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IgG glycation and function during continuous ambulatory peritoneal dialysis.

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Chapter 5

ABSTRACT

IgG in dialysate may have an important role in anti-infection mechanisms during continuous ambulatory peritoneal dialysis (CAPD). As Fc fragment oligosaccharidic chains are crucial for IgG effector functions, we have tested the hypothesis that IgG glycation might occur during CAPD and modify IgG properties.

Purified normal IgG was incubated with glucose solutions of different concentrations and pH. Separation of glycated IgG was performed by affinity chromatography. Complement activation (C3c deposition) and phagocytosis by polymorphonuclear leukocytes (PMN) were studied in vitro using Staphylococcus aureus Wood (STAW) as antigen. In addition, we compared the percentages of glycated IgG in IgG purified from sera and dialysates of 12 CAPD patients.

The percentage of glycated IgG after in vitro incubation of normal IgG with glucose solutions was directly proportional to glucose concentrations, incubation time and pH. Glycated IgG anti-STAW induced a higher C3c deposition than non-glycated IgG anti-STAW (C3c/IgG (mean ± SD) 0.96 ± 0.06 vs 0.79 ± 0.08; p=0.027). PMN phagocytosis was not affected by IgG glycation. The percentages of glycated IgG in dialysates of CAPD patients were greater than those in corresponding sera (5.38 ± 2.36% vs 4.56 ± 2.47%; p=0.006).

It is concluded that IgG glycation may take place in the peritoneal cavity during CAPD and lead to enhanced complement activation. This could explain the high degree of complement activation previously described in dialysate of CAPD patients and might theoretically result in a reduction of complement factors available in dialysate for adequate anti-infection mechanisms.
INTRODUCTION

The role of humoral immunity in the local anti-infection defence mechanism during continuous ambulatory peritoneal dialysis (CAPD) is still not elucidated. Some studies reported a relationship between a high peritonitis incidence and low IgG concentrations in effluent of overnight dialysis dwells [1,2] but such a relationship could not be confirmed by other groups [3-5]. IgG molecules contain polysaccharidic chains which play an important role in the Fc fragment functions such as IgG plasmatic clearance by the liver asialoprotein receptors [6], complement activation [7] and IgG binding to Fc receptors [8]. As glucose is still the more commonly used osmotic agent in peritoneal dialysis and as prolonged contact between glucose and proteins can lead to non-enzymatic glycation of the latter [9], we tested in the present study the hypothesis according to which the use of glucose in CAPD could induce the formation of glycated IgG and modify IgG ability to activate complement and promote phagocytosis in dialysate. Studies were performed in vitro and in patients.

PATIENTS AND METHODS

Twelve stable adult CAPD patients aged from 18 to 57 years were included in the study. They were treated with glucose-containing dialysis solutions (Dianeal, Baxter BV, Utrecht, The Netherlands) for overnight exchange. The highest dialysate glucose concentration commercially available (3.86%, 0.21M) was chosen according to in vitro experiments showing that increased IgG glycation is more likely to occur when IgGs are incubated with solutions containing high glucose concentrations. Patients had not experienced a peritonitis episode for at least 4 weeks before the study. Five diabetic patients not treated with CAPD and 5 healthy adult volunteers were used as controls.

Blood samples and dialysates

Dialysates from night dwells (7-11 hours) were collected and immediately stored at -20°C until determinations. Sera from patients with agammaglobulinaemia (and with normal haemolytic complement activity) were used as source of complement. All agammaglobulinemic samples were stored at -70°C and thawed at 4°C just before use. Other serum samples were flash-frozen and stored at -70°C. Serum samples and corresponding night dwells were collected on the same day.

Materials

HRP-conjugated rabbit anti-human IgG and HRP-conjugated anti-human C3c were purchased from Dako, Glostrup, Denmark. Unencapsulated protein A-deficient Staphylococcus aureus Wood (STAW) bacteria were kindly provided by L. van Alphen (Department of Microbiology, University of Amsterdam, Amsterdam, The Netherlands). STAW was cultured in brain - heart infusion broth containing hematin and NAD+. Bacteria were harvested in log phase, washed three times with phosphate-buffered saline (PBS) (140 mmol/L NaCl, 9.2 mmol/L
Na$_2$HPO$_4$, 1.3 mmol/L NaH$_2$PO$_4$; pH 7.4), and resuspended in coating buffer (0.05 mol/L NaHCO$_3$, pH 9.6). Phosphate buffer containing Ca$^{2+}$ and Mg$^{2+}$ (PiCM buffer; pH 7.2 - 7.4) consisted of 137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na$_2$HPO$_4$, 1.5 mmol/L KH$_2$PO$_4$, 1.0 mmol/L MgCl$_2$, 0.6 mmol/L CaCl$_2$, 1 % (w/v) glucose (all from Merck, Shuchardt, Hohenbrunn, Germany), and 2.5 % (v/v) human serum albumin (from CLB, Amsterdam, The Netherlands). Tetramethyl-benzidine was purchased from Sigma, St. Louis, Mo. Tween 20, NaHCO$_3$, citrate, and Na$_2$HPO$_4$ were also from Merck. Flat bottom, 96-well microtiter plates (Immunolo, M129A) were from Greiner, Kloten, Switzerland.

IgG purification

IgG was isolated from sera, plasma and dialysate using precipitation by ammonium sulphate at a final concentration of 40% (serum or plasma) or 50% (dialysate) saturation. The precipitate was centrifuged for 15 minutes at 4°C and the pellet was resuspended in PBS and dialysed against PBS at 4°C. If necessary the sample was concentrated until usable IgG concentrations were reached.

In vitro glycation

The influence of pH, glucose concentration and incubation time on IgG glycation was studied using commercial 16% IgG preparations for intramuscular injections (CLB, Amsterdam, The Netherlands). These were incubated at a final concentration of 10mg/mL at 37°C for varying times with different glucose solutions (0.05, 0.2, 0.4M) at different pHs (5.5, 7, 9).

Purification of glycated IgG and estimation of glycated IgG percentage

Glycated IgG was separated from non-glycated IgG in IgG purified from sera and dialysates by the method used for determining plasma HbA$_{1c}$ concentrations (Glyco test II 100, Pierce, Illinois), adapted for glycated IgG as described by Dolhofer-Bliesener and Gerbitz [10]. Briefly, 100 µl of purified IgG solutions in the range of 0.2 - 2 mg total IgG in PBS were applied to gel chromatography columns containing m-amino-phenyl-boric acid able to bind to glucose molecules. Non-glycated IgG was eluted with a wash buffer without sugars. Glycated IgG was then eluted from the columns using a sorbitol-containing buffer. Glycated IgG percentages were determined by measuring the absorbance at 280 nm of both fractions with a spectrophotometer. All measurements were performed in duplicate. Within-run and between-day variation coefficients were calculated using classical formulae, and were always less than 10%.

IgG ELISA assay

The IgG ELISA assay was performed as previously described by Rümke et al. [11]. Antibodies to STA W (IgG and IgG subclasses) were measured according to Ruths et al. [12].
Complement deposition assay

The ability of IgG to induce complement activation was studied by measuring the deposition of complement split products, C3b, C3bi, C3c on bacteria according to the method described by Bredius et al. [13], adapted from Gordon et al. [14]. Four different preparations of commercial 16% IgG for intramuscular injections and presenting with high anti-STAW antibody titers (CLB Amsterdam, The Netherlands) were used. Complement activation was expressed per amount of antibacterial antibody bound to the antigen, expressed as a ratio. Antibacterial antibodies were measured by ELISA [12] and expressed in µg bound antibody/mL as described above, and complement activation in the C3c deposition ELISA was expressed as percentage of normal human serum, measured in an A₄₅₀ range of 0.5-1.0. Standards were normal human sera presenting with high titers of anti-bacterial IgG and titration curves relatively parallel to those of IgG fractions. Complement activation was tested by using 10 mmol/L MgEGTA in the assay.

Phagocytosis assay

Polymorphonuclear leucocytes (PMN) were isolated from blood obtained from healthy volunteers. Briefly, PMN were separated from platelets and mononuclear cells by density gradient centrifugation over 1.076 g/mL Percoll (Pharmacia). Erythrocytes were removed by isotonic lysis at 0°C, and PMN (> 95% neutrophils) were washed once with a buffer containing 20 mmol/L Hepes, 132 mmol/L NaCl, 6 mmol/L KCl, 1.2 mmol/L KH₂PO₄, 1 mmol/L MgCl₂, 1.5 mmol/L EGTA (Merck-Schuchardt), 0.1% w/v gelatin and 5.6 mmol/L glucose, pH 7.4 (Ca²⁺-free Hepes), and adjusted to 1 X 10⁷ cells/mL. EGTA was added to this buffer to bind free Ca²⁺, to prevent granulocyte aggregation during phagocytosis.

To assay phagocytosis, we employed a flow cytometric method [15] based on that of Martin and Bahkdi [16]. Phagocytosis was expressed as the percentage of total PMN that were fluorescent green.

Statistical analysis

Comparison of percentages of glycated IgG and results of complement activation were done with the Wilcoxon signed rank test and Student’s t-test, respectively.

RESULTS

Using the modified Gluco test 100 II method for separation of glycated from non-glycated IgG, in vitro experiments of IgG incubation with glucose solutions at concentrations similar to those used for peritoneal dialysis treatment showed that the percentage of glycated IgG was directly related to the pH and glucose concentrations as well as to the incubation time (Figures 1 and 2). For example, for a 0.2 M glucose solution at pH 9, the percentage of glycated IgG increased from 5% to 16% after 8 h, 40% after 24 h and 70% after 72 h (Figure 1). After incubation times of 8, 24 and 72 h with a 0.2 M glucose solution, the percentage of glycated IgG was 5, 12 and 29 %, respectively at pH 7 and 5, 7 and 15 % at pH 5.5 (Figure 1). Finally, for
the same incubation time (72 h) and pH (5.5), the percentage of glycated IgG was, respectively, 20, 9 and 7 % with 0.4, 0.2 and 0.05 M glucose solutions (Figure 2). The analysis of IgG subclass content of both fractions showed no difference between glycated and non-glycated IgG fractions (data not shown).

**Figure 1.** Percentages of glycated IgG determined in samples of normal commercial IgG after incubation with 0.2M glucose solutions at pH 5.5, 7 and 9, for 8, 24 and 72 h. Glycated IgG was separated from non-glycated IgG by gel chromatography using affinity columns loaded with gel coated m-aminophenylboric acid.

**Figure 2.** Percentages of glycated IgG determined in samples of normal commercial IgG after incubation with 0.05, 0.2 and 0.4 M glucose solutions at pH 5.5 for 16, 72 and 144 h. Glycated IgG was separated from non-glycated IgG by gel chromatography using affinity columns loaded with gel coated with m-aminophenylboric acid.

**In vivo IgG glycation**

The percentage of glycated IgG found in sera of 12 CAPD patients was not statistically different from that found in 5 normal controls (4.56 ± 2.47% vs 4.34 ± 1.36 %). The values in
IgG glycation in CAPD fluid

sera of diabetic patients (12.94 ± 7.05 %; p<0.001) were higher than those found in normal controls. The percentages of glycated IgG in overnight dwell dialysates of 12 CAPD patients dialysed with 3.86% glucose solutions were significantly higher than those found in the corresponding sera, as shown in Figure 3 (5.38 ± 2.36 % vs 4.56 ± 2.47 %; P=0.006).

![Graph](https://via.placeholder.com/150)

**Figure 3.** Percentages of glycated IgG determined in night dwell dialysates and sera from 12 CAPD patients dialysed with 3.86% glucose solutions. Glycated IgG was separated from non-glycated IgG using affinity columns loaded with gel coated with m-aminophenylboric acid. Percentages of glycated IgG were higher in dialysate (P=0.006).

**Glycated IgG functions**

The ability of IgG to bind specifically to antigens after glycation was tested using commercial 16 % IgG (CLB, Amsterdam, The Netherlands) with high anti-STAW antibody titers. After separation of glycated from non-glycated IgG, identical amounts of both fractions were applied to ELISA assays using STAW-coated plates. The amounts of IgG binding to the plates were similar for both fractions (data not shown). The ability of glycated and non-glycated IgG to activate the complement cascade was tested with the same ELISA system using agammaglobulinemic sera as complement source. The local C3c deposition was measured and the results are expressed in C3c per IgG. Glycated IgG activated the complement cascade more than the corresponding non-glycated IgG in four different commercial IgG preparations, as shown in Figure 4 (C3c/IgG ratio: 0.96 ± 0.06 vs 0.79 ± 0.08; P=0.027). To specify the route of complement activation, the same experiments were performed in presence of MgEGTA. In these experiments, C3c production in the presence of glycated and of non-glycated IgG was completely abolished when 10 mmol/L MgEDTA was added to the wells (data not shown).
**Phagocytosis assay**

No differences were found between the percentages of green fluorescent PMN in the phagocytosis assay using glycated or non-glycated IgG, using trypan blue or not, and using a complement source (agammaglobulinemic sera) or not (data not shown).

![Graph](image)

**Figure 4.** Complement deposition (measured by ELISA) induced by glycated (100%) and non-glycated (0%) IgG separated from four commercially available normal IgG preparations by affinity gel filtration chromatography. Three samples were tested twice and the fourth was tested three times. Results are expressed in amount of deposited C3c per IgG. C3c deposition was higher for glycated IgG (P=0.027).

**DISCUSSION**

Dolhoffer-Bliesener and Gerbitz [10] reported that IgG glycation may could occur during *in vitro* incubation of IgG with glucose solutions. Indeed, they showed increased IgG glycation after incubation of normal purified IgG in glucose-containing solutions at concentrations ranging from 0.014 to 0.056 M, for 6 days at pH 9. As those incubation conditions were not comparable with those encountered in peritoneal dialysis, since pH 9 is not physiological and dwell times are shorter in CAPD, we have repeated those experiments using different incubation times (8, 24, 72 hours), different pH conditions (5.5, 7, 9), and glucose concentrations similar to those used in commercially available CAPD solutions (0.05, 0.1, 0.2 M). We have confirmed a direct relationship between the percentage of glycated IgG, glucose concentration and incubation time. Also, we have shown that the pH of the incubating solution is a major factor determining the degree of IgG glycation. Indeed, the percentage of glycated IgG was directly related to the pH of the solutions, being the greatest at pH 9. However, according to these *in vitro* experiments no increased IgG glycation should be expected *in vivo*. Indeed, significantly increased percentages of glycated IgG are observed only after at least 24 h incubations, whatever the experimental solutions, whereas maximal dwell times *in vivo* are about 11 h. The rapid increase of dialysate pH, which reaches physiological values half an hour after intra-abdominal infusion, does not explain this discrepancy [17]. This may bbe explained
by the observation of Krediet et al. [18], who demonstrated that the peritoneal interstitium may constitute a third compartment between blood and dialysate, in which equilibrium with blood and dialysate takes place for serum proteins. It may therefore be hypothesized that contact between glucose and IgG takes place in the latter compartment for longer periods than dwell times, allowing IgG glycation before its passage into the peritoneal cavity, and therefore explaining the higher glycated IgG percentages in dialysate than in serum.

As a reduction of the Fc fragment ability to activate complement was described by Dolhofer-Bliesener and Gerbitz [10] after in vitro glycation of IgG, we were surprised to find that glycated IgG separated from normal IgG induced more complement activation than non-glycated IgG. The reasons for this discrepancy are unknown. However, in glycation, glucose reacts with free amino groups 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 be increased in diabetes (for a review see [9]). The Amadori product very slowly undergoes a series of still unknown rearrangement reactions to form yellow-brown, fluorescent and cross-linking substances, called advanced glycosylation end (AGE) products. These products are thought to accumulate on proteins and play an important role in the development of late diabetic complications (for a review see [19]). In the experiments of Dolhofer-Bliesener and Gerbitz [10], the experiment conditions (long incubation time, high pH) led to the formation of AGE products as shown by the formation of non-enzymatic brown products revealed by measurement of absorption at 350 nm and fluorescence at 440 nm upon excitation at 370 nm. Glycated IgG used in our experiments were most probably early glycosylation products. Indeed, immunoreactivity for AGE products is not increased and almost non-existent in IgG fractions purified from normal controls and diabetic patients because of a rapid clearance by mononuclear phagocytes receptors for AGE [20]. It is therefore conceivable that, being chemically different, glycated IgG used in our experiments expresses different effector properties from those used by the latter authors. The more important complement activation by glycated IgG observed in our experiments is induced by the classical route, as shown by complete inhibition by MgEGTA and may be secondary to the increased capacity of glycated IgG to aggregate [10]. This hypothesis should be tested by comparing on gel chromatography columns the proportions of aggregates found in glycated and non-glycated fractions of our IgG preparations.

Contrary to complement activation, IgG mediated phagocytosis was similar when performed with glycated or non-glycated IgG. This observation is also in agreement with the hypothesis that glycated serum IgG contains early glycosylation products instead of AGE since AGE-containing IgG has a reduced ability to promote phagocytosis [16].

In conclusion, the moderately increased percentage of glycated IgG observed in dialysate of patients treated with CAPD most probably does not affect directly the anti-infection mechanisms in the peritoneal cavity, since no decrease in PMN phagocytosis was observed in experiments using in vitro assays comparing the function of glycated and non-glycated IgG. However, the higher complement activation induced by glycated IgG may contribute to the increased complement activation in dialysate, as compared with sera, reported by several
authors in adults and children treated with CAPD [21,22]. This could indirectly affect PMN function, since C5a can contribute to chemotactic unresponsiveness in CAPD [23] and reduce the dialysate concentration of complement factors necessary for adequate anti-infective mechanisms. The role of glycated IgG in dialysate complement activation should be further elucidated in experiments using IgG purified from dialysates of CAPD patients.

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REFERENCES


