Assignment of Weight-Based Immunoglobulin G1 (IgG1) and IgG2 Units in Antipneumococcal Reference Serum Lot 89-S(F) for Pneumococcal Polysaccharide Serotypes 1, 4, 5, 7F, 9V, and 18C

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Weight-based assignments for immunoglobulin G1 (IgG1) and IgG2 subclass antibodies to *Streptococcus pneumoniae* capsular polysaccharides (PnPs) in antipneumococcal standard reference serum lot 89-S (lot 89-S), also known as lot 89-SF, have been determined for serotypes 1, 4, 5, 7F, 9V, and 18C. This extends the usefulness of lot 89-S beyond the IgG1 and IgG2 subclass assignments for serotypes 3, 6B, 14, 19F, and 23F made previously (A. Soininen, H. Kayhty, I. Seppala, and T. Wuorimaa, Clin. Diagn. Lab. Immunol. 5:561–566, 1998) to cover 11 major serotypes associated with the highest percentage of pneumococcal disease worldwide.

A method of equivalence of absorbances in enzyme immunoassays was used to determine the IgG1 and IgG2 antibody concentrations for the additional serotypes in lot 89-S, based on the subclass values previously assigned for PnPs serotypes 6B, 14, and 23F. This cross-standardization method assures consistency with previous antibody assignments in that reference serum. The newly assigned subclass values for serotype 9V, and previously assigned values for serotype 14, were used to quantitate PnPs antibodies in sera from adult and pediatric subjects immunized with a pneumococcal conjugate vaccine. There was a predominance of IgG1 anti-PnPs antibodies in pediatric sera and IgG2 anti-PnPs antibodies in the adult sera. The IgG1 and IgG2 subclass assignments for the 11 PnPs serotypes in antipneumococcal standard reference serum lot 89-S are useful for quantitating and characterizing immune responses to pneumococcal infection and vaccination regimens.

*Streptococcus pneumoniae*, a gram-positive diplococcus expressing a serotype-specific capsular polysaccharide, is a major cause of pneumonia, otitis media, bacteremia, and meningitis (15, 18, 49). It has been established that antibodies to capsular polysaccharides provide protection against invasive disease (3, 6, 7, 38, 45). There are over 80 known serotypes of pneumococci, each distinguished by a unique polysaccharide capsule. While current 23-valent pneumococcal polysaccharide nonconjugate vaccines are of limited use in children <2 years of age, they afford adults coverage against those serotypes associated with approximately 90% of pneumococcal disease, with varied estimates of efficacy (3, 8, 14, 46). A 7-valent pneumococcal conjugate vaccine, capable of presenting polysaccharides to the immune system as T-cell-dependent antigens, has been efficacious in disease prevention in infants and toddlers (4, 28, 33, 36, 45). The seven serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) in the current conjugate vaccine provide approximately 80 to 90% coverage of the disease in the United States (11, 21, 22).

Development of pneumococcal conjugate vaccines with broader coverage and extending their use to adult populations will have a major impact on disease prevention worldwide (12, 45).

A human standard reference serum lot 89-S is available (as U.S. standard reference serum lot 89-SF from the Center for Biological Evaluation and Review at the U.S. Food and Drug Administration [Bethesda, Md.]) and has been characterized with respect to immunoglobulin isotype and subclass units for a number of pneumococcal serotypes (34, 35, 40). Lot 89-S has been useful in assessing efficacies of pneumococcal conjugate and nonconjugate polysaccharide vaccines in clinical trials (4, 13, 26, 29, 36).

Immunoglobulin G1 (IgG1) and IgG2 subclass assignments for serotypes 3, 6B, 14, 19F, and 23F in lot 89-S were determined previously, using an equivalence-of-absorbance method (40). Using a similar approach, we report here additional IgG1 and IgG2 assignments for serotypes 1, 4, 5, 7F, 9V, and 18C in standard reference serum lot 89-S, further extending the utility of this reference serum.

MATERIALS AND METHODS

Sera. Human antipneumococcal standard reference serum lot 89-S was described elsewhere (34). Previously determined pneumococcal polysaccharide (PnPs) serotype antibody concentrations for IgG1 and IgG2 (0.66 and 10.54 μg/ml for serotype 6B, 2.87 and 21.23 μg/ml for serotype 14, and 0.73 and 6.66 μg/ml for serotype 23F, respectively, in standard reference serum lot 89-S) were used to quantitate additional subclass serotypes in this reference serum (40). Total IgG antibody assignments for the 24 serotypes were reported by Quataert and coworkers (34, 35). Human sera were obtained with informed consent from healthy adults immunized with one dose of an experimental 9-valent pneumococcal polysaccharide (serotypes 1, 4, 5, 6B, 9V, 14, 18C, 19F, and 23F) vaccine conjugated to CRM197 (Wyeth) and from infants postimmunization with a third dose of 7-valent pneumococcal conjugate vaccine [Prev(e)mar; Wyeth]. These

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TABLE 1. Assignment of IgG1 and IgG2 antibody quantities for pneumococcal serotypes 1, 4, 5, 7F, 9V, and 18C in standard reference serum lot 89-S

| IgG subclass | Serotype-specific PnPs quantity for serotype: |
|--------------|---------------------------------------------|
|              | 1   | 4   | 5   | 7F  | 9V  | 18C |
| G1           | 0.85 ± 0.22 (25.7%) | 0.17 ± 0.03 (16.3%) | 0.19 ± 0.02 (13.0%) | 0.31 ± 0.03 (9.4%) | 0.78 ± 0.13 (17.4%) | 0.66 ± 0.06 (9.2%) |
| G2           | 4.35 ± 0.78 (18.0%) | 3.84 ± 0.78 (9.1%) | 4.84 ± 0.49 (10.2%) | 4.10 ± 0.47 (11.5%) | 7.55 ± 0.71 (9.4%) | 3.98 ± 0.37 (9.2%) |

*Values (in micrograms per milliliter) are means ± standard deviations, and the CVs are shown in parentheses (n = 9).*

Sera were organized into two panels representing the entire concentration range of anti-PnPs serotype antibodies previously observed (34).

PnPs were purchased from the American Type Culture Collection (Manassas, Va.); each lot was qualified for use in the enzyme immunoassay (EIA) (44). The PnPs were diluted to 0.01 M phosphate-buffered saline (PBS) with 0.02% sodium azide, and 100 μl of each serotype polysaccharide/well was used to coat 96-well microtiter plates (NUNC C96 Polysorp) for 5 h at 37°C at the following concentrations: 10 μg/ml for serotypes 1, 6B, and 23F; 5 μg/ml for serotype 5; 2 μg/ml for serotype 7F; 5 μg/ml for serotypes 4, 14, and 18C; and 0.5 μg/ml for serotype 4. Pneumococcal polysaccharide (PnA) for serotypes 6B, 14, and 23F, run on three separate assay days (9). For the cross-standardization method to be valid, the binding characteristics of the antibodies to their respective PnPs should be similar, as evidenced by parallelism (9). The PnPs were coated on 96-well microtiter plates (NUNC) for 5 h at 37°C. After washing, 100 μl of each antibody in each lot was added to each well and incubated for 2 h at room temperature. Plates were washed between each EIA step with 0.01 M Tris-buffered saline–0.1% Brij 35 at pH 7.2.

**RESULTS**

Optimal conditions and concentrations of reagents used in the IgG1 and IgG2 subclass EIAs were determined using standard reference serum lot-89S (data not shown). The subset-specific IgG1 and IgG2 concentrations for pneumococcal serotypes 1, 4, 5, 7F, 9V, and 18C in lot 89-S were determined in anti-PnPs IgG subclass EIAs using a cross-standardization method with three reference standard anti-PnPs EIAs, including serotypes 6B, 14, and 23F, run on three separate assay days (9). For the cross-standardization method to be valid, the binding characteristics of the antibodies to their respective PnPs should be similar, as evidenced by parallelism (9). Linear regression analysis of log of optical density versus log of serum dilution for IgG1 and IgG2 with all serotypes yielded similar slopes (mean slope, 1.05 ± 0.20). Thus, similar slopes for lot 89-S titration in between the reference and experimental anti-PnPs EIAs ensure accuracy in quantitation. Each of the three subset-specific reference EIAs demonstrated similar sensitivity for their three separate determinations, confirming equivalence in optical density values and consistency in assay performance (data not shown). Using a four-parameter logistic fit analysis program, antibody concentrations for either IgG1 or IgG2 for each serotype were calculated from the corresponding three reference EIA standard curves on each of the three assay days (i.e., n = 9). Descriptive statistics of the data set generated for each IgG1 and IgG2 subclass for pneumococcal serotypes 1, 4, 5, 7F, 9V, and 18C are shown in Table 1. The corresponding coefficient of variation (CV) for IgG1 and IgG2 in these six serotypes ranged from 9.1 to 25.7%, with an overall mean CV equal to 13.0%.

Attempts were made to quantitate the IgG3 and IgG4 subclass antibodies in standard reference serum lot 89-S, as was done for IgG1 and IgG2 EIAs. Using myeloma-derived human IgG3 and IgG4 to coat microtiter plate wells, the limit of detection for these IgG subclasses was approximately 1 μg/ml. Pneumococcal serotype-specific IgG3 or IgG4 quantities were below this level in standard reference serum lot 89-S. Thus, most of the pneumococcal IgG antibodies present in lot 89-S belong to either the IgG1 or IgG2 subclasses, consistent with the overall proportion of these IgG subclasses (greater than 90%) in total human serum (17, 20). Accordingly, for each serotype, the sum of the IgG1 and IgG2 concentrations agreed very well with the total IgG concentrations previously assigned (34), ranging from 83 to 121% of the total IgG values, with an average of 96.2% (Table 2).

The newly assigned subclass values for serotype 9V in reference serum lot 89-S (this report) were used to estimate concentrations of serotype 9V-specific IgG1 and IgG2 antibodies in panels of sera from adult and children immunized with 9-valent and 7-valent pneumococcal conjugate vaccines, respectively. While IgG3 and IgG4 assignments could not be made to lot 89-S, these subclasses were also tested with the
TABLE 2. Comparison of the sum of IgG1 and IgG2 with total IgG in standard reference serum lot 89-S

| PnPs serotype | Conc (μg/ml) | IgG1 | IgG2 | IgG1 + IgG2 | Assigned total IgG | % Agreement |
|---------------|-------------|------|------|-------------|------------------|-------------|
| 1             | 0.85        | 4.35 | 5.20 | 6.32        | 83               |
| 4             | 0.17        | 3.84 | 4.01 | 4.07        | 98               |
| 5             | 0.19        | 4.84 | 5.03 | 5.75        | 87               |
| 7F            | 0.31        | 4.10 | 4.41 | 5.21        | 85               |
| 9V            | 0.78        | 7.55 | 8.33 | 6.90        | 121              |
| 18C           | 0.66        | 3.98 | 4.64 | 4.46        | 103              |

*From Quataert et al. (34).*

*Percent agreement = 100 × [(IgG1 + IgG2)/(total IgG)].

panels of sera. The sera were quantitated for total IgG concentration by the EIA method used to assess the licensed 7-valent conjugate vaccine, as described previously (34). For comparison purposes, the panels of sera were tested in parallel for serotype 14-specific IgG1 and IgG2 antibody quantities, using the previously assigned serotype 14 subclass values for lot 89-S (40). The correlation of independently determined serotype-specific total IgG quantity and the sum of IgG1 and IgG2 quantities was assessed by linear correlation analysis as described elsewhere (34, 40). For pediatric sera (n = 32), the correlation coefficients were 0.89 and 0.91 for serotypes 9V and 14, respectively (Fig. 1); for the adult sera (n = 78), the correlation coefficients were 0.87 and 0.93, respectively (Fig. 2). These data are indicative of good linearity over a wide concentration range for both IgG subclasses and both pneumococcal serotypes. Furthermore, the slopes of the regression lines using the adult and pediatric sera in each assay were near 1, ranging between 0.87 and 1.02, indicating good concordance between the sum of the individual IgG subclasses and the total IgG concentration.

The various subclasses of IgG may have differential functional properties, especially regarding their potential to effect opsonization and complement activation (1, 2, 5, 24). Furthermore, because of differences in immune status and in natural exposure to *S. pneumoniae* and cross-reactive antigens, the subclass response of adults and children to pneumococcal conjugate vaccines may differ (2, 41). To assess this, the ratios of IgG1 to IgG2 in the panels of adult and pediatric sera were examined in both the serotype 9V- and 14-specific EIAs (Table 3). Using the adult serum panel, the geometric mean IgG1/ IgG2 ratios were well less than 1 for serotypes 9V and serotype 14, 0.07 and 0.04, respectively. This predominance of IgG2 antibodies in adult sera postvaccination is consistent with results from prior postvaccination studies where the IgG subclass levels against serotype 14 and other serotypes were assessed (30, 41). In contrast, the geometric mean ratios for the pediatric serum panel of postimmunization sera showed a predominance of IgG1 (i.e., ratios greater than 1) for serotypes 9V and 14, 2.35 and 1.45, respectively. Previous studies of pediatric sera after either natural pneumococcal infection or pneumococcal vaccination indicated an increased quantity of IgG1 antibodies (16, 30, 47). As with lot 89-S reference serum, IgG3 or IgG4 quantities in the adult and pediatric sera analyzed here were below the lower limit of detection (approximately 1 μg/ml).

**DISCUSSION**

A multivalent pneumococcal conjugate vaccine is currently registered and licensed for use in pediatric populations and is effective in providing protection against the major diseases associated with *S. pneumoniae*, including acute otitis media, sinusitis, pneumonia, bacteremia, and meningitis (33, 45). As consideration is given to extend this vaccine to older age groups and to evaluate new candidate vaccines, serologic tools to assess the human immune response are needed (26, 27). In this study, IgG1 and IgG2 subclass-specific antibody concentrations to PnPs serotypes 1, 4, 5, 7F, 9V, and 18C in standard reference serum lot 89-S were assigned using an equivalence-of-absorbance, cross-standardization method (9). Specifically,
the cross-standardization method employed reference EIA assays for serotypes 6B, 14 and 23F, whose subclass-specific antipneumococcal antibody levels in lot 89-S were previously assigned (40). Our strategy, involving the use of several preassigned reference serotypes rather than just one reference serotype, minimizes the risk of assay bias when new weight-based assignments are made to the reference standard serum. Additionally, IgG1 and IgG2 quantities in two panels of postimmunization adult and pediatric sera were assessed. It has been reported that the specificity of the standardized EIA is improved, especially for sera obtained prior to immunization, by the addition of heterologous PnPs, such as PnPs from serotype 22F (10, 39, 48). PnPs 22F absorbent was not required, or recommended, for our studies of the reference serum, since pneumococcal antibodies in lot 89-S were shown previously to be highly serotype specific (34, 44). Furthermore, PnPs 22F absorbent, or another heterologous PnPs absorbent, has relatively minimal effect on pneumococcal serotype-specific antibody levels in postimmunization serum specimens, such as those from the adult and children panels used here (10, 42). Concentrations of IgG3 and IgG4 in reference serum lot 89-S and the postimmunization specimens were below the level of detection for all serotypes, consistent with the relatively low concentration of these subclasses (less than 10% combined) in human serum IgG (17, 20). Accordingly, the sum of the IgG1 and IgG2 assignments for IgG subclass antibodies specific to each pneumococcal serotype in lot 89-S and the postimmunization specimens were below the level of detection for all serotypes, consistent with the relatively low concentration of these subclasses (less than 10% combined) in human serum IgG (17, 20). Accordingly, the sum of the IgG1 and IgG2 assignments for IgG subclass antibodies specific to each pneumococcal serotype in lot 89-S and the postimmunization specimens correlate well with the independently determined total IgG quantity for that serotype. For lot 89-S, the sum of the IgG1 and IgG2 subclass quantities, relative to the total IgG quantity, for the additional serotypes reported herein ranged from 83 to 121%. For the postimmunization panels of sera from adults and children, linear correlation analysis of the sum of IgG1 and IgG2 to total IgG resulted in correlation coefficients ranging from 0.87 to 0.93 and slopes near 1, attesting to the accuracy of the subclass determinations for these specimens.

Potential functional differences between the subclasses of IgG antibodies have renewed interest in determining the subclass distribution of IgG antibodies to PnPs antigens in adults and children (1, 2, 5, 24). PnPs conjugate vaccines induce primarily T-cell-dependent IgG1 antibody responses in infants, while PnPs conjugate and nonconjugate vaccines induce predominantly IgG2 responses in adults (41, 43, 47). The ability to quantitate IgG1 and IgG2 subclasses is informative in understanding and evaluating the immune response(s) to *S. pneumoniae* polysaccharide conjugate and nonconjugate vaccines. As we show here, the assignment of weight-based units to the antipneumococcal reference standard serum allows the relative and absolute response levels of IgG1 and IgG2 to be assessed. When comparing the immune serologic status in adult and pediatric sera obtained from individuals following pneumococcal CRM197-conjugate vaccine immunization, there was a striking difference in the predominance of IgG subclass antibodies: infants showed a predominance of IgG1, while in adults IgG2 antibodies predominated. These results are consistent with prior studies using other panels of sera that concluded that IgG subclass antibody responses to pneumococcal

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**TABLE 3.** IgG1 and IgG2 quantities for anti-PnPs serotypes 9V and 14 in sera from adult and pediatric vaccinees

| Serum type | Pneumococcal serotype | Antibody quantity<sup>a</sup> | IgG1/IgG2<sup>b</sup> ratio |
|------------|-----------------------|-----------------------------|-----------------------------|
|            | 9V                    | 0.591                       | 8.911                       |
|            | 14                    | 0.660                       | 16.980                      | 0.07 |
|            | 9V                    | 0.926                       | 0.394                       | 2.35 |
|            | 14                    | 3.297                       | 2.280                       | 1.45 |

<sup>a</sup> Geometric mean (micrograms per milliliter).

<sup>b</sup> Ratio of geometric means.

<sup>c</sup> Immunized with an experimental 9-valent pneumococcal conjugate vaccine.

<sup>d</sup> Immunized with the 7-valent pneumococcal conjugate vaccine [Prev(escort)].
vaccines are age linked (30). Since preimmunization sera are not described in this study, the related issue of relative fold rises in these subclasses following immunization is not addressed. Soininen and coworkers have shown that adults have preexisting serotype-specific IgG2 antibody, due to prior natural exposure to pneumococci, and that both IgG1 and IgG2 responses may follow pneumococcal conjugate vaccine immunization (32, 41). Different vaccine formulations may elicit preferentially different subclass responses, or responses of different magnitudes (37, 41). In naive immune systems, as in infants, IgG1 antibodies are elicited by effective pneumococcal and Haemophilus polysaccharide conjugate vaccines, as expected for T-cell-dependent antigens (28, 31, 37). The ability to measure and characterize the antipneumococcal antibody response in all age groups and in immunocompromised individuals provides a useful tool to assess immune responses relative to protection from invasive pneumococcal disease and to performance of new generations of pneumococcal vaccines. The standard reference serum lot 89-S can be used for accurate and consistent quantitation of serotype-specific IgG subclass antibodies to S. pneumoniae in both adult and pediatric sera. Furthermore, the subclass assignments for IgG1 and IgG2 reported here, and by Soininen and coworkers, can be used for further cross-standardization for subclass assignment against additional pneumococcal serotypes (9, 40). A consensus guidance for a third-generation pneumococcal antibody ELISA, using commercially available reagents, for use in the evaluation of both pre- and postimmunization serum specimens was published recently (44).

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