MoAb glycated under the same conditions but with 50 mM glucose instead of 0·5 M glucose, was also significantly higher than control MoAb ($P < 0{\cdot}05$, $n = 5$). Lowering the phosphate concentration from 0·1 M to 15 mM, while keeping glucose at 0·5 M, did not significantly affect the K_d ($P > 0{\cdot}1$, $n = 6$). As an additional control, MoAb was incubated with 0·5 M α-methyl glucose instead of glucose to rule out the possibility that changes in K_d were as a consequence of the higher solute concentration in those buffers

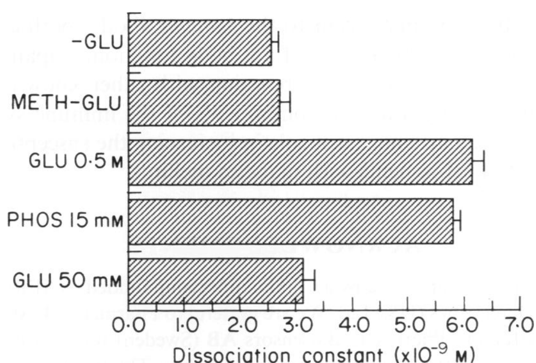

Fig. 1. The K_d of anti-human IgG MoAb was determined using an ELISA-based method. The figure shows the mean K_d (± 1 s.e.m., $n = 6$) of the MoAb after 21 days incubation in phosphate buffer pH 7·4 at 37°C. Incubation conditions were as follows; with no glucose added and 0·1 M phosphate (−GLU), with 50 mM glucose and 0·1 M phosphate (GLU 50 mM), with 0·5 M glucose and 0·1 M phosphate (GLU 0·5 M), with 0·5 M glucose and 15 mM phosphate (PHOS 15 mM), and with 0·5 M α-methyl glucose and 0·1 M phosphate (METH–GLU).

containing glucose. The K_d of the unglycated control antibody did not differ significantly from the K_d of the MoAb incubated in α-methyl glucose ($P > 0{\cdot}05$, $n = 5$).

The effect of glycation on the K_d of MoAb incubated with and without continuous exposure to 15 mM glucose was also investigated. Glycated MoAb was incubated as previously described for 21 days with 15 mM glucose and the K_d determined. During the first stage of the ELISA affinity method (see Materials and Methods), when the MoAb was diluted 1:17 000 in PBS-T, mixed with antigen and incubated overnight, glucose was added aseptically so that the concentration remained at 15 mM, thereby ensuring that no net dissociation of SB occurred. The unglycated MoAb was treated identically but with no glucose added during the 21-day incubation and no glucose added to the MoAb when it was diluted 1:17 000 and mixed with antigen. However, 15 mM glucose was added to the antigen–antibody mixture immediately before adding the mixture to an antigen-coated plate, to rule out the possibility that the presence of 15 mM glucose in the wells alters K_d rather than the extent of MoAb glycation. The mean K_d ($n = 5$) of glycated MoAb continually exposed to 15 mM glucose was significantly higher at $4{\cdot}0 \times 10^{-9}$/M (±0·12) compared with $3{\cdot}4 \times 10^{-9}$/M (± 0·11) for incubated control MoAb ($P < 0{\cdot}02$, $n = 5$).

DISCUSSION

The SPR measurements show that the k_{diss} of three different mouse MoAbs glycated in 0·5 M glucose was significantly higher than the k_{diss} of the same MoAbs treated identically but with no glucose added (see Table 2). However, for all three MoAbs the k_{ass} of glycated MoAb was not significantly different from unglycated MoAb (see Table 2). These data are consistent with the theory that glycation of antibody destabilizes the antigen–antibody complex. We postulate that the fall in pK_a of amino groups within the antibody due to glycation of those groups, or conformational changes occurring as a consequence of the binding of sugar residues, increases the repulsion between the antibody-bound antigen and the MoAb, thereby increasing k_{diss}.

To validate the SPR method for determining k_{diss}, we took glycated and unglycated forms of the anti-human IgG (Fab) MoAb, prepared exactly as described for use in the SPR experiments, immobilized both forms on microtitre plates and measured the dissociation of bound ^{125}I-labelled antigen in the presence of an excess of unlabelled antigen. Under these conditions we were able to determine the k_{diss} of both glycated and unglycated MoAb. We found the k_{diss} (mean ± 1 s.e.m., $n = 7$) of glycated MoAb to be $9{\cdot}6 \times 10^{-5}$/s (± 0·5) compared with $4{\cdot}0 \times 10^{-5}$/s (± 0·4) for unglycated control MoAb. These values compare well with the values for k_{diss} obtained by measuring SPR, which were $10{\cdot}0 \times 10^{-5}$/s for glycated MoAb and $6{\cdot}6 \times 10^{-5}$/s for the unglycated form (see Table 2, column 2). Thus, glycation of the anti-human IgG (Fab) MoAb resulted in a significant increase in k_{diss}.

If the k_{diss} of the glycated form of MoAb is significantly higher than the unglycated form, whilst the k_{ass} does not differ, this implies that the equilibrium dissociation constant (K_d) of glycated MoAb is significantly higher than unglycated MoAb. An approximation of the equilibrium constant K_d (net dissociation at equilibrium) can be obtained from the SPR measurements by calculating the ratio k_{diss}/k_{ass} (see Table 2, column 3), since the conditions under which k_{diss} and k_{ass} were measured are close to equilibrium conditions. The values of K_d obtained in this way for glycated and unglycated MoAb against human IgG (Fab) were approximately $4{\cdot}0 \times 10^{-9}$ M and $2{\cdot}9 \times 10^{-9}$ M, respectively. The K_d of the same MoAb was measured directly using the ELISA affinity assay of Friguet *et al.* [21], the data obtained being analysed using the method of Stevens [23]. Using the ELISA method, the value for K_d (mean ± 1 s.e.m., $n = 6$) of glycated MoAb was found to be $6{\cdot}2 \times 10^{-9}$ M (± 0·2) and the value for unglycated MoAb $2{\cdot}6 \times 10^{-9}$ M (± 0·2) (see Fig. 1). When MoAb was glycated in 15 mM glucose, the increase in K_d (mean ± 1 s.e.m., $n = 5$) was smaller but still significantly higher than for unglycated MoAb, increasing from $3{\cdot}4 \times 10^{-9}$ M (± 0·1) to $4{\cdot}0 \times 10^{-9}$ M (± 0·1) ($P < 0{\cdot}02$).

Our finding that the K_d of glycated MoAb is significantly higher than that of unglycated MoAb means that glycation of MoAb decreases the affinity of the antibody for its antigen, since the equilibrium affinity constant (K_a) is the reciprocal of K_d. This is in apparent contradiction to the findings of Morin *et al.*, who could find no evidence that glycation of antibodies significantly impaired their ability to bind antigen [8]. Morin *et al.* glycated several different antibodies used commercially in clinical assays and compared the calibration curves obtained with standard samples using either glycated or unglycated antibody. However, assessing antibody function by determining its performance in a commercial immunoassay does not reveal as much information about the interaction as measuring the kinetics and affinity parameters, and can sometimes be misleading. In a commercial assay, antibody is much in excess and sensitive changes in affinity may not be apparent. Also, antibodies in commercial assays are selected to have a relatively high k_{ass} and relatively low k_{diss}, since these are the important properties for antibodies to be used in an immunoassay. Therefore, subtle changes in k_{diss} may not be apparent. The assays we have used, which measure both the kinetic and affinity constants, are much more sensitive to small changes in k_{diss}.

Dolhofer *et al.* examined the functional properties of both the Fab region and the Fc region [13]. While they found

glycation significantly impaired Fc functions such as complement binding and binding to Protein A, they could find no evidence, using the Farr assay [24], that glycation significantly altered the affinity or concentration of a polyclonal rabbit antibody against human albumin. However, they did not rule out the possibility that antibodies with certain primary sequences of amino acids in the Fab region could be affected by glycation.

It is our view that as polyclonal antibodies are a mixed population of antibodies, some with high affinity and some with low affinity, the presence of a high-affinity antibody whose affinity is not impaired by glycation may mask the change in affinity of a low-affinity antibody that is affected by glycation, even if the low-affinity antibody is present at a higher titre. This view is supported by the work of Stanley *et al.* [25], who demonstrated that even a low proportion of high-affinity antibody had a marked effect on measurements of both the concentration and affinity of a predominant low-affinity antibody. Therefore, any investigation into the effect of glycation on the concentration and affinity of antibody which involves the use of a polyclonal antibody may result in misleading results, since the impairment of a high concentration but low-affinity antibody cannot be reliably measured in the presence of a high-affinity antibody that is not significantly impaired. For this reason, we have chosen to investigate the effect of glycation on the kinetics and affinity of MoAbs.

Recently, Sasaski *et al.* demonstrated that glycation of a mouse MoAb against UV-damaged DNA significantly reduced the relative binding capacity of the glycated antibody compared with unglycated antibody in an ELISA, although no kinetics or affinity parameters were measured [14]. Our results, in combination with those of Sasaski *et al.*, significantly strengthen the view that glycation of MoAbs can result in a demonstrable impairment of Fab function, and we have shown that k_{diss} and K_d are significantly increased even at relatively low levels of glucose (15 mM). Normal plasma glucose levels, in adults < 60 years, are typically between 3·9 and 5·8 mM [26], whereas significantly higher levels can be found in diabetics. A random selection of 137 diabetics, whose plasma glucose was determined at Nottingham City Hospital, were found to have a mean (±1 s.d.) plasma glucose of 13·2 mM (± 6·1). Of the 137 diabetics, 36% had a plasma glucose level of > 15 mM and 10% had a level > 20 mM.

We have found that glycation of different MoAbs alters k_{diss} by varying amounts, which may be as a consequence of variability in the primary structure of antibodies. It may be necessary for glycation of key amino acids within the antibody binding region to occur before K_d is significantly increased. We cannot therefore rule out the possibility that some MoAbs will be unaffected by glycation due to a lack of glycatable residues lying within the binding site. Significantly, however, all three MoAbs that we have investigated show significant differences in k_{diss} between glycated and unglycated forms. A larger number of antibodies will need to be investigated in order to determine how widespread this phenomenon is.

Our finding that the Fab region of antibody can be impaired by glycation only partially explains why complement fixation of glycated antibody is so markedly impaired as demonstrated by Dolhofer *et al.* [13]. It is our view that impairment of both Fab and Fc function may occur to the same antibody, both effects contributing to impairment of antibody function. Impairment of antibody function in combination with other known consequences of glycation on components of the immune system [27] may play an important role in increasing the susceptibility to infection of patients with diabetes mellitus.

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