Radioimmunoassay for Immunoglobulin G in Serum and Urine

Jannie Woo, Michael Floyd, Mary Ann Longley, and Donald C. Cannon

We describe a radioimmunoassay for immunoglobulin G (IgG) in serum and urine. Aliquots of diluted samples and $^{125}$I-labeled IgG were incubated in antibody-coated tubes at 37 °C for 24 h, the supernates were decanted, and the radioactivity in tubes containing the bound fraction was counted. The dose–response curve in the range of 0.4–500 mg/L of urine or 640–40 000 mg/L of serum was linear on logit-log transformation and iterative weighted regression. Assay sensitivity was 10 ng of IgG. Validation studies included testing for precision, accuracy, antibody specificity, and parallelism of the dose–response curves for standard and unknown. In a study of 14 apparently normal individuals, serum IgG = 4.0–10.9 g/L, urine IgG = 1.1–4.8 mg/24 h, and IgG clearance = $0.2 \times 10^{-4}$ to $4.8 \times 10^{-4}$ mL/min. In 20 patients with renal allografts, serum IgG = 15.8–66 g/L, urine IgG = 9.8–626 mg/24 h, and IgG clearance = $9 \times 10^{-4}$ to $1.99 \times 10^{-1}$ mL/min. IgG values correlated well with severity of renal allograft rejection.

Additional Keyphrases: renal allograft rejection assessment • glomerular permeability • renal metabolism of IgG • kidney disease

The amount of immunoglobulin G (IgG)$^2$ in urine reflects glomerular permeability. In a classic study of differential protein clearance (1), the relative urine/plasma concentration ratio of albumin (Mf $98$ 000) or transferrin (Mf $79$ 000) to IgG (Mf $154$ 000) was used as an index of the degree of "leakiness" of the glomerular capillary wall, the molecular form of urinary IgG being for this purpose assumed to be monomeric. However, other studies indicate that this assumption may be unjustified in some cases. Hardwicke and White (2) reported the presence of low-Mf, fragments of IgG in several patients with focal glomerulosclerosis. In contrast, we have found IgG with an unexpectedly high Mf, (presumably aggregated IgG) in two patients with proteinuria associated with acute allograft rejection (unpublished data). Further study would appear justified to unravel the renal metabolism of IgG in different pathological conditions, particularly with respect to its antigen specificity in the aggregated form. In addition, certain immunologic observations point to the need for a sensitive and specific quantitative assay for low concentrations of urinary IgG and its metabolic degradation products. Examples include the extremely low concentration of antibodies to glomerular basement membrane in certain forms of glomerulonephritis, the transient nature of the immune complexes in various glomerular diseases, and the role of the renal tubule in further reducing the concentration of urinary IgG and its fragments by protein reabsorption.

Advances have been made in the quantitation of IgG in human serum to include sensitive techniques such as nephelometry (3), radioimmunoassay (RIA) (4–8), and enzyme-linked immunassay (9, 10). However, the study of urinary proteins has been confined primarily to single radioimmunoassay (11) and more recently sodium dodecyl sulfate/polyacrylamide gel electrophoresis (12); both of these procedures are time consuming and at best only semiquantitative. We describe a solid-phase RIA for IgG in urine and serum with use of an antisera specific for human IgG. The procedure is simple, sensitive, and precise. We also describe a preliminary study of IgG clearance, in which we used this assay to assess glomerular permeability in patients undergoing renal allograft.

Materials and Methods

Reagents and Solutions

Phosphate-buffered saline, 10 mmol/L. Ten millimoles of Na$_2$HPO$_4$, 150 mmol of NaCl, and 1.5 mmol of NaN$_3$ (1.41 g, 8.8 g, and 0.1 g, respectively) were dissolved in 900 mL of reagent-grade water. The pH was adjusted to 7.3, and the solution was diluted to 1 L.

Bovine serum albumin solution, 10 g/L. Five grams of bovine serum albumin (Calbiochem, La Jolla, CA 92112) was mixed with 500 mL of phosphate-buffered saline.

Sodium bicarbonate buffer, 100 mmol/L. Twenty-five millimoles of NaHCO$_3$, 2.1 g, was dissolved in 200 mL of reagent-grade water, the pH adjusted to 9.2, and the solution diluted to 250 mL.

Human IgG coating solution. Human IgG (Miles Laboratories, Elkhart, IN 46515) was used to coat antibody tubes. A stock solution was prepared by dissolving 50 mg of IgG in 5 mL of reagent-grade water. Two milliliters of the stock solution was further diluted to 200 mL with sodium bicarbonate buffer.

Human IgG standards. Human IgG was prepared from freshly collected normal human serum. The serum, clotted at 37 °C, was complemented by heating at 56 °C for 20 min. The immunoglobulin fraction was precipitated with saturated ammonium sulfate (final concentration, 550 g/L). The precipitate was redissolved in phosphate-buffered saline and reprecipitated with ammonium sulfate. After dialysis against phosphate buffer (1 mmol/L, pH 7.4) for 18 h, the protein was applied to a 10 × 2.5 cm column packed with "DEAE Bio-Gel
Igecoating determined and its purity was confirmed both by radial immunodiffusion and by immunoelectrophoresis (IEP) in agarose (10 g/L of barbiturate buffer, pH 8.2), developed with rabbit antinormal human serum and with antisera to IgG, IgM, IgA, and kappa and lambda light chains. All IEP testing was performed with the antigen well filled twice. The fractions of column eluate containing IgG were combined and their IgG content was determined spectrophotometrically at 280 and 280 nm (13). Working standard solutions, prepared freshly before each assay, were obtained by diluting the combined eluate with bovine albumin solution to yield solutions containing 500, 250, 125, 62.5, 31.3, 15.6, and 7.8 ng/100 μL.

Human IgG antiserum, rabbit. Two milligrams of human IgG, purified by the procedure just described, was dissolved in 1 mL of a phosphate buffer (5 mmol/L, pH 7.4) and mixed with an equal volume of Freund's complete adjuvant. The mixture was injected bimonthly for three months, subcutaneously, into four New Zealand albino rabbits. Ten days before the final bleeding, each rabbit was given the same amount of IgG, but without Freund's complete adjuvant.

We determined the optimum dilution of the antiserum for coating antibody tubes by performing an antiserum-dilution study, using the procedure described below. Antiserum diluted 1600-fold yielded an optimal initial binding (B/T)₀ of 50%. The phosphate-buffered saline was used as the antiserum diluent.

Procedures

Preparation of antibody-coated tubes. The method of Salmon et al. (5) was modified as briefly described: 1 mL of human IgG coating solution was incubated in polystyrene tubes, 12 mm × 75 mm (Falcon, Oxnard, CA 93030) at room temperature for 18 to 24 h. The solution was decanted and the tubes were washed twice, each time with 2 mL of 150 mmol/L saline, then decanted and drained for 5 to 10 min. Two milliliters of bovine albumin solution was incubated in each tube for 18 to 24 h at room temperature, the solution was decanted, and the tubes were washed twice with the 150 mmol/L saline as before. The bovine albumin solution served to coat any remaining vacant sites on the polystyrene tubes. One milliliter of a freshly prepared antibody solution (1600-fold dilution) was then incubated in each tube for 18 h at room temperature. The antibody solution was decanted and the tubes were again washed twice with 2 mL of 150 mmol/L saline as before. Finally, the coated tubes were dried under a stream of air. These tubes were stable for at least a week when stored at room temperature.

Radioiodination of human IgG. Human IgG was iodinated by the Chloramine T procedure of Hunter and Greenwood (14), modified as follows. Human IgG prepared from human serum as described previously, 40 μg in 10 μL of phosphate buffer (400 mmol/L, pH 7.6), and 5 μL of carrier-free Na¹²⁵I (500 μCi; Amersham Searle, Arlington Heights, IL 60005) were mixed gently in a 500-μL plastic AutoAnalyzer cup. Ten microliters (25 μg) of a freshly prepared Chloramine T solution was added, and the mixture was allowed to react for 30 s. The reaction was stopped by adding 25 μL (62.5 μg) of sodium metabisulfite and 100 μL of a 1 mol/L solution of KI. Before purification of the labeled IgG, its specific activity was measured by reacting 5–10 μL of the iodination mixture with 0.5 mL of a 150 g/L trichloroacetic acid solution, which precipitates the protein fraction. The iodinated preparation was purified by gel-filtration chromatography on a 0.9 cm × 15 cm column packed with Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, NJ 08854). The column was eluted with the phosphate-buffered saline and 500-μL fractions were collected in 16 × 100 mm tubes, each coated previously with 50 μL of bovine albumin solution. Fractions containing the iodinated IgG (tubes 17–20) were combined. Just before assay, an aliquot of the iodinated preparation was diluted with phosphate-buffered saline to give a concentration of 1.8 ng/100 μL (about 20 000 cpm/100 μL).

Radioimmunoassay. Assay-standard incubation mixtures in antibody-coated tubes were made up of (a) 100 μL of working IgG standard solution (0–500 ng), (b) 100 μL of ¹²⁵I-labeled IgG solution containing 20 000 cpm, and (c) 800 μL of bovine albumin solution. All urine (fivefold and 100-fold) and serum (8000-fold) samples were diluted with an
"Autodiluter" (LKB Instruments, Inc., Rockville, MD 20852; Model 2075) with phosphate-buffered saline as the diluent. Sample incubation mixtures in antibody-coated tubes were prepared similarly, except that a was replaced with 100 μL of diluted urine or serum samples. All standards and samples were assayed in duplicate. The contents of each of these tubes were well mixed and allowed to incubate for 24 h at 37 °C. The tubes were inverted and drained for at least 15 min, then washed three times with 2 mL of 150 mmol/L saline, and allowed to drain for at least 10 min after each wash. Radioactivity in each of the tubes containing the bound fraction was counted for 1 min in a gamma scintillation spectrometer (Packard Instrument Co., Downers Grove, IL 60515; Model 52300).

Radioimmunoassay data obtained from the bound fraction were processed with a Model 9830A programmable calculator and a Model 9862 plotter (both from Hewlett-Packard, Fort Collins, CO 80525). The dose–response variables were subjected to logit-log transformation and iterative weighted regression analysis to yield a linear slope, an 80% intercept, and a 50% intercept, all of which serve as quality-control data for assay monitoring. In urine sample calculation, an averaged IgG value obtained from both urine dilutions was converted to the unit reported: mg/24 h. Where the binding for one of the dilutions fell beyond the acceptable limits of precision—i.e., 85% < B/B₀ < 15%—the final calculation was performed on one dilution only.

Specimens. Timed 12-h or 24-h urines were collected from apparently healthy laboratory individuals known to be taking no drugs and from patients undergoing kidney transplantation. Serum was sampled from the same individuals within the first 30 min of the urine collection.

IgG clearance (CᵢG, mL/min) was calculated in both normal subjects and in patients as follows:

$$CᵢG = \frac{(UᵢG \times V)}{(SᵢG \times t)}$$

where UᵢG = urinary IgG excretion, in mg/L; SᵢG = serum IgG concentration, in mg/L; V = volume of timed urine, in mL; and t = duration of urine collection, in min.

Results

Iodination of human IgG. The specific activity of the iodinated IgG, as determined after trichloroacetic acid precipitation, was 10–12 Ci/g. Iodination efficiency was 67%. When stored frozen in aliquots, the preparation being diluted to its working concentration just before assay, each iodination was stable for at least five weeks.

Standard curve. Figure 1 shows a representative dose–response curve after logit-log transformation and iterative weighted regression analysis. The relation was linear over an absolute range of 8–500 ng of human IgG per assay tube, equivalent to 0.4–500 mg/L of urine at sample dilutions of fivefold and 100-fold, and 640–40 000 mg/L of serum at a sample dilution of 8000-fold. The assay sensitivity, as defined by B/B₀ of 85%, was 10 ng of IgG, or 0.5 mg/L of urine at a sample dilution of fivefold, and 640 mg/L of serum at a sample dilution of 8000-fold. The upper limit of precision, as defined by B/B₀ of 15%, was 400 ng of IgG, or 400 mg/L of urine at a sample dilution of 100-fold and 32 000 mg/L of serum at a sample dilution of 8000-fold.
Fig. 3. Quality-control chart for RIA for IgG showing the between-run variations in 20 assays

**Precision.** We assayed 37 urine samples with IgG values ranging from 9.6 to 798 mg/L, each on two days, to assess the between-run precision. The CV was 15.3%. Another precision study in which two serum specimens were used as controls in 20 individual assays resulted in a CV of 12.9% at 550 mg/L and 11.0% at 1220 mg/L. These serum controls were assayed at sample dilutions of 1000-fold.

**Accuracy.** Known amounts of purified human IgG standard (25–400 ng/tube) were added to aliquots of a normal serum specimen for which the IgG concentration had previously been determined. These aliquots were then assayed for human IgG. Analytical recovery of IgG ranged from 90.8 to 114%.

**Specificity.** The cross reactivity of this antisera to other plasma proteins was evaluated by reacting the following immunoglobulins with the assay system for measuring IgG: (a) IgA, (b) IgM, (c) kappa and lambda light chains, (d) kappa light chain isolated from an urine sample of a myeloma patient by gel filtration chromatography with DEAE Bio-Gel A, and (e) myeloma urine after removal of IgG by adsorption with "Pansorbin" (Calbiochem). As shown in Figure 2, this antisera cross reacted 6.6% with IgA, 2.4% with IgM, and virtually not at all with either light chain.

**Assay validation.** Figure 2 also shows the standard curves obtained with (a) human IgG as the standard, (b) a serial twofold diluted urine specimen, and (c) a serial twofold diluted serum specimen, both from a renal-transplant patient. All three curves can be superimposed in the range 10.0–500 ng/assay tube, which is equivalent to 0.5–25.0 mg/L for a fivefold diluted urine sample, 10.0–500 mg/L for a 100-fold diluted urine sample, and 800–40 000 mg/L for a 8000-fold diluted serum. To test for possible interference with bloody and highly proteinuric specimens, we mixed urine samples from renal transplant patients separately with (a) intact erythrocytes (50 μL/mL), (b) hemolyzate (50 μL/mL), and (c) human serum albumin (10 mg/mL). The IgG values obtained were all well within the coefficients of variation as defined for this method. The standard curves obtained with serial twofold dilution of these treated samples showed parallelism with that obtained with IgG as standard. This indicates that the determination of IgG in both urine and serum is specific, un influenced by the other substances in concentrations of 0.5–500 mg/L for urine and 900–40 000 mg/L for serum.

**Quality-control parameters.** Radioimmunoassay functions that are useful in quality-control monitoring are: [(B/T)/b] % nonspecific binding, slope of the logit-log transformed standard curve, the 80% intercept, and the 50% intercept (15). Between-run variations in these are graphically presented in Figure 3. The mean and standard deviations for each function for 20 runs are: [(B/T)/b] = 54.2 ± 6.5, percent nonspecific binding = 1.1 ± 0.6, slope = 1.00 ± 0.12, 80% intercept = 16.3 ± 3.6, and 50% intercept = 65.2 ± 10.1.

**Normal range and data on patients.** The normal ranges for IgG in serum and urine, based on data from 14 healthy laboratory subjects, were 4.0–10.9 g/L and 1.1–4.8 mg/24 h, respectively; the corresponding IgG clearance was 0.2 × 10⁻⁴ to 4.8 × 10⁻⁴ ml/min. Serum and urine IgG for 20 patients after renal allografts ranged from 15.8 to 66 g/L and 9.6 to 626 mg/24 h, respectively, and the corresponding IgG clearance was 9 × 10⁻⁴ to 1.99 × 10⁻¹ ml/min.

**Discussion**

The ranges of IgG concentration covered in this assay, 0.5–500 mg/L of urine and 800–40 000 mg/L of serum, are sufficient to include individuals with normal urine function, patients with minimal glomerular disease, and patients with acute episodes of allograft rejection. This procedure is simple to operate and the solid-phase antibody-coated tubes are convenient to prepare in large batches, the actual technician time required being about 1 h/100 tubes. Although the antisera cross reacts slightly with IgA (6.6%) and with IgM (2.4%), this should be of little practical concern in view of the low concentrations of these immunoglobulins in serum and their virtual absence in urine.

Our finding of aggregated IgG in the urines of two patients with acute allografts rejection has prompted us to investigate similar patients in the hope of gaining further information regarding IgG metabolism in the kidney. The investigation of possible IgG fragments associated with renal disease other than focal glomerulosclerosis may also be brought to focus by use of this assay. Finally, determination of IgG clearance in conjunction with estimation of albumin clearances by our recently developed radioimmunoassay (16) may provide a rapid means of determining the selectivity of glomerular proteinuria (17), which should be clinically useful in diagnosis and treatment of some renal diseases.

**References**


2018 CLINICAL CHEMISTRY, Vol. 25, No. 12, 1979