Monoclonal Antibody-Based Solid-Phase Immunoenzymometric Assays for Quantifying Human Immunoglobulin G and Its Subclasses in Serum

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We developed quantitative immunoenzymometric assays for human IgG and its subclasses by using monoclonal antibodies, an avidin–biotin detection system and, as the calibrant, the U.S. National Reference Preparation for Specific Human Proteins. The assays are sensitive (detecting as little as 6 \( \mu \text{g/L} \)), precise (average inter-assay CV <11%), and vary linearly with concentrations over a five- to 10-fold range, depending on the monoclonal antibody. We evaluated 22 different monoclonal antibodies, many of which remained highly reactive when immobilized in wells of microtiter plates coated with bovine serum albumin–glutaraldehyde to “capture” total IgG or subclasses of IgG in the sample. We demonstrated the specificity of the most reactive antibodies by using a panel of 20 purified myeloma proteins. The sum of IgG subclass concentrations correlated well (\( r = 0.84, p <0.001 \)) with the total IgG measured in sera from 63 apparently healthy adults (26 men, 37 women). We estimated 95 percentile reference intervals for the immunoglobulins in these subjects and determined the following mean percentage distributions of IgG subclasses: IgG1 49, IgG2 33, IgG3 9, and IgG4 7. The availability of these assays should facilitate studies of the clinical significance of the subclasses.

Additional Keyphrases: avidin–biotin labels • panels of myeloma proteins • reference interval • monoclonal antibodies • distribution of IgG subclasses • pediatric clinical chemistry • assay of culture supernatants, lavage fluid

Human immunoglobulin G (IgG) comprises four subclasses—IgG1, IgG2, IgG3, and IgG4—which differ in their heavy-chain amino acid sequence and their biological function (1). In sera from healthy adults the order of subclass concentration is IgG1 > IgG2 > IgG3 > IgG4, although the ranges and proportions vary (2–4). Alterations in the concentrations of several IgG subclasses have been associated with numerous diseases (5–8). Results of different clinical studies are difficult to compare, however, because many different methods and reagents have been used to quantify the subclasses. For example, the non-standardized materials used to calibrate the assays have ranged from “purified” myeloma proteins to pooled human sera (2–4). Furthermore, many studies have involved use of polyclonal antibodies to the subclasses; not only are these antisera weakly reactive, they are also difficult to prepare and to standardize from batch to batch. In contrast, monoclonal antibodies, the products of stable hybridoma clones, provide a source of consistently specific and avid immunoenzymochemical reagents.

Here we describe immunoenzymometric assays (EIA) for quantifying human IgG and its subclasses through the use of a panel of previously characterized monoclonal antibodies (9) immobilized on a solid-phase surface for reaction with antigen in solution. Using the U.S. National Reference Preparation for Specific Human Serum Proteins (USNRP) as the calibrant, we have measured the concentration of IgG and that of each subclass in 63 normal adult sera to estimate normal reference intervals.

Materials and Methods

Chemicals and prepared reagents. Polyoxyethyleneboronate monolaurate (Tween-20), anhydrous citric acid, o-phenylenediamine, glutaraldehyde (grade II, 250 g/L aqueous solution), and bovine serum albumin (BSA; 98–99% pure, cat. no. 7030) were obtained from Sigma Chemical Co., St. Louis, MO 63178. Other reagents, including hydrogen peroxide, 300 mL/L solution, and concd. \( \text{H}_2\text{SO}_4 \) were analytical-grade products from Mallinkrodt, Inc., Paris, KY 40361. Normal mouse serum was purchased from Cappel Laboratories, Cochranville, PA 19330.

Prepared reagents were (a) phosphate-buffered saline (PBS) containing, per liter, 10 mmol of sodium phosphate and 150 mmol of NaCl, pH 7.2; (b) peroxidase substrate, per liter, 3.5 mmol of \( \text{H}_2\text{O}_2 \), 2.3 mmol of o-phenylenediamine, 50 mmol of citric acid, and 100 mmol of disodium phosphate, pH 5; (c) glutaraldehyde, 2 mL/L of PBS; and (d) BSA, 0.2 g/L in water and 0.5 g/L in PBS. We used glass-distilled water throughout the analyses.

Immunochromaceutical reagents. Preparations of affinity-puriﬁed biotinylated goat anti-human IgG, avidin, and biotinylated horseradish peroxidase (EC 1.11.1.7) were obtained as components of a kit for determining human IgG ["Vectastain ABC" (avidin–biotinylated peroxidase complex]; Vector Laboratories, Burlingame, CA 94010].

Monoclonal antibody reagents. These mouse hybridoma products have been characterized earlier (9). After preliminary testing of 22 monoclonal antibodies for use in the assay (see Table 1 below), we selected five of them: HP6012 (anti-IgG1), HP6014 (anti-IgG2), HP6048 (anti-IgG3), HP6022 (anti-IgG4), and HP6046 (anti-IgG). Filtered acetic acid fluid that had been lyophilized in 0.2-mL borosilicate glass vaccine vials were rehydrated in distilled water, then either diluted 300-fold in PBS for immediate use in tests or dispensed in 66-\( \mu \text{L} \) aliquots into polypropylene microcentrifuge tubes and stored at -40 °C.

USNRP for calibration of subclass tests. To generate standard curves for the IgG subclass and total IgG assays, we used the USNRP (lot no. 120575C, cat. no. IS1644; Centers for Disease Control, Atlanta, GA 30333 (10), which had been calibrated for IgG subclasses by immunofluorometric assay (11). When reconstituted according to the instruc-

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5 Nonstandard abbreviations: EIA, immunoenzymometric assay; USNRP, U.S. National Reference Preparation for Specific Human Serum Proteins; BSA, bovine serum albumin; and PBS, phosphate-buffered isotonic saline.
6 Use of trade names and commercial source is for identification purposes only and does not constitute endorsement by the Public Health Service or any of its agencies.
tions accompanying the product, the concentrations (g/L) of analytes in this material are: IgG 11.4, IgG1 6.95, IgG2 3.15, IgG3 0.70, and IgG4 0.61. Aliquots of the rehydrated preparation were stored at −70 °C, until the day of the assay, when they were diluted 1000-, 10 000-, and 100 000-fold in PBS, yielding total IgG concentrations of 11.4 mg/L, 1140 μg/L, and 114 μg/L, respectively, depending on the assay. These standards were then further diluted to obtain the following range of working standard concentrations (μg/L): IgG1, 7–50; IgG2, 8–78; IgG3, 7–35; IgG4, 6–30; total IgG, 11–81.

Purified IgG myeloma proteins. To study assay specificities, we used 20 IgG myeloma proteins, previously purified by chromatography on diethylaminoethyl-Sephadex or Staphylococcus Protein A–Sepharose (12). The light chain type and IgG subclass had been determined by immunofluorometry involving a panel of monoclonal antibodies (9). These preparations contained 2 to 3 g of total protein per liter, as determined by spectrophotometry ([ε280] of a 10 g/L solution = 13.8 (12)).

Sera. Samples were obtained, with informed consent, from 63 American Red Cross blood donors (37 women and 26 men, ages 22 to 66 years). Sera were stored at −70 °C until the day before the assay, when they were thawed and kept at 4 °C until analysis. Samples were diluted in PBS analogously to the preparation of USNR dilutions. For each immunoglobulin assay, we tested each sample at three different dilutions, such that the range of concentrations obtained were expected to yield absorbances overlapping the linear portion of the appropriate standard curve.

Supplies and apparatus. Polystyrene flat-bottom 96-well microtiter plates, a “Titertek” Multiwash automatic plate washer, a "Titertek" Multiscan filter-photometer plate reader, and “Titertek” fixed-volume (0.1-mL) multichannel pipettes were all obtained from Flow Laboratories, Inc., McLean, VA 22102. Micropipettes and automatic diluters were from SMJ, Emeryville, CA 94608, and Micromedic Systems, Huntsville, AL 35810, respectively.

Immunossay protocol. We coated microtiter plate wells with BSA–glutaraldehyde as described by Saunders (13). Briefly, the procedure is as follows. Allow 20 μg of BSA in 0.1 mL of distilled water to air-dry in each well at 37 °C, then add 0.1 mL of 2 mL/L glutaraldehyde solution (pH 7.2, verified) and let sit for 30 min at 37 °C. Wash the plates by machine five times with distilled water, allow to air-dry at 37 °C, then store in sealed plastic envelopes in a desiccator at room temperature for no longer than six months.

Optimal reaction conditions had been determined in preliminary experiments. All reagents and samples were added to the wells in 0.1-mL volumes. After each step in the procedure, plates were washed by machine five times with PBS containing 0.5 mL of Tween-20 per liter, unless otherwise stated. Drying and incubation steps were done at 37 °C, unless otherwise stated.

Every assay plate included four or five dilutions of USNR for the calibration curves, background reactions (with only the sample omitted), and dilutions of a sample of pooled human sera for monitoring reproducibility. All reactions were performed in quadruplicate.

The selected monoclonal antibodies, still in ascitic fluid and unpurified, were conjugated to precoated BSA–glutaraldehyde microtiter plates by diluting the antibodies 300-fold and letting them react until the plates were completely dry, typically 20–24 h. We then added BSA (50 μg in 0.1 mL of PBS) to each well and let this react for 1 h to neutralize any unoccupied reactive sites. Appropriate dilutions of the sample—either standards, control, or test sera—were then added and allowed to react for 2 h.

Biotinylated anti-human IgG was diluted 2000-fold in PBS containing 2 mL of normal mouse serum per liter at room temperature 30 min before we added it to the plate and allowed it to react for 1 h. The biotinylated anti-human IgG is known to cross react with mouse globulin (personal communication, Dr. L. J. McIntyre, Vector Laboratories); adding the normal mouse serum substantially decreased the nonspecific background.

We prepared the avidin–biotinylated peroxidase complex reagent (“Vectastain ABC”) by first diluting it 100-fold in PBS at room temperature 30 min before use; just before use, we further diluted it 40-fold in PBS and let it react with the samples on the plate for 45 min. We then added peroxidase substrate to the wells and incubated with continuous mixing in a water bath at 23 ± 2 °C for 8 min. The reaction was stopped by adding H2SO4, 2.5 mol/L. We measured the absorbance of the chromagen at 492 nm.

Data reduction and analysis. Standard dose–response curves were constructed from averaged absorbance values and concentrations that had been entered into a curve-fitting four-parameter logistic program, available in a desktop computer software package from the Corning Inform Diagnostics III Data Management System (Corning Medical and Scientific, Medfield, MA 02052). The mathematical model has been described elsewhere (14). The concentration of each analyte in each sample was obtained by interpolating the averaged absorbance value from the standard curve and multiplying the result by the dilution factor; only values from the linear portions of the curves were considered valid.

We used linear regression analysis to estimate the relationship between the IgG subclasses and IgG, and to compare total IgG as measured with a monoclonal antibody vs the total IgG calculated as the sum of IgG subclass measurements. Decisions for significance were based on the F-test. We used the two-sample Student’s t-test at the 0.05 probability level to estimate the significance of sex-related differences of the immunoglobulin concentrations. For all statistical tests we used the ABSTAT™ programs (Anderson-Bell, Denver, CO 80236) in an Altos-8000 microcomputer (Altos, San Jose, CA 95134).

Results

Reactivity and Specificity of Monoclonal Antibodies

We evaluated 22 unpurified monoclonal antibodies (in ascitic fluid) that are known to react with various portions of human IgG or IgG subclasses in order to determine their reactivity as capture antibodies (Table 1). Although two of the anti-IgG1 antibodies, HP6001 and HP6012, had reacted strongly in an immunofluorometric assay against solid-phase antigens (9), only HP6012 reacted as a capture antibody in this EIA against antigen in solution. Similarly, the reactivity of the anti-IgG2 and anti-IgG4 as capture antibodies ranged widely: anti-IgG2 HP6014 was strongly reactive, but anti-IgG2 HP6002 was unreactive. Of the anti-IgG4 hybridoma products, three reacted well, four reacted moderately well, and two did not react as EIA capture antibodies. Both anti-IgG3 antibodies and all four anti-IgG antibodies performed well. For subsequent studies, we selected the most reactive monoclonal antibody in each group: anti-IgG1, HP6012; anti-IgG2, HP6014; anti-IgG3, HP6048; anti-IgG4, HP6022; and anti-IgG, HP6046.

We confirmed the specificity of the selected monoclonal antibodies in this assay configuration by using a panel of 20 purified IgG myeloma proteins (Figure 1). All paraproteins reacted specifically in the appropriate corresponding subclass assay and in the total IgG test, but very little in the
Table 1. Mouse Monoclonal Antibodies* to Human IgG and IgG Subclasses Used as "Capture" Antibodies for Total and Subclass Solid-Phase Immunoassays: Reactivity Demonstrated with Dilutions of Reference Serum

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Antigen specificity</th>
<th>Mouse isotype</th>
<th>A \textsubscript{ave} *</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP6012*</td>
<td>IgG1,Fc</td>
<td>IgG1</td>
<td>.880</td>
</tr>
<tr>
<td>HP6068*</td>
<td>IgG1,Fc</td>
<td>IgG1</td>
<td>.880</td>
</tr>
<tr>
<td>HP6091*</td>
<td>IgG1,Fc</td>
<td>IgG1</td>
<td>.040</td>
</tr>
<tr>
<td>HP6055</td>
<td>IgG1,Fc</td>
<td>IgG1</td>
<td>.020</td>
</tr>
<tr>
<td>HP6001</td>
<td>IgG1,Fc</td>
<td>IgG2b</td>
<td>.000</td>
</tr>
<tr>
<td>HP6014</td>
<td>IgG2,Fab</td>
<td>IgG1</td>
<td>.685</td>
</tr>
<tr>
<td>HP6002</td>
<td>IgG2,Fc</td>
<td>IgG1</td>
<td>.000</td>
</tr>
<tr>
<td>HP6048*</td>
<td>IgG3,hinge</td>
<td>IgG1</td>
<td>1.215</td>
</tr>
<tr>
<td>HP6047</td>
<td>IgG3,hinge</td>
<td>IgG1</td>
<td>1.100</td>
</tr>
<tr>
<td>HP6022</td>
<td>IgG4,Fc</td>
<td>IgG1</td>
<td>.810</td>
</tr>
<tr>
<td>HP6023</td>
<td>IgG4,Fc</td>
<td>IgG3</td>
<td>.800</td>
</tr>
<tr>
<td>HP6024</td>
<td>IgG4,Fc</td>
<td>IgG3</td>
<td>.700</td>
</tr>
<tr>
<td>HP6020</td>
<td>IgG4,Fab</td>
<td>IgG2a</td>
<td>.470</td>
</tr>
<tr>
<td>HP6021</td>
<td>IgG4,Fab</td>
<td>IgG1</td>
<td>.450</td>
</tr>
<tr>
<td>HP6026</td>
<td>IgG4,Fab</td>
<td>IgG2a</td>
<td>.350</td>
</tr>
<tr>
<td>HP6006</td>
<td>IgG4,Fab</td>
<td>IgG3</td>
<td>.300</td>
</tr>
<tr>
<td>HP6011</td>
<td>IgG4,Fc</td>
<td>IgG1</td>
<td>.000</td>
</tr>
<tr>
<td>HP6025</td>
<td>IgG4,Fc</td>
<td>IgG1</td>
<td>.000</td>
</tr>
<tr>
<td>HP6046</td>
<td>IgG,Fd</td>
<td>IgG1</td>
<td>1.690</td>
</tr>
<tr>
<td>HP6043</td>
<td>IgG,Fc</td>
<td>IgG2b</td>
<td>1.820</td>
</tr>
<tr>
<td>HP6017</td>
<td>IgG,Fc</td>
<td>IgG2a</td>
<td>1.250</td>
</tr>
<tr>
<td>HP6000</td>
<td>IgG,Fc</td>
<td>IgG2b</td>
<td>1.210</td>
</tr>
</tbody>
</table>

*Except for HP6046, HP6068, and HP6091, these antibodies have been characterized in detail elsewhere (9).

*Absorbance, after subtracting background, of antibody reaction with USNRP (antigen) diluted 10 000-fold to obtain concentrations (μg/L) of total IgG 1140, IgG1 695, IgG2 315; IgG3 70; IgG4 61. Assay protocol as described in Materials and Methods, except that standard curves were not used here.

Monoclonal antibodies described in ref. 15; HP numbers were previously assigned for coding. The original clones and their sources are NL16 (HP6012), Dr. R. Jeffers, University of Birmingham, Birmingham, U.K.; 2C8 (HP6068), Dr. O. Mekela and A. Sarneesto, University of Helsinki, Helsinki, Finland; and 8C6-39 (HP6091), Dr. J. Gergely, Lorand Eotvos University, Geo-physical Institute, Budapest, Hungary. HP6048 is from the Centers for Disease Control.

Three heterologous subclass tests. The low heterologous reactivity of (e.g.) IgG2 and IgG3 paraproteins in the IgG1 assay (Figure 1) are almost certainly attributable to the presence of non-paraprotein IgG, because we did not attempt to eliminate these from our preparations of the myeloma proteins.

Standard Curves

Representative standard curves for the five assays, constructed point-to-point before computer transformation, are shown in Figure 2. The detection limit for each assay corresponds to the concentration of the lowest standard. Nonspecific background, or zero concentration, absorbances were acceptably low, usually 0.15–0.25, and thereby permitted nearly full-scale (2.0 A) photometric detection of specifically formed chromogen. Figure 2a shows a typical standard curve and the computer-transformed curve for total IgG; Figure 2b illustrates representative curvilinear standard curves for each IgG subclass assay.

Precision

Table 2 summarizes the inter- and intra-assay precision determined with pooled human sera. The between-assay CV, averaged for the five tests, was 10.8%; the average within-assay CV was 2.6%.

Correlation between Measured and Calculated Total IgG in Normal Sera

We quantified the four IgG subclasses and total IgG in 63 healthy adult sera. In each case, values for the IgG subclass-
Fig. 2. Typical standard curves for monoclonal antibody-based immunosays for (A) total IgG, and (B) IgG subclasses determined with monoclonal antibodies HP6046 (anti-IgG), HP6012 (anti-IgG1), HP6014 (anti-IgG2), HP6046 (anti-IgG3), and HP6022 (anti-IgG4)

USNRP was used as the calibrant in each case, diluted four or five times starting with 10 000-fold or 100 000-fold dilutions, depending on the monoclonal antibody and the immunoglobulin subclass or total IgG. Concentrations (μg/L) refer to the working standards. Data points (and bars) represent means (and SD) of quadruplicate measurements. For clarity, bars were omitted in B, but in all cases, 1 SD was <10% of the mean. The line in A is the standard curve for total IgG, computer-transformed (see Data Reduction and Analysis) to fit the four-parameter logistic equation. IgG subclass data in B were similarly transformed for data reduction.

Table 2. Precision of Assays of Total IgG and IgG Subclasses in Pooled Sera

<table>
<thead>
<tr>
<th>Assay</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgG3</th>
<th>IgG4</th>
<th>Total IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-assay (n = 4)</td>
<td>Mean, g/L</td>
<td>5.5</td>
<td>3.1</td>
<td>0.24</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>SD, g/L</td>
<td>0.19</td>
<td>0.11</td>
<td>0.006</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>CV, %</td>
<td>3.5</td>
<td>3.4</td>
<td>2.5</td>
<td>1.7</td>
</tr>
<tr>
<td>Between assays (n = 14)</td>
<td>Mean, g/L</td>
<td>5.3</td>
<td>2.7</td>
<td>0.25</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>SD, g/L</td>
<td>0.64</td>
<td>0.14</td>
<td>0.04</td>
<td>0.075</td>
</tr>
<tr>
<td></td>
<td>CV, %</td>
<td>12.1</td>
<td>5.2</td>
<td>18.8</td>
<td>7.7</td>
</tr>
</tbody>
</table>

Human sera from 50 subjects were pooled and stored at -70 °C in single-use aliquots. On assay days, quadruplicate measurements of the pooled sera were performed in parallel with the tested sera. The between-assay values were obtained on 14 different days during six weeks; the within-assay data were all obtained on one day.

Fig. 3. Scattergram of data points, showing the comparison between measured total IgG, using monoclonal antibody HP6046, and calculated IgG (sum of IgG1 + IgG2 + IgG3 + IgG4) as measured with the antibodies listed in Fig. 2B in sera from 63 adults.

Regression analysis of the data gives: slope = 0.98; intercept = 6.7; standard error of the estimate = 13.3; correlation coefficient = 0.94, p < 0.001

Fig. 4. Frequency polygons showing the distributions of total IgG and IgG subclasses in sera from 63 healthy adults.

(all t-test values <2.000, p > 0.05), but the sera obtained from men had marginally more IgG (t statistic = 2.485, 0.01 < p < 0.05). We obtained 95 percentile ranges by a nonparametric method (16) to estimate normal reference limits for the immunoglobulins (Table 3). We also expressed the distributions of the concentrations of each immunoglobulin in terms of quartiles of the values measured for our 63 sera. Finally, we calculated the mean percentage of each subclass in the sera.

Discussion

Development of these assays required us to confront several problems that have limited both clinical and research applications of IgG-subclass measurements. Five well-characterized monoclonal antibodies to human IgG heavy-chain epitopes (9) have been adapted to a solid-phase EIA system for measuring total IgG and IgG subclasses in
Table 3. Reference Values for Measured Total IgG and IgG Subclasses in Sera from 63 Healthy Adults

<table>
<thead>
<tr>
<th>Subclass</th>
<th>Total IgG</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgG3</th>
<th>IgG4</th>
</tr>
</thead>
<tbody>
<tr>
<td>percentage of total, mean (and SEM)</td>
<td>49(1.6)</td>
<td>33(1.4)</td>
<td>9(0.4)</td>
<td>7(0.7)</td>
<td></td>
</tr>
<tr>
<td>95 percentile, g/L</td>
<td>3.8-15.0</td>
<td>1.8-7.8</td>
<td>1.0-4.6</td>
<td>0.3-1.4</td>
<td>0.08-1.8</td>
</tr>
</tbody>
</table>

*Range from 2.5 percentile to 97.5 percentile.

To normalize the concentrations, we first expressed each immunoglobulin in relation to the respective mean in the population sample. After ranking the normalized values in ascending order, we established the quartiles from the minimum and maximum values (g/L) for each IgG subclass.

human serum. Use of monoclonal antibodies provides consistency and specificity not possible with polyclonal reagents, while a detection system based on the avidin–biotin reaction enhances sensitivity. Our assay plates routinely include calibration preparations of the USNRP, for which assigned IgG subclass analyte values are traceable to unofficial calibrations (2,3) of the World Health Organization’s "primary" reference serum for IgG, IgA, and IgM (batch no. 67/97). Such standardization with a serum preparation that is available to other investigators should provide a basis for uniform calibration and interconversion of various immunoassay results (17).

Tests using 22 different monoclonal antibodies as solid-phase EIA capture antibodies revealed, as with immunofluorometric assays (9), a wide range of responses within the same anti-IgG subclass group. Use of purified monoclonal antibody instead of ascitic fluid frequently improves the results (Reimer et al., unpublished observations).

A key element in the success of these assays was that the antibodies retained their activity after being attached to a plastic surface. In previous experiments, we observed low and non-reproducible reactivity when the monoclonal antibodies were directly adsorbed onto the microtiter plates, presumably because of denaturation upon contact with the hydrophobic polystyrene surface. Precoating the plates with BSA, which forms a hydrophilic surface (13, 18)—i.e., a hydrogel—may help prevent such denaturation and thus improves the results. The addition of glutaraldehyde provides cross-linking sites for covalent attachment of antibody to the solid phase.

Performing subclass and total IgG assays in identical configurations with use of aliquots of the same sample dilutions facilitates validation of the subclass results. Unusual discrepancies between the sum of the IgG subclass concentration and the measured IgG values are immediately apparent, and this may assist in detecting technical errors such as incorrect sample dilutions. This method is preferable to comparing monoclonal antibody-based subclass data with total IgG values measured with use of a polyclonal reagent and of a method based on a different principle, such as nephelometry or radial immunodiffusion.

We found a higher value for IgG2 when calculated as the sum of the IgG subclasses than when measured in our pooled normal serum (Table 2). Although the slope of the regression line between the calculated and measured total IgG concentrations of the different normal sera was nearly 1.0 (Figure 3), there was also a slight positive bias. This may simply reflect the fact that the calculated value includes the errors of four assays, or it may indicate that the assigned mean values for the IgG or IgG subclass concentrations in the USNRP, each of which has an inter-run CV between 8.3 and 14.6% (11), are biased.

Our sample of the normal population was limited to adults. Other studies have shown significantly different IgG subclass ranges for normal children (3,19,20). The percentage subclass distributions that we found are similar to those from two large studies of adults; these studies used polyclonal antisera and different calibrators and assay procedures (2,3). We found a slightly lower mean percentage of IgG1 (49%), higher IgG3 (9%) and IgG4 (7%), and a similar IgG2 percentage (33%) as compared with those previously reported (Morrell and Skvaril (2): IgG1, 61%; IgG2, 30%; IgG3, 5%; IgG4, 4%; van der Geissen et al. (3): IgG1, 58%; IgG2, 31%; IgG3, 5%; IgG4, 5%). These differences may reflect the use of different antibodies, calibrators, or methods, or intrinsic differences in the subjects studied, or both. Our use of the USNRP eliminates at least one of these variables and allows comparison among subsequent studies in which this same calibrator is used.

The normal reference interval for total IgG and IgG1, IgG2, and IgG3 spanned approximately one order of magnitude, while that for IgG4 spanned approximately two orders of magnitude. Since our detection limit for IgG4 is 6 μg/L, approximately one order of magnitude lower than in several other studies (3,21-23), and the coefficient of variation of our IgG4 assay is comparable with that for the other subclasses, this broad range may reflect greater biological variation in the immunogenetics or immunoregulation of this subclass.

These precise immunoassays should facilitate studies of the clinical significance of IgG subclasses. Fifty microliters of serum is sufficient to complete all five immunoglobulin tests. This micro-sample volume requirement, stemming from the assay sensitivity, is especially desirable for testing sera from children. In addition, such samples as bronchoalveolar lavage fluid and cell culture supernatants, which contain very low concentrations of IgG, may be assayed.

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