IgG Subclass Concentrations in Certified Reference Material 470 and Reference Values for Children and Adults Determined with The Binding Site Reagents

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Background: There is currently no international reference preparation for IgG subclass (IgGSc) quantification. This situation has led to calibration differences among assays and a variety of reference interval values with consequential difficulties in comparing results. We therefore evaluated IgGSc concentrations in Certified Reference Material 470 (CRM 470).

Methods: Pure, polyclonal IgG1, -2, -3, and -4 were prepared from a large serum pool for use as primary standards. The IgG mass in each preparation was calculated from amino-acid analysis data. IgGSc concentrations were assessed in CRM 470 by nephelometry with modern analytical techniques, using these reference preparations. Subsequently, IgGSc concentrations were measured in 380 healthy individuals (250 males and 130 females), and age-dependent reference intervals were established.

Results: IgGSc concentrations in CRM 470 were as follows: IgG1, 5028 mg/L; IgG2, 3418 mg/L; IgG3, 579 mg/L, and IgG4, 381 mg/L, with a total IgG concentration of 9406 mg/L, 2.83% below the certified total IgG value of 9680 mg/L. Age-dependent percentile curves for the four IgGSc were constructed using a Box–Cox transformation. Maximum median values were as follows: IgG1, 6.02 g/L at 11 years; IgG2, 3.45 g/L at 31 years; IgG3, 0.63 g/L at 17 years; and IgG4, 0.48 g/L at 14 years. No significant sex-related differences were observed.

Conclusions: The correlation between the summation of individual IgGSc and separate measurements of total IgG concentrations was good and supports the accuracy of the results. The results are based on The Binding Site assays and should not be considered appropriate for other assays unless so demonstrated.

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In 1970, selective IgG subclass (IgGSc)9 deficiency was shown to be associated with increased susceptibility to upper respiratory tract infections (1). Since then, IgGSc measurements have become an established tool in the differential diagnosis of immunologic deficiencies. However, no universally accepted reference material currently exists, and therefore, standardization of results is unsatisfactory. This has led to poor consensus with regard to reference interval data and assessment of abnormal results.

The published IgGSc values in serum and reference materials and the methods used for their determination

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9 Nonstandard abbreviations: IgGSc, IgG subclass; CRM, Certified Reference Material; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide electrophoresis; RID, radial immunodiffusion assay; and BNA, Behring Nephelometric Analyzer.
are shown in Table 1. The results indicate considerable variation in both the concentrations of the individual IgGSc and their proportion as a percentage of the total IgG in each protein preparation. Some of these variations might be attributable to the use of monoclonal immunoglobulins, which by their very nature may be idiosyncratic, as primary standards (2–5). Another cause of inaccuracy has been the use of relatively impure primary standards. These factors may have led to disparity in results that should be substantially similar, such as those based for WHO 67/97. Of particular note are the values ascertained by Klein et al. in 1985. These were ratified by the WHO in 1987 (6). They may be attributable to the use of relatively impure primary standards. These factors may have led to disparity in results that should be substantially similar, such as those based for WHO 67/97. Of particular note are the values ascertained by Klein et al. (5) in 1985. These were ratified by the WHO in 1987 (6) and are used in many centers. However, the IgG3 concentrations are the lowest of all reported values and lead to an inverted IgG3/IgG4 ratio. This is unlikely to be correct, based on the reported frequencies of the different IgGSc in patients with multiple myeloma (7, 8) and the discordance with other studies. Because there is evidence of IgG3 instability in WHO 67/97 (5), this could account for the results. Turbidity after reconstitution (9) also makes the material unsatisfactory for standardization of nephelometric or turbidimetric assays.

In 1992, the College of American Pathologists and The Bureau Communitaire de Référence released “The Reference Preparation for Proteins in Human Serum” or “Certified Reference Material 470” (CRM 470). This has been accepted as The International Reference Preparation for proteins in human serum (9–12). In this material, each of the 15 calibrated analytes, including IgG, has demonstrated long-term stability. On the assumption that the IgGSc proteins are also stable, CRM 470 should be a suitable reference preparation for these proteins. The purpose of this study was to assess IgGSc concentrations in CRM 470, using modern analytical techniques, and to evaluate reference intervals for healthy children and adults.

### Materials and Methods

**IgGSc value assignment to CRM 470**

**Purification of primary standards.** IgGSc proteins were purified from a pool of serum from >200 healthy blood donors. Semipurified IgG was passed through a protein A-Sepharose column. This bound the IgG1, -2, and -4, whereas the bulk of the IgG3 passed through the column. IgG1, -2, and -4 were eluted from the protein A and further purified by positive immunoaffinity chromatography. Subsequently, the IgGSc preparations were subjected to repeated negative immunoaffinity chromatography until the purity was >98% (see below). IgG3 was purified in a similar manner except that the first step was positive immunoaffinity chromatography followed by negative immunoaffinity chromatography. IgG3 was not purified from the protein A column flow-through because G3(m)s and G3(m)3,t allotypes bind to the column (13).

**Purity assessment.** Sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS-PAGE) (14) was used to assess the purity of the IgGSc preparations. The proteins were visualized by staining with Coomassie Brilliant Blue R 250, and the percentage purity of each preparation was determined by scanning densitometry. Contamination of each IgGSc preparation by other IgGSc proteins was assessed by sandwich ELISA using monoclonal IgGSc antibodies. The capture antibodies were as follows: IgG1, clone C8C6; IgG2, clone HP017; IgG3, clone DAG3A; IgG4, clone GB7B (15, 16).

**Stability.** The stability of each protein preparation was assessed by radial immunodiffusion assays (RIDs) using
specific polyclonal antibodies (The Binding Site). The concentration of each preparation was measured 12 times at day 0 and after storage for 4 weeks at 4 and −20 °C, and the mean values were compared.

Amino acid analysis. The preparations were subjected to acid hydrolysis (6 mol/L HCl) at 110 °C for 24 h (17), and the concentrations of each amino acid were quantified by the ninhydrin/ion-exchange method (18). This was performed on 14 amino acids, and the data from the remaining 6 were disregarded because they produced unreliable results.

IgGSc amino acid composition and calculation of IgGSc concentrations. All amino acid data were obtained from the Internet-based database Swiss-Prot (19). There is no single correct amino acid sequence for immunoglobulin molecules because of allotypic variations (20) and genetic differences in the variable region domains. A mean amino acid composition was therefore calculated from sequences for the constant region domains and 30 sequences for the variable region domains. We also made allowance for allotypic variations by assessing their respective frequencies and weighting the average results appropriately. We also assumed that the κ/λ ratio was 0.7 for each IgGSc. Because of their similar amino acid composition, changes in the ratio made no significant difference to the final results. The concentration of each IgGSc in each pure preparation was calculated independently from the 14 amino acid determinations, and mean results were obtained.

IgGSc value assignment to CRM 470. The protocol used for IgGSc value assignment was based on the IFCC value-transfer protocol (12). Dilutions were monitored on a balance, and compensations for errors in dilution were made. Using CRM 470 with arbitrary values for each IgGSc, we constructed a calibration curve for each IgGSc assay on a Behring Nephelometric 228 Analyzer (BNA; Behringwerke AG), using antisera designed for use on the BNA (The Binding Site Ltd.). The pure IgGSc preparations were assayed in duplicate at five dilutions, and this was repeated three times on 5 separate days. The mean values for these measurements were then used to calculate the concentration of each IgGSc in CRM 470.

Measurement of IgGSc in healthy individuals. Before assays all samples were stored at −20 °C. Total IgG and individual IgGSc were assayed with an IgGSc BNA Kit (The Binding Site). Measurements were standardized against the CRM 470 IgGSc concentrations described above.

Study population. The study population included 312 clinically healthy children (214 males and 98 females) from 6 months to 18 years of age who were admitted to hospital for minor surgery. Informed consent was obtained from the parents for all participants. The adult group included 68 healthy blood donors (36 males and 32 females), 20–61 years of age. Only sera from individuals who were free of recurrent infections or inflammation were used. Good health was confirmed by a clinical questionnaire and serum C-reactive protein concentrations within the reference interval.

Statistics. The Box–Cox transformation method was used to adjust data to a gaussian distribution (21). An iterative process for each set of results identified the appropriate transformation. These were then organized into 20 groups according to the ages of the participants. Percentiles (2.5, 10, 25, 50, 75, 90, 97.5) were determined from the mean and the SD of the transformed data and transformed to the original scale. Polynomial regressions of the percentiles with age were then produced for each IgGSc.

Results. SDS-PAGE analysis of the IgGSc preparations indicated that none had any visible contamination with non-IgG molecules (Fig. 1). By ELISA the contamination for each preparation was as follows: IgG1, 0.5%; IgG2, 1.9%; IgG3, 0.5%; IgG4, 1.5%. None showed significant deterioration during storage for 1 month at −20 or 4 °C. The IgGSc concentrations in CRM 470 were as follows: IgG1, 5028 mg/L; IgG2, 3418 mg/L; IgG3, 579 mg/L; IgG4, 381 mg/L. The sum for the IgGSc concentrations was 9406 mg/L, 2.83% below the certified IgG value of 9680 mg/L.

The reference interval studies indicated that the concentrations of each IgGSc and total IgG were strongly age-dependent as described previously (Fig. 2). Concentrations increased with age and then reached a maximum.
and a plateau for IgG2 and -3, whereas IgG1 and -4 subsequently decreased. On the basis of the 50th percentile curve, the maximum values for each subclass were as follows: IgG1, 6.02 g/L at 11 years; IgG2, 3.45 g/L at 31 years; IgG3, 0.63 g/L at 17 years; and IgG4, 0.48 g/L at 14 years. We observed good correlation ($r = 0.96$) between the sum of the four subclasses and total IgG (Fig. 3). There were no significant sex-related differences.
Discussion

We ascribed IgGSc values to CRM 470 by use of nephelometry and highly purified preparations of polyclonal IgGSc proteins. Evidence for the accuracy of the results is based on several factors. The high purity of the IgGSc preparations allowed an accurate estimation of protein content. The purity was assessed by SDS-PAGE and ELISA analysis, methods that are considerably more sensitive than those used in previous studies. Protein content was, for the first time, determined by amino acid analysis. Variations in the amino acid sequences of IgG were taken into account by use of the mean amino acid composition of all available variable region sequences and all allotypes of IgG1, IgG2, and IgG3. Finally, modern nephelometric assays were used to provide good precision in the value assignment protocol, which was demonstrated by the low CVs for assigned values on 15 replicates: IgG1, 4.1%; IgG2, 3.4%; IgG3, 3.9%; and IgG4, 6.3%. The sum of the IgGSc values compared well with the certified total IgG ascribed to CRM 470, with a difference of 2.83%.

Several studies have reported IgGSc concentrations in healthy children and adults. Most of the studies used RIDs and various reference materials (22–27). In this study, we used automated nephelometry to measure IgGSc values in healthy children and adults, using the described CRM 470 calibration. The results were broadly similar to those reported for previous studies with the concentrations of IgG3 being greater than those of IgG4. The largest difference was seen for IgG1. The peak median IgG1 in this study was 6.0 g/L (Table 2) compared with >7.0 g/L in previous studies. The good correlation between the sum of the IgGSc and the measured value of total IgG supports the accuracy of the analysis produced.
in this study. It should be noted that the results are based on The Binding Site assays and should not be considered appropriate for other assays unless so demonstrated.

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References