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Glycation increases the vascular clearance rate of IgG in mice

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SUMMARY

As elevated levels of glycated IgG have been detected in the plasma of diabetics we have investigated whether glycation of IgG affects its vascular clearance rate, using a mouse model system. Polyclonal mouse IgG was aseptically incubated for 14–19 days with 0·5 M glucose in 0·1 M phosphate buffer (pH 7·4) at 37°C. As control, IgG was incubated under identical conditions but with no added glucose. After incubation, both forms were labelled with ^{125}I and injected intravenously into BALB/c mice. The rate of vascular clearance of the glycated IgG was found to be significantly higher than the control IgG in the periods 5–24 h ($P < 0{\cdot}001$, $n = 6$) and 24–48 h ($P < 0{\cdot}01$, $n = 6$) after injection. After 2–3 days the mice were killed and the major organs were harvested. With glycated IgG there was a significant increase in the ^{125}I accumulated in the kidney ($P < 0{\cdot}02$). In later experiments, dual labelling with ^{131}I and ^{125}I allowed mixtures of glycated and unglycated IgG to be injected into the same mouse so that the vascular clearance of both forms of IgG could be followed simultaneously. These experiments confirmed that glycation of the IgG significantly increases its vascular clearance rate.

Keywords glycation vascular clearance IgG iodination diabetes mellitus

INTRODUCTION

Since a correlation between the prevalence of infection in diabetic patients and their mean plasma glucose level was observed in a large study of diabetics by Rayfield *et al.* [1], the consensus view has been that poorly controlled diabetics are predisposed to infection [2]. The underlying mechanism is yet to be established, but the effect of increased glycation of vital components of the immune system, as a result of prolonged hyperglycaemia, is thought to play a major role.

Glycation is the spontaneous reaction of reducing sugars with amino groups which occurs *in vivo* [3] and is quite distinct from glycosylation which is under the control of specific glycosyl transferase enzymes. Glycation is a non-enzymic process with sugar molecules becoming covalently attached to amino groups on lysine residues or the N-terminal amino acids of proteins so as to form a labile Schiff base [4]. Over a period of days a proportion of the Schiff base undergoes rearrangement to form a much more stable sugar adduct known as an Amadori product (AP) [5], and in later stages of glycation, cross-linking of proteins occurs which leads to the formation of advanced glycosylation end-products (AGE-proteins) [6].

The presence of increased amounts of glycated IgG in the serum of diabetics is well established [7–10]; the mean per cent glycation of IgG ranges from 6·6% to 9·3% for non-diabetics and 14·6% to 21·6% for diabetics. Other immunoglobulins such as IgA and IgM also show increased levels of glycation [11].

Previous *in vitro* studies have linked glycation with structural and functional changes to IgG. For example, glycation significantly decreases the ability of IgG to fix complement [7,12] and glycated IgG has been shown to have impaired binding to Protein A [12]. High molecular weight aggregates of IgG have also been detected after 22 days glycation, suggesting that AGE formation is occurring [13]. Furthermore, it has been demonstrated that mouse macrophages possess receptors with a high affinity for AGE-proteins, thereby providing an *in vivo* mechanism for their removal [14]. If the rate of clearance of immunoglobulins is increased as a result of glycation this may contribute to a compromised immune response in the diabetic individual.

The purpose of this study was to use a mouse model system to study the effect of *in vitro* glycation of IgG on its vascular clearance. Our results suggest that glycation significantly increases the vascular clearance, and we discuss the significance of these findings in relation to those with diabetes mellitus.

MATERIALS AND METHODS

Commercially purified polyclonal mouse IgG was purchased from Sigma Chemical Co. (Poole, UK). Glucose was purchased from Sigma and other reagents from BDH (Poole, UK). All reagents were of reagent grade. Iodination was carried out using Iodogen from Pierce and Warriner (UK) Ltd. (Chester, UK)

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with ^{125}I from ICN Biomedicals Ltd. (Irvine, UK) and ^{131}I from Dupont (UK) Ltd. (Stevenage, UK). PD-10 gel filtration columns were purchased from Pharmacia (Milton Keynes, UK). Male BALB/c mice were obtained from the Comparative Biology Centre (University of Newcastle upon Tyne, UK). The age range of the mice was 9–16 weeks and the weight (mean ± 1 s.d.) was 28·5 ± 2·2 g. Groups of mice within each experiment were of similar age and size. Heparinized haemacrit centrifuge tubes (Gelman-Hawkesley, Lancing, UK) were used for blood collection. Radioactivity was measured using an Intertechnique CG-4000 Gamma counter (Kontron Instruments Ltd., Watford, UK).

Glycation

Polyclonal mouse IgG was incubated for 14–19 days with and without 0·5 M glucose in 0·1 M phosphate buffer (pH 7·4) at 37°C. At all stages sterile procedures were used and the solutions were sterilized by filtration through a 0·22 μm filter. The extent of glycation of the IgG was determined by a periodate assay for glycoproteins developed by us [15]. The extent of glycation for the unglycated form of IgG was within the range 1·65–1·74 moles of Amadori product/mole of IgG, increasing to 6·2–6·6 moles of Amadori product/mole of IgG for the glycated form.

Protein labelling

Labelling of the IgG with ^{131}I and ^{125}I was carried out using the Iodogen method [16]. 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 the vials with 0·1 M phosphate buffer (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 the free iodine. Fractions were eluted with PBS at pH 7·4 and analysed by SDS-PAGE electrophoresis. Autoradiography revealed that no significant damage had occurred to the IgG (data not shown). Fractions containing protein were pooled and diluted in PBS (pH 7·4) to 1×10^7 ct/ min per ml.

Initially, both glycated and unglycated forms of mouse IgG were labelled with ^{125}I and injected into two groups of BALB/c mice. To confirm the results from these experiments, dual labelling with ^{131}I and ^{125}I was used so that simultaneous vascular clearance of glycated and unglycated IgG could be followed in the same mouse. The mice were injected with a mixture of glycated and unglycated IgG in approximately equal proportions. In one group the glycated IgG was labelled with ^{125}I and the unglycated IgG labelled with ^{131}I. In the other group the labels were reversed.

Injection of IgG

Radiolabelled IgG was administered by injection into the tail vein. Injection volume was 100 μl and a maximum of 1×10^6 ct/ min was injected into each mouse.

Vascular clearance rate measurements

Samples of blood (approximately 25 μl) were taken from the tail vein into a heparinized haemacrit centrifuge tube. The blood was immediately transferred to previously weighed polystyrene tubes which were then sealed. After weighing to determine the mass of blood, the insides of the tubes were washed with 500 μl of PBS and the radioactivity in each tube measured.

Measurement of organ uptake of radiolabel

Mice were killed 2–3 days after injection with radiolabel by cervical dislocation. The major organs in the abdomen and chest cavity were removed, weighed wet after blotting and the radioactivity in each organ determined.

Radioactivity measurements

In the single-label experiments and organ distribution study ^{125}I was measured using a preset 'gross' window counting over the entire isotope spectrum. For dual labelling both isotopes were counted using preset windows at the peak energies of each isotope (35/65 KeV for ^{125}I and 364 KeV for ^{131}I). ^{125}I counts were adjusted to account for ^{131}I counts appearing within the ^{125}I window. Negligible ^{125}I counts were found within the ^{131}I window. ^{131}I counts were also adjusted to account for decay during the experiment assuming a half-life of 8·07 days.

Statistical analysis

Vascular clearance rates for the two different forms of IgG and for each label were compared by calculating the difference between the means of each sample using an unpaired *t*-test. Because of the small sample sizes, a combined standard deviation was calculated for the *t*-test.

RESULTS

The effect of glycation on IgG vascular clearance using ^{125}I labelling

Groups of six mice were injected with either glycated or unglycated IgG. Both forms of IgG were labelled with ^{125}I. The aim was to investigate whether glycation altered the vascular clearance rate. Blood samples were removed at 5–10 min ($t=0$) and 2, 4, 6, 24 and 48 h after injection. Radioactivity was expressed as ct/min per mg of whole blood. Figure 1 shows the ct/min per mg for each sample as a percentage of the $t=0$ value. In common with a previous clearance study in rats [17], an initial phase of rapid vascular clearance up to 6 h after injection was observed due to distribution of the radiolabelled IgG throughout the tissues. This was followed by a less rapid second phase.

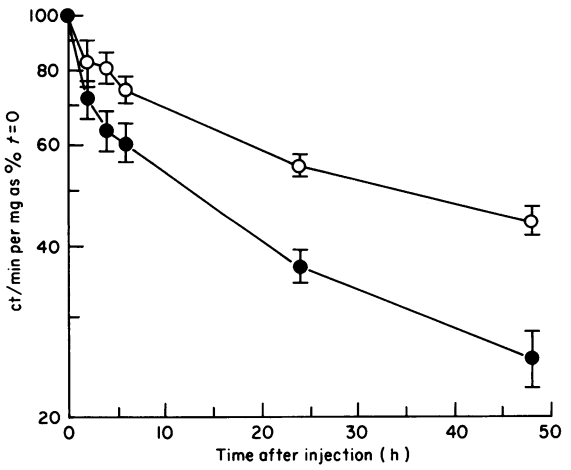

Fig. 1. The amount of radioactivity per mg of whole blood over time, up to 48 h after injection. The values are expressed as a percentage of the $t=0$ values for both glycated (●) and unglycated IgG (○). Points shown are obtained from the mean of six animals and bars show ±1 s.e.m.

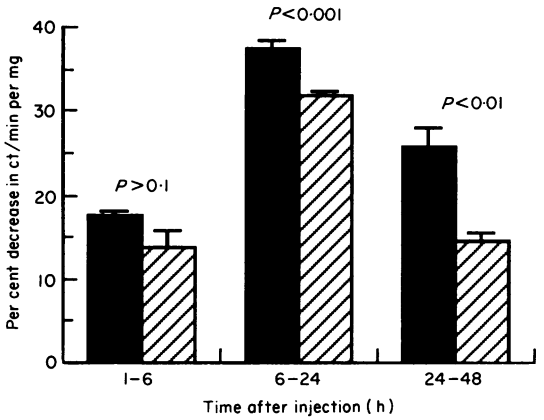

Fig. 2. The percentage decrease in radioactivity per mg of blood over the periods 1–6 h, 6–24 h and 24–48 h after injection with either glycated (■) or unglycated radiolabelled IgG (■). Figures shown are obtained from the mean of six animals, with bars showing ±1 s.e.m.

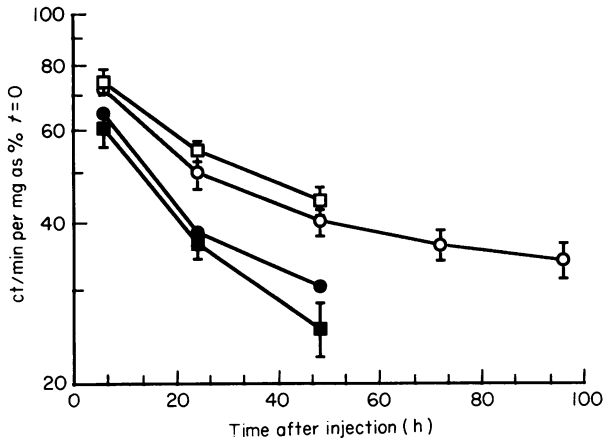

Fig. 3. Comparison of the results obtained from similar clearance experiments but using different batches of antibody and mice. For one batch of mice data were obtained for unglycated IgG up to 96 h. Points were obtained using the mean from six mice, bars showing ±1 s.e.m. ○, Unglycated IgG (1); □, unglycated IgG (2); ●, glycated IgG (1); ■, glycated IgG (2).

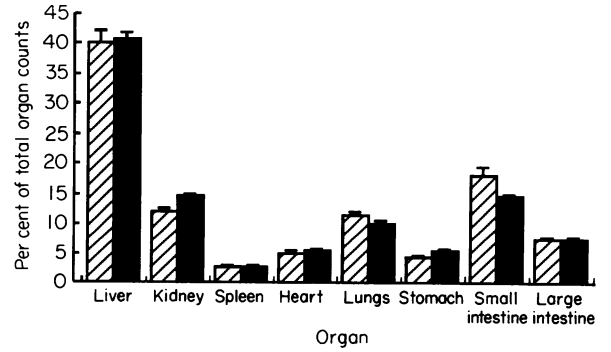

Fig. 4. The amount of radioactivity accumulated in each organ as a percentage of the total radioactivity accumulated in all the organs 48 h after injection with either glycated (■) or unglycated radiolabelled IgG (■). Figures are obtained from the means of six mice, with bars showing ±1 s.e.m.

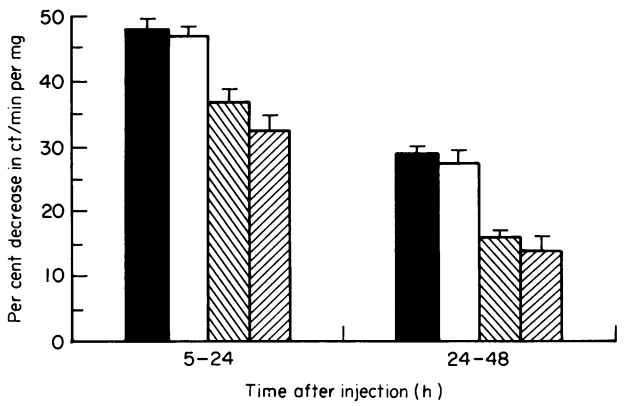

Fig. 5. The percentage decrease in radioactivity per mg of blood over the periods 6–24 h and 24–48 h after injection with either glycated or unglycated radiolabelled IgG. A mixture of glycated and unglycated IgG was injected into each mouse so that clearance of both forms could be followed in the same mouse. Figures shown are obtained from the mean of six animals, with bars showing ±1 s.e.m. ■, Unglycated (^{125}I); ▨, unglycated (^{131}I); □, glycated (^{125}I); ■, glycated (^{131}I).

After 24 h the rate began to fall as background levels were reached. Using these data we have calculated the mean decrease (±1 s.e.m.) in counts over each of three periods: 1–6 h, 6–24 h and 24–48 h after injection (see Fig. 2). From this we found that the vascular clearance rate of glycated IgG was significantly higher than that of unglycated IgG in the second ($P<0·001$, $n=6$) and third ($P<0·01$, $n=6$) periods. Figure 3 shows results from two single labelling experiments carried out using a different batch of IgG and different batches of mice. Data were obtained for unglycated IgG up to 96 h on one occasion only.

Distribution of counts in major organs 48 h after injection

The two groups of six mice from the single labelling vascular clearance study were killed 48 h after injection and the total counts per organ calculated as a percentage of the total radioactivity incorporated into all the organs (see Fig. 4). The overall distribution was similar in both groups, but with glycated IgG significantly more of the label was recovered in the kidneys ($P<0·02$).

The effect of glycation on IgG vascular clearance using dual labelling of glycated and unglycated IgG

Two groups of mice ($n=8$ and $n=9$) were injected with a mixture of glycated and unglycated IgG. In one group the glycated IgG was labelled with ^{131}I and unglycated with ^{125}I. In the other group the labels were reversed. Figure 5 shows the vascular clearance rates for each labelled form of IgG. For both groups of mice the vascular clearance rates of glycated IgG were significantly higher than those of unglycated IgG over the periods 5–24 h ($P<0·001$) and 24–48 h ($P<0·001$). For one group the vascular clearance rate for glycated IgG in the period 1–5 h was also significantly increased ($P<0·001$). Reversing the isotope used for protein labelling had no effect on its vascular clearance rate.

DISCUSSION

The results from the single-labelling experiments show that the vascular clearance rate, or rate of disappearance of radio-

labelled IgG from the blood, was significantly higher in mice injected with glycated IgG (see Figs 1 and 2). The mean t_{50} for unglycated IgG was calculated as 34 h and for glycated IgG 13 h. These figures represent the time taken for 50% of the radiolabel to disappear from the blood, and includes IgG taken up into the extravascular pool. The mean t_{50} of 34 h for unglycated IgG is significantly higher than the value of 14–20 h found by Eccles *et al.* [17] for unglycated rat IgG, suggesting a different rate of vascular clearance for the two species.

The amount of radiolabel incorporated into the major organs was also measured (see Fig. 4). Radiolabel may become incorporated into the tissue as a result of uptake or deposition, or alternatively as a consequence of degradation of the radiolabelled IgG and subsequent reincorporation of the radiolabel into other proteins. In addition, we have calculated the ratio between the amount of radioactivity in the blood shortly after injection ($t=0$) and the total amount of radiolabel incorporated into the organs after 48 h. This ratio (mean $\pm$ 1 s.d.) was found to be 0·073 ($\pm$0·013) for unglycated IgG and 0·041 ($\pm$0·009) for glycated IgG. Thus, a significantly smaller proportion of radiolabel becomes incorporated into the organs when glycated IgG is injected than is the case for unglycated IgG ($P<0·001$). Because the amount of radiolabel at 48 h after injection in both the major organs and the blood is lower for glycated IgG than for unglycated IgG, this evidence suggests that for glycated IgG the radiolabel is cleared more quickly from the body in the 48 h after injection.

Further studies are necessary to explain by what mechanism the glycated IgG is removed from the blood, although one possibility is that it is taken up by the reticuloendothelial system, as has been suggested for denatured and aggregated antibodies [18,19]. Macrophages are also known to have specific receptors for the uptake of highly glycated proteins [14].

Previous studies on the kinetics of disappearance and tissue distribution of IgG in rats and mice have shown that, after i.v. injection of radiolabelled IgG, initial clearance is rapid, with 30% or more of the administered antibody being removed from the intravascular space within 24 h [17–19]. Our results in Fig. 1 also show that 30–40% of the injected radiolabel of unglycated antibody disappears within the first 6 h, and this is comparable to the rapid initial clearance phase of 2–4 h IgG observed by Eccles *et al.* in rats [17].

This initial rapid clearance phase was followed by a second phase such as seen in previous studies, which all used the rate of clearance after 24–48 h after injection, i.e. in the second phase, to calculate the half-life of the IgG. However, IgG which is aggregated or damaged, such as has been observed to occur when IgG is glycated *in vitro* [13], is likely to be cleared in the first 24–48 h [18,19]. Since previous studies have not published data for the clearance of mouse IgG with time points in the first 24–48 h, this made comparison of our results with those of other groups difficult. Therefore, to aid comparison, we obtained clearance data up to 96 h for unglycated IgG (see Fig. 3) and calculated the half-life of the IgG using the same method as Pollock *et al.* [18]. The half-life of polyclonal mouse IgG (mean $\pm$1 s.e.m., $n=6$) was calculated as 156·4$\pm$19·5 h, which compares well with half-lives within the range 120·8–228·3 h obtained by Pollock *et al.* for mouse MoAbs [18].

In order to ensure that the different vascular clearance rates were due to differences in the level of glycation, and not differences between batches of animals, dual labelling was employed so that the vascular clearance of both glycated and unglycated IgG could be followed in the same animal (see Fig. 5). The vascular clearance rates obtained were similar to those in single-labelling experiments, and confirmed that glycation significantly increases the vascular clearance rate of IgG. By using two groups of mice, one with glycated IgG labelled with ^{125}I and unglycated with ^{131}I and the other group with the labels reversed, the possibility that differences in the vascular clearance rate were due to differences in the labelling procedure or the label used was discounted.

The subsequent distribution of radiolabel in the organs was similar with both glycated and unglycated IgG (see Fig. 4). However, with glycated IgG there was a significant increase in the radiolabel accumulated in the kidney, which may be the result of deposition of glycated IgG on the basement membrane, which has been shown to occur in diabetics [20]. Any increased uptake of glycated IgG by the kidney will contribute to the increase in vascular clearance rate of glycated IgG, but is insufficient to explain the total increase.

We have yet to identify the mechanism by which glycated IgG is cleared from the vascular system at a greater rate than the unglycated form. However, a potential mechanism for the removal of long-term glycated proteins (AGE-proteins) has been proposed by Vlassara *et al.* [14]. High-affinity receptors for AGE-proteins have been identified on the surface of mouse macrophages. If these receptors are responsible for the removal of senescent proteins, they may be the route by which glycated proteins are removed from the vascular circulation.

We have found that glycation of IgG significantly increases its vascular clearance rate. This process, in combination with other known effects of glycation on IgG function such as impaired antigen binding and complement-fixing activity [12,21], suggests that IgG may be functionally compromised in diabetics. Such functional impairment of IgG by glycation may play a significant role in increasing the prevalence of infection amongst diabetics.

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