References

Nephelometry of Human IgG Subclass Concentration in Serum
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An immunoassay method for determination of human IgG subclass concentration by rate nephelometry was developed by using the Beckman Immunochemistry Analyzer, subclass-specific antisera, and a human serum standard. Twelve sera derived from normal as well as gammopathological patients were analyzed. The total IgG concentration, as determined by using a readily available kit, correlated with the sum of the concentration of the individual subclasses. Most of the gammopathological cases were shown to be of the IgG1 subclass, which was confirmed by radial immunodiffusion.

Additional Keyphrases: rate nephelometry - immunglobulins - radial immunodiffusion - Immunochemistry Analyzer

The concentration and percentage distribution of the human IgG subclasses in serum has heretofore been determined by radial immunodiffusion (1–5). This method is not only costly because of the amount of antiserum required, but also quite time-consuming.

Human IgG subclasses have been quantitatively determined by radioimmunoassay (6), which affords a savings on antiserum but is still rather costly. Mann et al. (7) were able to test the IgG subclass distribution by means of a radioimmunoassay whereby a greater degree of accuracy can be obtained with a lesser amount of specific antiserum, but the absorbancy of the antigen posed problems then and still does so today.

All of the above-cited studies have contributed to a better understanding of the function of the IgG subclasses, and in some cases have aided diagnosis. In other instances the correlation of a specific subclass to a specific antigen has led to interesting and stimulating results, which could explain the recurrent infections that occur in patients with low concentrations of a specific subclass.

Schur et al. (8) reported that recurrent pyogenic infections were due to low concentrations of IgG1, -2, and -4. In addition, the question regarding which subclass contains the antibodies against tetanus toxoid has not been completely clarified. Carrel et al. (9) showed that IgG1 carried the major part of the antibody activity, followed by IgG2, -3, and -4, whereas van der Giessen et al. (10) restricted this activity to IgG1. Recently, Beck (11) was able to confirm previous reports and show that there is a high antiviral activity among the IgG3 subclasses, and that IgG3 exhibits no anti-tetanus activity.

All of these studies carried out by the gel diffusion method have an inherent error of at least 10%, if not more.

It is clear from this cursory overview that a reliable, relatively inexpensive, fast method for determining IgG subclass concentrations would soon find ready application in the clinical laboratory, where early diagnosis of gammopathological states would become possible if sufficient subclass-specific antisera could be produced. Also, these studies could shed light on deficiency states, which could explain the antigen-binding characteristics of the various subclasses. In addition, the percentage subclass distribution in the various commercial immunoglobulin preparations—our main task at the Paul Ehrlich Institute—would validate or perhaps even invalidate their use in immunoglobulin therapy. One such study has been reported (4), and our recent study (12) has been accepted for publication.

Materials and Methods
The Immunochemistry Analyzer, reagent test kits, and nephelometric-grade isotonic saline were purchased from Beckman Instruments, Munich.

Subclass-specific antisera and the human serum standard H00-02 were purchased from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and diluted with nephelometric-grade isotonic saline.

Serum samples were collected from donors at the Paul
Ehrlich Institute. The gammapathological sera were supplied by Dr. Stephanescu (Frankfurt University Clinic).

Serum. All normal sera were collected without preservatives and allowed to clot overnight before centrifugation at 357 × g for 15 min. Preliminary trials whereby the serum lipids were removed by Frigen 113-TR-T treatment did not improve the accuracy of determination of the subclasses.

Ouchterlony plates. Agarose gels (10 g/L) containing polyethylene glycol (40 mL/L) were run, with use of subclass-specific antisera for qualitative identification of the subclasses in all of the samples, and to identify the specific subclass affected in the gammapathological sera.

Total IgG. IgG Reagent Test Kits (Beckman) were used to determine the total IgG concentration of the sample sera. A sample mean was calculated from at least 10 trial runs.

Manual mode of rate nephelometry. Using the method reported by Baldwin et al. (13) as a guideline, we calculated optimal sample, standard, and antibody dilutions (see manual for Manual Mode Operations, Beckman) and also experimentally determined them. The appropriate manual mode card (M-44) was selected because it produced a range of 100 to 700 peak rate units for the lowest calibrator (standard) concentration and the highest calibrator concentration, respectively.

All tests were done at a 10-fold calibrator dilution with nephelometric-grade saline, polyethylene glycol (PEG), 40 mL/L in phosphate buffer (Beckman), in the reaction cell, and sterile filtration of the standard and sample preparations after dilution. Subclass specific antisera were diluted with Beckman nephelometric-grade isotonic saline as prescribed by that firm, and sterile-filtered.

Results

Because formation of light-scattering complexes depends on the presence of antigen and antibody molecules in optimal proportions, for a constant amount of antibody the degree of complexation increases with the amount of antigen present up to a maximum, beyond which larger amounts of antigen result in progressively less complexation. This phenomenon is exploited in rate nephelometry by maintaining a constant...
antibody concentration and measuring the peak rate signal generated in the presence of PEG (40 mL/L) with increasing concentrations of antigen, in order to construct a standard reference curve based on peak rate units for the calibrator (standard) (13). By definition, a peak rate unit represents the degree of light-scattering generated per unit time during the formation of immune complexes.

Using the manual for manual mode operations, one can calculate the concentration of standard for each of the subclasses from the values supplied by the source of the serum standard, thereby obtaining the actual concentration in the reaction cell.

The human serum standard (HO0-02)—designated as containing, per liter, 6.20 g of IgG1, 3.20 g of IgG2, 0.54 g of IgG3, and 0.64 g of IgG4—was diluted serially from 1:5 through 1:625. By the calculation methods stated above, the total IgG concentration range in the reaction cell was from 130 mg/L to 1 mg/L. Through the use of selected manual mode cards designed for given levels of signal amplification, and by varying the concentration of the standard (antigen) while the antibody concentration is maintained constant, a standard reference curve can be developed for each of the subclasses.

Reference curves so obtained for IgG1, IgG2, IgG3, and IgG4 are shown in Figure 1. The curves for each subclass reflect their percentage distribution in the standard, i.e., they exhibit a characteristic shape for each subclass, depending on their reactivity as measured in the presence of PEG (40 mL/L) in the Beckman Immunochemistry Analyzer. At a 10-fold dilution of the standard a total IgG sample concentration of 65 mg/L being injected into the reaction cell was calculated, which, when converted by the percentage distribution of the subclasses in the standard, becomes 38 mg/L for IgG1; 19.6 mg/L for IgG2; 3.3 mg/L for IgG3; and 3.9 mg/L for IgG4.

Using the antibody (anti-IgG1, etc.) dilutions of six- and 36-fold for IgG1 and varying the concentration of the standard from 1:1 through 1:45, we constructed the reference curve for IgG1 and determined the measuring range. In a like manner an antibody dilution of 1:36 to 1:216 was used for IgG2 and 1:216 to 1:1296 for IgG3 and IgG4. The manual mode card M-44 produced the best signal amplification, lying in the range of 100 to 700 peak rate units throughout the dilution series for all subclass reference curves. Two antibody dilutions are necessary to determine the lower and upper limits of the measuring range based on peak rate units.

The upper portion of the manual mode calibration curves—i.e., between 500 and 700 peak rate units for each subclass—approaches linearity and can serve as a measuring range for determining the individual concentrations of each subclass measured against the standard and run at the same dilution for the standard and the sample, while maintaining dilutions of the antisera as shown in the figures.

By selecting the dilution range hereafter designated measuring range (m.r.), wherein each of the curves approaches linearity, the best sample dilution for determining IgG1, 2, 3, is between five- and 10-fold, whereas fivefold is the only sample dilution that could be used to determine the concentration of IgG4.

The overall measuring range of dilutions for IgG1 was found to lie between 1:14 and 1:10; for IgG2 1:12 to 1:4; for IgG3 1:12 to 1:5; and for IgG4 1:10 to 1:5, as shown in the figure

Table 1 shows results obtained from serum samples taken from normal donors. The IgG1 and IgG2 subclasses varied most, with IgG3 being fairly constant. The age of the donors ranged from 20 to 60 years, a factor we consider important in developing a reliable method.

Table 2 is a compilation of results obtained from patients in various stages of gammopathy. Not only the IgG gammapathological probands were chosen but also those presenting with above-normal values for IgA and IgM. Table 2 also shows the concentration of all three gamma globulins, IgG, -A, and -M.

**Discussion**

One of the drawbacks involved in rate-nephelometric de-
termination of the concentration of IgG subclasses in some sera, and especially gammapathological sera, centers around the fact that monoclonal subclass species do not react with their equivalent antibody in the antiserum as readily as the same subclass does in normal sera.

It was found during this study that if one waits at least 30 to 45 seconds after injecting the serum sample into the reaction cell before injecting the antiserum sample, this produces more accessibility or reactivity of the monoclonal protein subclass antigen determinants, resulting in faster and more reliable and reproducible peak verification. A similar finding was reported by Baldwin et al. (13) in determining the concentration of low-density lipoprotein. Also, the excess scatter signal has to be overridden by removing and replacing the reaction cell before injecting the antiserum sample.

Utilizing a standard recorder in conjunction with the analyzer, the curves shown in Figures 1 can readily be constructed by retracing the original trial run for each dilution and, once constructed, this curve can be used for determining the concentration of the sample when the appropriate dilution is found and converted to mg/L. A recorder with a low noise level should be chosen for this purpose.

Once the standard reference values have been established, and the appropriate curves, sampling can proceed at one per minute. No difference was observed whether the serum samples had been frozen or were freshly collected. Delipidization of the samples was not found to be necessary and did not produce better results.

A series of at least 150 samples can be run with one batch of antiserum (1 mL per subclass) and the corresponding standard. Filtration through a 0.45-μm sterile filter tends to speed up the peak-verifying process without affecting accuracy.

The probands chosen for this study represented an age cross-section, which was considered an important factor in establishing a reliable method to be used in clinical settings because of a probable difference in the reactivity of the subclasses with age.

The coefficients of variation (CV) shown in the tables were calculated from a series of 10 trials and represent within-run variation.

In summary, it can be said that any immunochemistry analyzer that utilizes rate nephelometry can be adapted to determine IgG subclass concentrations if subclass-specific antisera with a high degree of monospecificity can be procured.

Routine clinical determination of IgG subclass concentrations will undoubtedly contribute to the early diagnostic procedures.

We are grateful to Dr. Gerhard Treitler, Product Line Manager, Beckman Instruments, Munich, for his cooperation and helpful assistance in adapting the Beckman Immunochemistry Analyzer to subclass determination. We thank Dr. Stéphaneescu for supplying the serum samples from the Frankfurt University Clinic.

References