CHAPTER 10

INCREASED URINARY IGG/ALBUMIN INDEX IN NORMOALBUMINURIC IDDM PATIENTS: A LABORATORY ARTEFACT

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The previous observation that urinary IgG excretion is increased in normoalbuminuric IDDM patients is unexplained and could possibly be related to a laboratory phenomenon. When untreated urine samples were stored at -20°C for 2 to 4 weeks, the IgG/albumin index (IgG clearance divided by albumin clearance) was higher in normoalbuminuric IDDM patients than in control subjects (0.91(0.68-1.54), n=27 vs 0.72(0.55-0.79), n=15 (median(interquartile range)), p<0.05). In normo- and microalbuminuric IDDM patients the IgG/albumin index was higher in urine samples with glucose than without glucose (1.16(0.93-1.68), n=11 vs 0.73(0.50-0.91), n=16; p<0.05, and 0.33(0.23-0.60), n=17 vs. 0.15(0.10-0.26), n=14, p<0.02 for normo- and microalbuminuric patients, respectively). We, therefore, evaluated the preserving effects of glucose and bovine serum albumin (BSA) on urinary IgG after 1 hour to 16 weeks of freezing at -20°C in 4 non-diabetic subjects (proteinuria ranging from 0.05 to 8.0 g/day). Urine samples were either stored without precautions or treated with addition of phosphate buffer, BSA (1%) and glucose (100 and 300 mM). The weekly decline from 1 to 16 weeks of IgG in the urine aliquots diluted 1:1 with buffered glucose 300 mM and glucose 300 mM + BSA 1% was insignificant, whereas urinary IgG declined with all other storage regimes (p<0.05). These results suggest that glucose in urinary specimens of IDDM patients prevents at least in part the loss of urinary IgG and may thus explain the higher urinary IgG/albumin index when unprocessed urine is stored frozen before assay. Laboratory precautions are necessary when urinary IgG cannot be measured immediately.

Introduction

The glomerular filtration of macromolecules is dependent on their charge, size and structure, on the properties of the glomerular filtration barrier which govern charge and size selectivity, as well as on renal haemodynamic factors [1]. In the microalbuminuric stage of diabetic renal disease an important role is attributed to a loss of negative charge of the glomerular basement membrane, which is probably due to a decrease in the negatively charged proteoglycan, heparansulphate [2-5]. This impaired charge selectivity leads to enhanced glomerular passage and urinary excretion of negatively charged molecules like albumin and IgG4 [1,6,7]. In more advanced stages of diabetic nephropathy a concomitant defect in glomerular size selectivity is thought to result in a grossly increased urinary excretion of neutrally charged IgG [1]. Therefore, the observation that the urinary IgG excretion [3,8] or the IgG/albumin clearance ratio [9] is elevated in

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normoalbuminuric diabetic patients compared to healthy subjects is puzzling and awaits further explanation. This phenomenon could possibly be related to laboratory conditions of urine storage before IgG assay. In this respect it is important to note that urinary IgG concentration decreases within several weeks after freezing of samples at -20°C and that addition of bovine serum albumin (BSA) to the urine specimens and buffering urine to neutral pH diminishes this decline in IgG concentration [10].

In the present study we describe the apparent increasing effect of glucosuria on urinary IgG measurement when aliquots were stored at -20°C without precautions. Laboratory experiments were carried out to determine the effect of different storage conditions, including addition of glucose and BSA, on urinary IgG measurement.

**Patients and methods**

**Study A:** Urinary IgG (UIgG.V) and albumin (Ualb.V) excretion were determined in overnight and in day-time urine collections of 15 healthy controls (age 25±5 years, group C), in 27 normoalbuminuric (age 30±9 years, diabetes duration 15±10 years, group D1) and 31 microalbuminuric insulin-dependent diabetic patients (IDDM) (age 43±11 years, diabetes duration 22±9 years, group D2). Microalbuminuria was defined as an Ualb.V>20 µg/min in an exactly timed overnight urine collection. On the following morning, directly after completion of the overnight collection, a one hour urine collection was made and venous blood was taken for measurements of blood glucose, serum albumin and IgG at the beginning of this period. In the overnight samples, the IgG/albumin index was calculated as the IgG clearance divided by the albumin clearance.

**Study B:** The effects of laboratory storage conditions on urinary IgG measurement was evaluated in 4 non-diabetic subjects with a total urinary protein excretion of 0.05, 0.2, 2.0 and 8.0 g/day.

In all participants of both studies urinary tract infection was excluded by Dipslide tests. Urine samples for IgG measurement were stored at -20°C without precautions for 2 to 4 weeks in study A. Freshly collected samples were processed in study B (see below).

Urinary IgG was measured by an in-house developed enzyme linked immunosorbent assay (ELISA), essentially according to Fomsgaard et al. [11]. The following materials were used: coating: polyclonal goat-antihuman-IgG (gammachain) (Tago, Burlingame, CA, cat no 4100), as conjugate: goat-antihuman-IgG peroxidase (GAHu/IgG (H+L)/PO, Nordic, Tilburg, The Netherlands), enzyme substrate: o-phenylenediamine di-HCl (Eastman Kodak, Rochester, NY, USA). The IgG standard (standard human serum ORDT 06/07, Behringwerke AG, Marburg, FRG) was diluted providing a range from 1.5 to 200 µg/l. Incubations were carried out at 37°C. The colour development was stopped by adding 100 µl H₂SO₄ (0.5 mol/l). The absorbance was measured at 492 nm using a Titertek Multiscan (Flow Laboratories, Irvine, UK). Results were computed according to Rodbard et al. [12]. The intra- and interassay coefficients of variation (CV) were 5% and 15%, respectively. The lower detection limit was 1.5 µg/l.

To evaluate the effect of laboratory storage conditions on urine IgG measurement (study B) the following experiments were carried out: within 4 hours after voiding urine aliquots were treated (A) undiluted; (B) diluted 1:1 with 40 mM phosphate buffer, 0.1 M NaCl adjusted to pH 7.4 and sodium azide (NaN₃ 0.2%); (C) diluted 1:1 with buffer and
BSA 1% (10 g/l); (D) diluted 1:1 with buffer and glucose 100 mM; (E) diluted 1:1 with buffer and glucose 300 mM; (F) diluted 1:1 with buffer, glucose 100 mM and BSA 1%; (G) diluted 1:1 with buffer, glucose 300 mM and BSA 1%. IgG was measured in these samples before freezing and after 1 hour, and 1, 2, 4, 8 and 16 weeks of storage at -20°C. All IgG measurements were performed in quadruplicate.

In both studies urine aliquots for albumin measurement were stored at 4°C and analyzed within 24 hours. Urinary albumin was measured in duplicate using a commercially available double antibody radioimmunoassay (Diagnostic Products Corporation, Apeldoorn, The Netherlands, cat no KHAD). The intra- and interassay CV’s were 2.3% and 7.7%, respectively. The lower detection limit was 0.07 mg/l. Serum IgG was measured by nephelometry (Behring Nephelometer Analyzer™, Behringwerke AG, Marburg, FRG). The intra- and interassay CV’s were 2.4 to 3.6% and 2.5% respectively. Serum albumin was measured on a SMAC-II autoanalyser (Technicon Instruments Inc., Tarry Town, NY, USA). The intra- and interassay CV were 1.9% and 2.5%, respectively. Blood glucose was measured using a Yellow Springs glucose Analyzer (Model 23A, Yellow Springs Inc., Yellow Springs, Ohio, USA). Urinary glucose was measured using a Polarimeter Type 243 (Thorn Automation, Nottingham, UK).

Statistical analysis

Results are expressed as mean±SD or as median (interquartile range) for parametrically and non-parametrically analysed data, respectively. In study A, differences in urinary albumin excretion, urinary IgG excretion and the albumin/IgG index were evaluated by Kruskal-Wallis oneway analysis of variance with Duncan’s method to correct for multiple comparisons. Correlation coefficients between urinary and blood glucose and protein excretion were calculated using Spearman’s rank analysis. In study B, mean (±SD) recovery of IgG at each interval was calculated as percentage of the freshly analysed samples for each of the four urine collections. The overall means and their common SD's are given in the Table, and the data are compared by Student t-tests. Using linear regression analysis, the weekly percentage decline (±SD) from 1 to 16 weeks was calculated for each collection. The overall mean declines (± SD) are given in the Table. A two-sided p-value less than 0.05 was considered to be significant.

Results

Urinary albumin and IgG excretion (study A)

Overnight Ualb.V and urinary IgG excretion (UIgG.V) were significantly higher in group D2 (54.6(41.6-109.5) and 3.10(2.35-8.81) µg/min, respectively) than in groups D1 and C (7.1(4.6-10.1) and 4.6(3.0-7.2) for Ualb.V; 1.64(1.02-2.37) and 0.46(0.35-0.66) µg/min for UIgG.V, p<0.01 for all comparisons). Ualb.V was similar in group D1 and group C, but UIgG.V was higher in group D1 compared to group C (p<0.05). Since no differences were present in serum albumin and IgG concentrations, the overnight IgG/albumin index was higher in group D1 (0.91(0.68-1.54)) than in group C (0.72(0.55-0.79), p<0.05). This index was lower in group D2 (0.27(0.14-0.40), p<0.01) than in group C. As shown in Figure 1, in both diabetic groups the IgG/albumin index was higher in urine samples with glucose as compared to those without glucose (1.16(0.93-1.68) (n=11)
vs 0.73 (0.50-0.91) (n=16), p<0.05, for group D1; 0.33 (0.23-0.60) (n=17) vs 0.15 (0.10-0.26) (n=14), p<0.02, for group D2). In group D1, the IgG/albumin index in samples without glucose was not different from group C (Figure 1). In the day-time urine collections similar differences were present (not shown). In the combined diabetic patients (n=58), the day-time IgG/albumin index was positively correlated with urinary glucose concentration ($r_2 = 0.33$, $p<0.05$), urinary glucose excretion ($r_2 = 0.44$, $p<0.01$) and blood glucose ($r_2 = 0.56$, $p<0.01$).

**Figure 1.** The overnight IgG/albumin index in non-diabetic control subjects (group C, ○), in normoalbuminuric IDDM patients (group D1) without (□) and with glucosuria (■), and in microalbuminuric IDDM patients (group D2) without (▲) and with glucosuria (●). Bars represent median values. * denotes $p<0.05$ from group C and group D1 without glucosuria; † denotes $p<0.01$ from groups C and D1 with and without glucosuria ‡ denotes $p<0.02$ from group D2 without glucosuria.

**Effects of laboratory storage condition on urinary IgG measurement (study B)**

In the 4 non-diabetic patients the urinary IgG concentration was 0.68, 1.52, 12.1 and 109 mg/l in the immediately processed samples. The IgG concentrations after storage at
**Table 1.** Recovery of urinary IgG after different storage conditions during variable intervals at -20°C in 4 non-diabetic subjects with proteinuria.

<table>
<thead>
<tr>
<th>Dilution (1:1)</th>
<th>Storage time</th>
<th>weekly decline %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fresh</td>
<td>1h</td>
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<tr>
<td>A undiluted urine</td>
<td>mean</td>
<td>100</td>
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<tr>
<td></td>
<td>SD</td>
<td>6.2</td>
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<tr>
<td>B buffer alone</td>
<td>mean</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>8.3</td>
</tr>
<tr>
<td>C +BSA 1%</td>
<td>mean</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>3.8</td>
</tr>
<tr>
<td>D +glucose 100 mM</td>
<td>mean</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>3.6</td>
</tr>
<tr>
<td>E +glucose 300 mM</td>
<td>mean</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>3.8</td>
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<tr>
<td>F +BSA 1% +glucose 100 mM</td>
<td>mean</td>
<td>100</td>
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<tr>
<td></td>
<td>SD</td>
<td>3.1</td>
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<tr>
<td>G +BSA 1% +glucose 300 mM</td>
<td>mean</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>4.3</td>
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</tbody>
</table>

Data are given as overall mean and common SD in % of measurements in fresh urine and percentage decline per week (from 1 to 16 weeks) and common SD. * denotes $p<0.05$ as compared to fresh urine or as compared to zero-decline.

-20°C are given as percentage recovery of the initial value (Table 1). No immediate effect of 1 hour of freezing could be demonstrated. In contrast, the IgG content after 1 week of storage declined significantly in the untreated ($p<0.01$, A), buffered ($p<0.005$, B), buffered and BSA 1% diluted ($p<0.01$, C), buffered and glucose 100 mM diluted ($p<0.05$, D) urine specimens (Table 1). Moreover, this decline was progressive, since the weekly decline from 1 to 16 weeks of storage was significant in these storage regimes ($p<0.01$ for A,B,C and D). In glucose 300 mM diluted (E), glucose 100mM and BSA 1% diluted (F) and glucose 300 mM and BSA 1% diluted (G) urine specimens a small but significant decline in IgG was noted after 16 weeks. The weekly decline in IgG was not significant in the urine specimens diluted with glucose 300 mM ($p<0.20$, E) or glucose 300 mM and BSA 1% ($p<0.20$, G), but significant if diluted with glucose 100 mM and BSA 1% ($p<0.05$, F).

**Discussion**

In addition to documentation of microalbuminuria, measurement of IgG with sensitive assays will gain insight in the glomerular permelectivity defects underlying the development and evolution of diabetic renal disease. In a cross-sectional study we confirmed recent unexplained findings showing that normoalbuminuric IDDM patients...
have a higher urinary IgG excretion and a higher IgG/albumin index compared to controls [3,8,9]. Of importance, urine samples for IgG measurement were stored at -20°C without precautions in the study by Deckert et al [3] and in the present study, whereas in the other reports [8,9] the urinary storage conditions were not mentioned. The present data showed that among both normo- and microalbuminuric IDDM patients the IgG/albumin index was higher in samples with glucose compared to samples without glucose. Moreover, the IgG/albumin index was positively correlated with glucosuria. This unexpected finding prompted us to evaluate the effect of laboratory storage conditions on IgG measurement. In agreement with other data, storage at -20°C without precautions resulted in a pronounced loss of IgG [10], but in contrast with that study, addition of BSA alone did not prevent the decline in IgG. Only addition of 300 mM glucose (preferably in combination with BSA 1%) to the specimens prevented a rapid decline in IgG. Moreover, such high urinary glucose concentrations can be encountered in vivo during periods of severe hyperglycaemia. It is, therefore, plausible that the apparently increasing effect of glucose on IgG excretion in IDDM is attributable to a diminished or absent loss of IgG during storage without precautions. An alternative explanation, such as a general abnormality in glomerular or tubular IgG handling in diabetes mellitus, is difficult to envisage, since the glomerular permselectivity is considered to be intact [13] and β₂-microglobulin excretion is unchanged [1] in normo-albuminuric IDDM patients. Indeed, we were unable to reproduce an elevated IgG/albumin index in normoalbuminuric IDDM patients when using adequate storage procedures [unpublished observations]. Deckert et al. were also unable to find a difference in the IgG/albumin index in normoalbuminuric IDDM patients compared to controls if urine samples were stored at -40°C after predilution with 5% BSA in phosphate buffer [14, personal communication B. Feldt-Rasmussen].

A gradual loss in urinary IgG during storage at -20°C was observed, without an immediate effect of freezing and thawing. Denaturation or aggregation of globulins into multimers of 2 to 4 molecules at low temperatures, which might interfere with epitope recognition in the enzyme-linked immunosorbent assay, can possibly explain this loss in IgG. The employed glucose concentrations of 100 to 300 mM in the specimens were very high compared with the measured IgG concentrations that ranged from 0.0045 to 0.716 mM. This excess of glucose molecules could prevent IgG denaturation or aggregation during prolonged freezing. In contrast to IgG, urinary albumin does not decline after freezing for 1 week [15], although a period of longer storage is also associated with a loss of albumin [16]. Of note, addition of glucose does not influence the albumin measurement in samples assayed within one week after collection [15].

In conclusion, the presence of glucose in urine samples of IDDM patients most likely prevents, at least in part, the loss of urinary IgG when aliquots are frozen without precautions. This may explain the apparently higher IgG/albumin index in normoalbuminuric IDDM patients as compared to healthy subjects. Addition of glucose 300 mM plus BSA 1% (1:1) to the urine samples is an appropriate measure to prevent a substantial decline in IgG. It is, therefore, necessary to use such precautions when it is not possible to assay IgG immediately.

References
Glucose and urinary IgG in IDDM.