

Methodologic problems in establishing normal values for IgG subclass concentrations in a pediatric population; comparison of radial immunodiffusion and ELISA methods

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The aim of this study was to establish an enzyme-linked immunosorbent assay (ELISA) to measure IgG subclasses by means of monoclonal antibodies. The distribution of IgG subclass protein concentrations in sera from 227 healthy Danish children and 90 adults was measured. Furthermore, this newly established ELISA was compared with different assay systems for determination of IgG subclasses: two radial immunodiffusion methods (RID), one using polyclonal and one using monoclonal antibodies, as well as a commercially available ELISA kit. There was good agreement of results obtained by the different methods of measuring IgG3 and IgG4 concentrations. There was good correlation between results obtained by both RID methods. Despite good correlation between the assays, the ELISA kit showed higher levels of IgG1 in all investigated sera, and the ELISA kit showed no correlation with the other methods, when IgG2 was measured. Analysis of the normal ranges measured by ELISA developed in our laboratory and by RID with polyclonal antibodies showed that the levels obtained by RID were higher than those obtained by our ELISA in sera with low levels of both IgG1 and IgG2, and lower in sera with high concentrations of these two immunoglobulins. Our results emphasize the importance of establishing age-related normal limits for any novel assay measuring IgG subclass concentrations.

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Human IgG comprises four subclasses based on antigenic differences in their heavy polypeptide chains (11). The quantitation of IgG subclass proteins in human sera is of interest in the evaluation of patients with recurrent pyogenic infections or other symptoms of immunodeficiencies, atopic diseases, and certain respiratory disorders (7, 12, 14). Measurement of IgG subclass levels has mainly relied on methods of radial immunodiffusion (RID). Production of murine monoclonal antibodies specific for unique determinants on four IgG subclasses has made it possible to establish a more sensitive method, particularly useful to measure IgG subclasses in body fluids such as cerebrospinal fluid, tears, etc. (4, 6). Defining the normal reference interval for a given population from published IgG subclass values is

difficult. Study groups and methods vary in reports. Different statistical analyses are used, especially for the reported differences in the frequency distributions of the IgG subclasses (3, 5).

The purposes of the present study were as follows: 1) to establish an enzyme-linked immunosorbent assay (ELISA) using IgG subclass-specific monoclonal antibodies for the quantitation of IgG subclasses, 2) to study the distribution of IgG subclass concentrations in sera from normal Danish children and adults, and 3) to compare values obtained by this method with those obtained by a commercially available ELISA kit, as well as with those obtained by two RID methods, one using monoclonal, and one using polyclonal subclass-specific antibodies.

Material and methods

Sera

Sera from healthy children and adults were kindly supplied by the Tissue-Type Laboratory and Blood Bank, Rigshospitalet, Copenhagen, Denmark. All sera were collected during the years 1982–90 and stored at -20°C . Serum samples from 227 children aged 6 months to 15 years and 90 adults were examined by ELISA developed in our laboratory, and 170 of these sera were also assayed by RID with polyclonal antibodies. Among these sera, 34 were randomly selected for additional testing in RID with monoclonal antibodies. For investigation of the commercially available ELISA kit for IgG subclass determination, 17 of these 34 sera were tested. In all the assays, a reference serum (Janssen Biochimica, Beerse, Belgium), calibrated against the World Health Organization standard 67/97, was used as the standard.

Quantitation of IgG subclass proteins by RID

The IgG subclass concentrations were determined by RID with sheep IgG subclass-specific antisera (Janssen Biochimica), as described earlier (3).

The RID method using commercially available monoclonal IgG subclass antisera (Unipath, Bedford, UK) – HP6007, HP6008, HP6010 for IgG1–3, respectively, and a mixture of HP6011 and HP6013 for IgG4 – was applied as described by the manufacturer, with some modifications. Briefly, 66 μl anti-IgG1, 66 μl anti-IgG2, 30 μl anti-IgG3, and 33 + 33 μl of the two different anti-IgG4 were mixed with 5 ml 1% (w/v) LSM agarose (Litex, Glostrup, Denmark) containing 3.5% (w/v) polyethylene glycol (PEG) 6000 (Merck, Darmstadt, Germany) in 0.15 mol/l NaCl. The gels were cast on 5 × 5 cm glass plates, and 16 holes were punched with a 2-mm puncher. A four-point calibration was used, and the standard serum was diluted 1 + 1, 1 + 4, 1 + 9, 1 + 19 for IgG1 determination; 1 + 1, 1 + 5, 1 + 11, 1 + 23 for IgG2; and undiluted, 1 + 1, 1 + 3, 1 + 7 for IgG3 and IgG4 determinations. Test sera were diluted 1 + 4 for IgG1 and IgG2 determinations, and were used undiluted for IgG3 and IgG4 determinations. A volume of 3 μl standard serum dilution or diluted serum was used. Only single determinations were done. The plates were incubated for 72 h at 4°C in a humid chamber, fixed with 1% glutaraldehyde for 30 min, and washed with 0.15 mol/l NaCl. After pressing, the gels were dried and stained with Coomassie brilliant blue. The vertical and horizontal diameters of each precipitate were read before and after staining on a Behring Messprojector[®], and a calibration curve was constructed.

Quantitation of IgG subclasses by ELISA

Polystyrene 96-well microtiter plates (Maxisorb, Nunc A/S, Roskilde, Denmark) and reagent volumes of 100 μl were used. All incubation steps were done at 22°C . Between each step, plates were washed in phosphate-buffered saline (PBS) with 0.1% (v/v) Tween 20 (PBS-Tween). Plates were coated with rabbit antibody against mouse IgG (Z259, Dakopatts A/S, Glostrup, Denmark), diluted 1:2000 in PBS, and incubated for 1 h.

Additional binding sites were blocked by incubation overnight with PBS-Tween containing 1% (v/v) fetal calf serum (inactivated at 56°C for 30 min) and 0.5 M NaCl. This blocking solution was also used as dilution buffer. The assay conditions were chosen to minimize nonspecific binding.

Monoclonal antihuman IgG1 (HP6012), IgG2 (HP6008), IgG3 (HP6010), and IgG4 (HP6011) from Unipath (Bedford, UK) were diluted 1:1000 in dilution buffer and incubated for 1 h.

Standard serum and test serum samples were diluted in dilution buffer with 1% (v/v) mouse serum added and incubated for 1 h with shaking. All samples were tested in duplicate and at two different dilutions.

Horseradish peroxidase-labeled rabbit antibody against human IgG (P214, Dakopatts A/S) was diluted in dilution buffer with 1% (v/v) mouse serum, and the dilutions used were 1:2000 for IgG2 assay, 1:3000 for IgG1 and IgG4 assays, and 1:10000 for IgG3 assay.

Sodium citrate chloride (0.1 M, pH 5.0), containing 1,2-phenylenediamine-dihydrochloride (2.2 mM) (Sigma) and H_2O_2 (6.5 mM), was added to each well, and the enzyme reaction was stopped by addition of H_2SO_4 (1M) after incubation in the dark for 30 min.

The optical density at 492 nm (OD_{492}) was read on an automatic plate reader (Bio-Rad, Richmond, CA, USA). Calibration curves for each IgG subclass were constructed.

Both the monoclonal antisubclass antibodies and polyclonal rabbit antihuman IgG antibodies used in the assay are directed toward the Fc part of human IgG, and competition for binding sites may exist. To ensure that competition for binding sites on the IgG Fc part did not influence the measurement of IgG subclass concentration, we tested our assay with antibodies against light chains on IgG molecules (kappa and lambda, P129 and P130, Dakopatts A/S). Twenty sera were tested by ELISA with the antibody against the Fc part of IgG, and, in another assay, with the antibody against the kappa and lambda chains, and the results were compared. The correlation coefficient between results obtained by these different antibodies was 0.69 ($P = 0.0025$).

The human IgG subclass ELISA kit (code 25.661.53, Janssen Biochimica, Belgium) was used for IgG subclass determinations in 17 human sera. The kit was used according to the manufacturer's instructions.

Quantitation of total IgG concentration

The total IgG concentration was determined by RID in plates with 1.5 µl rabbit antihuman IgG, γ-chain-specific antibodies/cm² (Dakopatts, code no. 090 (3)).

Assessment of specificity of the monoclonal antibodies against human IgG subclasses

The specificity of the monoclonal antisubclass antibodies has been established previously (8). We confirmed the specificity of the selected monoclonal antibodies in our ELISA assay by using a panel of eight purified IgG myeloma proteins (Binding Site, Birmingham, UK). Monoclonal antisera against IgG subclasses have been found to be restrictive and not to detect all determinants within the IgG subclass (13). The monoclonal antibodies used in this study have been tested for their ability to react with a wide assortment of myeloma proteins with different allotypes (8). To ensure that different assay conditions did not alter the specificity of the monoclonal antibodies used, we determined IgG subclass concentration in sera with different allotypes in our ELISA assay. We used seven sera homozygous for Gm(3;5), seven sera homozygous for Gm(1,17;21), and six sera heterozygous for these markers; nine sera positive for Gm(23) and 11 negative for that marker.

Statistics

Correlations between IgG subclass protein concentrations measured by different assays were assessed by the Spearman rank sum correlation test. The level of significance was 5% (two-tailed). The day-to-day variations were determined for the assay developed in our laboratory by the formula $SD = \sqrt{(\Sigma d^2/2n)}$, where SD is the standard deviation, Σd^2 is the sum of squared differences of double determinations of the same sample, and n is the number of observations.

Results

Specificity, detection limits, and precision of the IgG subclass ELISA assay

All paraproteins reacted specifically in the appropriate corresponding subclass assay, but only marginally in the heterologous subclass tests (Fig. 1). The low heterologous reactivity of paraproteins was due

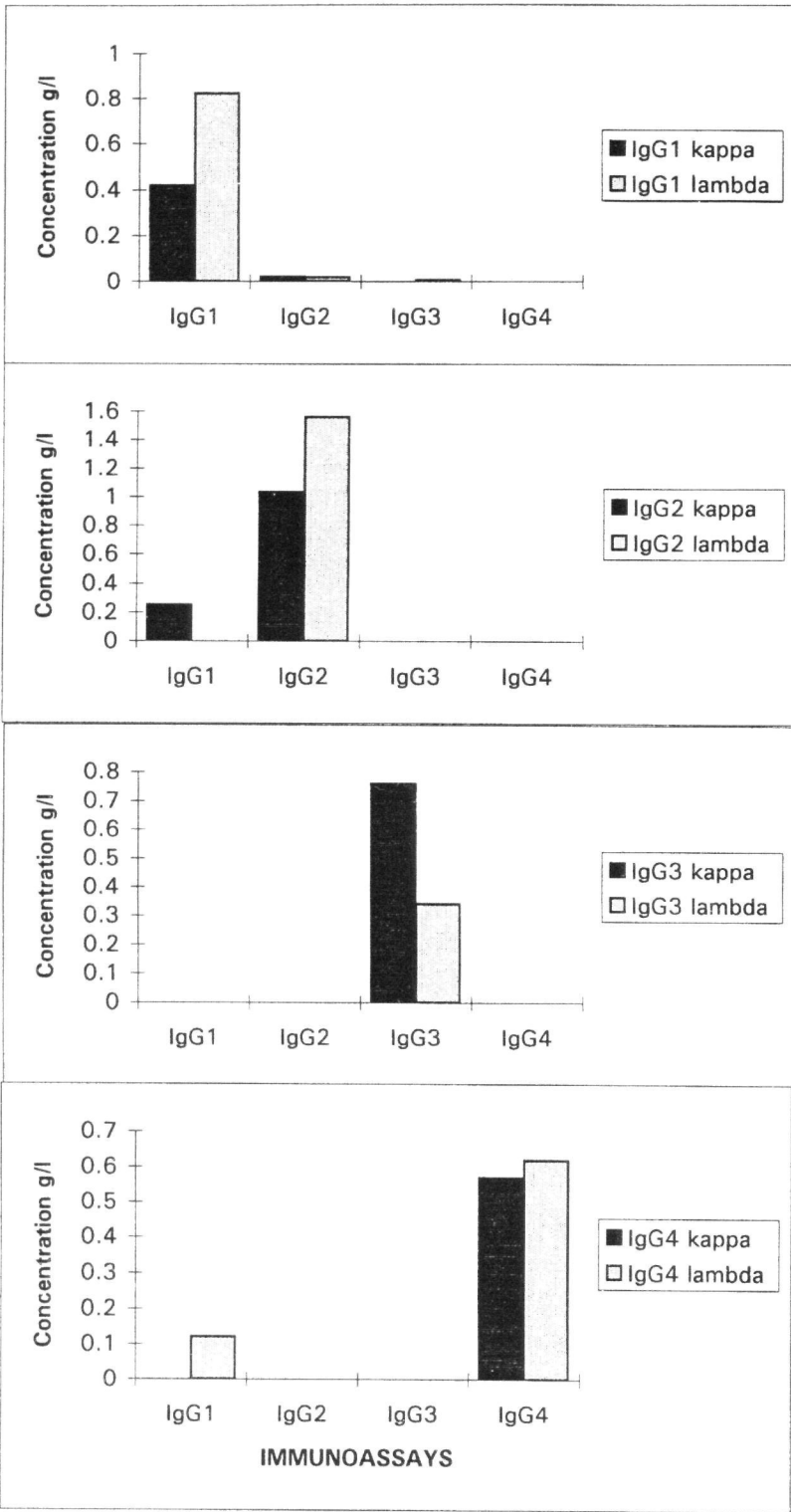


Fig. 1. Specificity of immunoassays for IgG subclasses. Eight purified IgG myeloma proteins of known subclass were assayed.

to the presence of small amounts of nonparaprotein IgG. Twenty sera with different Gm allotypes have been measured in our ELISA assay. No skewness in IgG subclass distribution in different Gm allotype groups was observed (data not shown). The detection limits for each assay are summarized in Table 1.

Table 1. IgG subclass ELISA characteristics

Assay specificity	Antibodies	Sensitivity limits (mg/ml)	Day-to-day variation
IgG1	HP6012	0.07	10.3%
IgG2	HP6008	0.13	12.2%
IgG3	HP6010	0.002	9.4%
IgG4	HP6011	0.006	16.2%

The day-to-day variation for determinations of subclass concentration in 10 different sera was between 9.4% and 16.2% (Table 1).

Correlations between IgG subclass concentrations determined by four different methods

The sum of the four IgG subclass measurements obtained by all investigated assays correlated significantly with the total IgG concentration ($P < 0.03$) (data not shown). The correlation coefficients (r) between IgG subclass determinations are shown in Table 2.

IgG1 assays. There were significant correlations among results obtained in all investigated methods, although the correlation coefficients were low between results obtained by our ELISA and the other methods. Despite good correlation, results obtained by the commercial ELISA kit overestimated the

concentration of IgG1 in all sera investigated, as compared with results from the other assays (data not shown).

IgG2 assays. The results obtained by the commercial ELISA kit did not correlate with results obtained by any other methods. There was a good correlation between results obtained by our ELISA, the RID using polyclonal antibodies, and the RID using monoclonal antibodies.

IgG3 assays. There was significant correlation among IgG3 results obtained by all methods investigated.

IgG4 assays. There was highly significant correlation among IgG4 results obtained by all methods investigated.

Distribution of IgG subclass levels in healthy children and adults measured by ELISA

The distribution of IgG subclass protein concentrations expressed as medians and 90th percentiles is shown in Table 3. IgG of all subclasses was detectable in all subjects. IgG1 and IgG3 concentrations started to increase from the first year of life and continued to increase with increasing age. The maturation of serum IgG2 and IgG4 concentration was much slower. In the age group of 10–14 years, the IgG2 and IgG4 levels were significantly higher than in younger children. A comparison of IgG subclass ranges in males and females showed no significant differences.

Distribution of IgG subclass levels in healthy children and adults measured by RID with polyclonal antibodies

The distribution of IgG subclass protein concentrations in sera from 117 healthy children and 53 healthy

Table 2. Correlation of IgG subclass determinations by different assays

	RID (Polyclonal Ab) $n=170$	RID (Monoclonal Ab) $n=34$	ELISA kit $n=17$
IgG1 assays			
ELISA	0.53 ^a (0.0003)	0.35 (0.04)	0.58 (0.02)
RID (Polyclonal Ab)		0.84 (0.0001)	0.93 (0.002)
RID (Monoclonal Ab)			0.84 (0.0002)
IgG2 assays			
ELISA	0.72 ^a (0.0001)	0.48 (0.008)	0.41 NS
RID (Polyclonal Ab)		0.68 (0.0001)	0.46 (0.07)
RID (Monoclonal Ab)			0.47 (0.07)
IgG3 assays			
ELISA	0.81 ^a (0.0001)	0.78 (0.0001)	0.72 (0.05)
RID (Polyclonal Ab)		0.84 (0.0001)	0.91 (0.0004)
RID (Monoclonal Ab)			0.92 (0.004)
IgG4 assays			
ELISA	0.98 ^a (0.003)	0.92 (0.0001)	0.82 (0.016)
RID (Polyclonal Ab)		0.65 (0.0002)	0.99 (0.003)
RID (Monoclonal Ab)			0.91 (0.0004)

^a r (P value).

NS=not significant.

Table 3. Medians and 90th percentiles in mg/ml of IgG subclass protein concentrations in sera from 233 healthy children and 90 adults measured by ELISA

Age (years)	n	IgG1	IgG2	IgG3	IgG4
< 1	12	2.7 1.6–5.4	0.83 0.2–1.8	0.25 0.11–0.37	0.05 0.01–0.45
1–2	36	3.3 1.8–5.1	1.12 0.4–2.2	0.3 0.24–0.64	0.12 0.04–0.70
3–5	41	4.4 1.5–13.6	1.5 0.7–4.1	0.28 0.19–0.75	0.28 0.06–1.24
6–9	51	4.0 2.4–9.6	1.8 0.6–4.5	0.38 0.20–0.60	0.35 0.05–0.87
10–14	93	5.3 2.1–12.6	2.8 1.0–7.2	0.50 0.15–1.06	0.51 0.11–1.43
≥ 15	90	7.1 3.8–14.6	3.8 1.5–8.2	0.51 0.28–0.96	0.40 0.09–1.76

Table 4. Medians and 90th percentiles in mg/ml of IgG subclass protein concentrations in sera from 117 healthy children and 53 adults measured by RID using polyclonal antibodies

Age (years)	n	IgG1	IgG2	IgG3	IgG4
<1					
1-2	13	6.2 5.0-8.7	1.1 0.5-1.9	0.45 0.29-0.58	0.19 0.07-0.24
3-5	13	8.0 5.6-9.6	1.5 0.7-2.7	0.37 0.16-0.65	0.23 0.09-1.32
6-9	32	8.3 6.0-11.3	1.9 1.1-3.5	0.40 0.23-0.74	0.25 0.07-0.82
10-14	59	9.0 6.0-12.5	2.7 1.8-5.0	0.50 0.25-0.91	0.52 0.14-1.16
≥15	53	6.7 4.1-10.6	2.9 1.4-4.4	0.58 0.33-1.01	0.54 0.20-1.10

adults, expressed as medians and 90th percentiles, is shown in Table 4.

Comparison of the ranges provided by RID and ELISA assays

The range for IgG1 determination, as assessed by the ELISA assay, was much wider than the range assessed by the RID method (Tables 3 and 4). Moreover, the medians for different age groups were lower when concentrations were measured by ELISA. Comparison of percentiles shows that ELISA provided lower concentrations than the RID assay when

sera with IgG1 concentrations lower than 10 g/l were compared. When sera with IgG1 concentrations higher than 10 g/l were assayed, ELISA measured higher concentrations than did the RID assay (Fig. 2). The medians were similar in both IgG2 assays, but again the range was wider when concentrations were measured by ELISA. Again, when percentiles were compared, the ELISA measured higher concentrations than did the RID in sera with IgG2 concentrations higher than 4 g/l. Both methods showed similar results when sera with concentrations lower than 4 g/l were assayed. The medians and ranges for IgG3 and IgG4 agreed well when the ELISA and RID were compared (Tables 3 and 4 and Fig. 2), but here also ELISA showed higher results when sera with IgG3 and IgG4 concentrations higher than 1 g/l were assayed.

Discussion

The measurement of serum IgG subclass concentrations is increasingly used to assess the immunologic competence of infection-prone subjects. Before establishing the laboratory diagnosis of IgG subclass deficiency, certain technical issues must be addressed. The choice of antibodies – monoclonal or polyclonal? Which IgG subclass method is to be used – RID or ELISA? Can we use the commercially available kits? Can we use the published normal range? or do patients' IgG subclass levels need to be compared to a normal range established by the same method, in the same laboratory, in the same race, and with the same reagents?

One of the aims of this study was to establish an IgG subclass ELISA with four well-characterized monoclonal antibodies against human IgG heavy-chain epitopes (8). In previous experiments, we observed that monoclonal antibodies lose their activity when they are directly adsorbed onto the plastic plates, presumably because of denaturation upon contact with the hydrophobic polystyrene surface. Precoating the plates with polyclonal antibodies prevents denaturation and improves the results (2). Our assay for IgG subclass determinations has been shown to be highly sensitive and to have good reproducibility. When different methods for IgG subclass determination were compared, some differences, especially in IgG1 measurements, were observed. Despite good correlation between IgG1 measurements obtained by the commercially available ELISA kit and both RID methods investigated, the ELISA kit showed higher values in all investigated samples. IgG1 determinations by both the ELISA developed in our laboratory and the two RID methods showed a significant correlation. However, some of the correlation coefficients were low. Similar problems in IgG1 measurements, even

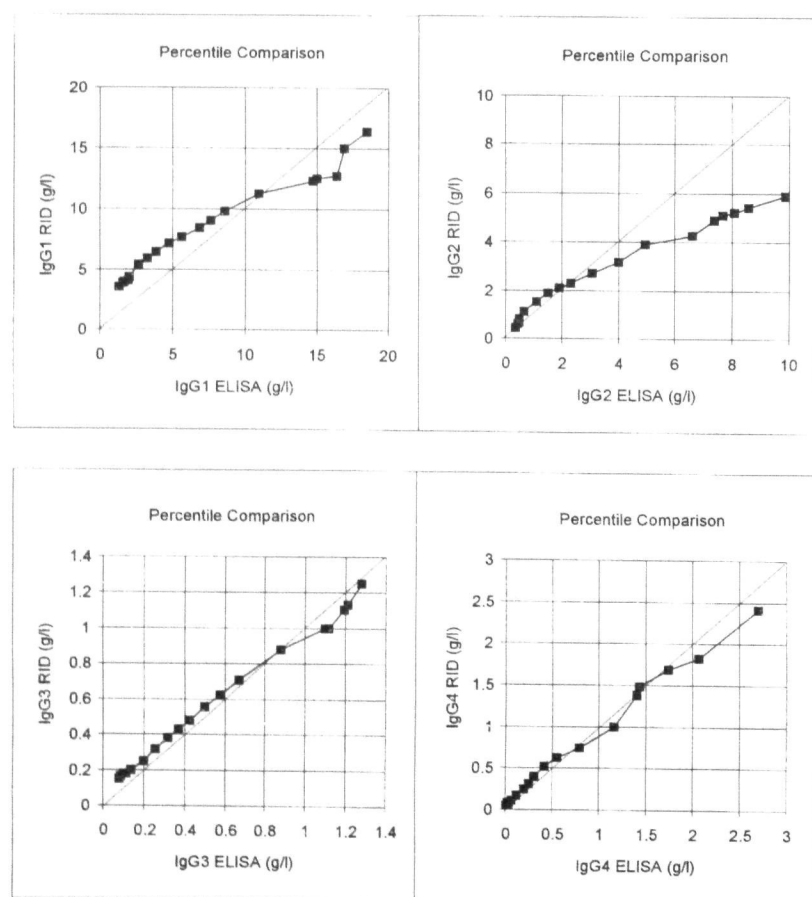


Fig. 2. Percentile comparison of normal values for IgG subclasses determined by ELISA and RID with polyclonal antibodies.

when the same method, the same subclass-specific antisera, and the same calibrator were used in two different laboratories, have been reported by others (3). Determination of IgG2 levels showed good agreement between results obtained by our ELISA and those of both RID methods. The commercially available ELISA kit showed no correlation with any of the other methods investigated. Comparison of four different methods of IgG subclass measurement showed good correlation between IgG3 and IgG4 results in all assays investigated (Table 2).

Analysis of normal ranges established by ELISA and RID with polyclonal antisera showed some interesting features; in the lower part of a normal range, ELISA results were lower than results obtained by RID. This observation indicates that the RID method may have some limitations in measuring IgG1 and IgG2 concentrations in sera with low levels of these immunoglobulins. This may be important when pediatric serum samples are investigated. When the sera with high levels of IgG1 and IgG2 were investigated, the results obtained by ELISA were higher than those obtained by RID. Aggregation of IgG molecules may occur, because of spontaneous interactions (1). Aggregation of IgG may influence the IgG subclass determinations by ELISA more than those by RID.

Comparisons of different assay systems for IgG subclass determinations in human sera have been previously published (9, 10), showing differences in IgG subclass determinations with different methods; these authors emphasized the importance of establishing the age-related normal limits for any assay measuring IgG subclass concentrations.

The importance of IgG subclass assay validity and interpretation becomes greater as newly defined immunodeficient groups are studied and therapeutic interventions are attempted. The IgG subclass deficiencies have been associated with recurrent infections, and measurement of IgG subclass concentrations is used to direct the management of these patients; therefore, it is important for each particular subclass assay that age-specific normal limits be determined with sera from a large group of healthy children. The use of published standards involving different assays and different laboratories is not sat-

isfactory, because the normal values established for one assay may not apply to others.

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