Characterization of Specific Immunoglobulin G (IgG) and Its Subclasses (IgG1 and IgG2) against the 23-Valent Pneumococcal Vaccine in a Healthy Adult Population: Proposal for Response Criteria

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The aim of the study was to standardize an enzyme-linked immunosorbent assay (ELISA) method for the quantification of immunoglobulin G (IgG) and its subclasses (IgG1 and IgG2) against the 23-valent pneumococcal vaccine and to establish the criteria for a normal response to the vaccine. Forty healthy individuals (20 women and 20 men; mean age, 29 years) were studied. All were vaccinated with the 23-valent pneumococcal vaccine; blood samples were drawn just prior to and 3 weeks after immunization. Quantification of specific IgG and its subclasses was performed by an ELISA with the vaccine as the antigen. The linearity of the ELISA method was demonstrated by the similar slopes of the linear regression lines generated from the titration of sera with different antibody concentrations. The specificity of the antibodies against the vaccine was demonstrated by (i) an absorption test with pneumococcal vaccine, (ii) a cross-reactivity experiment with Haemophilus influenzae type b polysaccharide, and (iii) affinity chromatography with protein A-Sepharose. Response to the vaccine was defined by using the lower level of the 90% probability interval (one-tailed) for postimmunization-specific IgG, IgG1, and IgG2. By using this cutoff, responders were considered to be those with an absolute increase in antibody titers higher than 395 arbitrary units/ml for IgG, 0.350 A450 units for IgG1, and 0.314 A450 units for IgG2. Overall, 20 (50%) subjects had IgG, IgG1, and IgG2 responses, 9 (22.5%) had IgG and IgG2 responses, 4 (10%) had IgG1 responses, 3 (7.5%) had IgG and IgG1 responses, and 4 (10%) were nonresponders. Ninety percent of our population responded to the 23-valent pneumococcal vaccine. Up to 10% of healthy individuals may respond to an IgG subclass without significant increases in total IgG titers. The ELISA method that is described may be useful for evaluating the specific antibody response against polysaccharides.

The latest review of humoral immunodeficiencies by the World Health Organization includes a syndrome characterized by a lack of response to polysaccharide antigens (30). This impaired antibody response has also been observed to be associated with common variable immunodeficiency such as cryopyrin-associated periodic syndrome (1, 30) and (ii) evaluate antibody responses in certain risk groups. The criteria could be used to (i) identify patients with impaired antibody responses to polysaccharides (1, 30) and (ii) evaluate antibody responses in certain risk groups that are candidates for the vaccine (22).

MATERIALS AND METHODS

Study population. Forty healthy nonrelated volunteers (20 women and 20 men; age range, 21 to 48 years; mean age, 29.5 years) were studied.

Immunization. All individuals were vaccinated with 0.5 ml of PNU-Immune 23 polysaccharide vaccine (Lederle Laboratories Division, Pearl River, N.Y.) administered intramuscularly in the deltoids. This vaccine contained 25 μg of each of the 23 specific capsular polysaccharides. Blood samples were obtained from each individual prior to and 21 days postvaccine administration. These samples were centrifuged at 3,000 rpm for 15 min, and the sera were obtained, stored in aliquots, and frozen at −20°C until they were studied.

Immunoglobulin quantification. IgG, IgA, and IgM levels were determined by kinetic nephelometry (Array Protein System; Beckman Instruments, Brea, Calif.). The reference values established in our laboratory were as follows: IgG, 8.5 to 16 g/liter; IgA, 0.75 to 3.5 g/liter; and IgM, 0.58 to 2.5 g/liter.

The levels of the IgG subclasses were determined by an ELISA technique. The reference values established in our laboratory were as follows: IgG1, 2.61 to 16 g/liter; IgA, 0.75 to 3.5 g/liter; and IgM, 0.58 to 2.5 g/liter (18).

Specific IgG and IgG subclasses to pneumococcal vaccine. Specific IgG to Streptococcus pneumoniae was determined by an ELISA based on the method described by Metzger et al. (15) but that was modified by using the pneumococcal vaccine as the antigen. Each well of high-level-binding microtiter plates (Costar, Cambridge, Mass.) contained 10 μg of antigen in 0.2 M carbonate-bicarbonate buffer (pH 9.6), and the plates were incubated at 4°C overnight. Washing (0.1 M phosphate-buffered saline [pH 7.5], 0.005% Tween 20) and blocking (phosphate-buffered saline, 1% bovine serum albumin) were then performed. After the washing, the plates were incubated with sera by using a standard curve at appropriate dilutions for 2 h at 37°C, in duplicate. The results were then compared with those on the standard curve. After the washings, horseradish peroxidase-labeled anti-human IgG (clone M16-1ME; 0.5 μg/ml) was incubated for 2 h at 37°C. Following the washing, the reaction was developed with 3,3′,5,5′-...
tetramethylbenzidine–3% H₂O₂ for 20 minutes at room temperature in the dark. The reaction was stopped with 2 M H₂SO₄, and the results were read at 450 nm with a plate reader (Titer tek Multiskan Plus MKII).

The results were expressed as arbitrary units by using a reference serum containing 2,240 U/ml calibrated against a pneumococcal reference preparation labelled PN-A, with an assigned value of 70 pneumococcal IgG antibody U/ml, from the European Quality Scheme for specific antibodies (Oxfordshire Health Authority, John Radcliffe Hospital, Oxford, United Kingdom), kindly donated by N. P. Johnson (Hospital Son Dureta, Palma de Mallorca, Spain). To study the behavior of sera compared to that of the reference serum, we performed titrations of five serum samples with antipneumococcal antibody levels of between 150 and 2,250 U/ml. The curves obtained for the sera and the reference serum were plotted for linear regression analysis.

The amounts of specific IgG1 and IgG2 were determined by the same method used to determine the amount of specific total IgG by using horseradish peroxidase-labelled anti-human IgG1 (clone MH161-1) and IgG2 (clone HP6014), and the results were expressed as A₄₅₀ units. For specific total IgG, the minimal amount of antibody detectable by this assay was 0.11 U/ml. This value was obtained as the mean plus 3 standard deviations of the value for the blank also performed for 20 determinations. Within-run and day-to-day coefficients of variation were 7.6 and 10.5%, respectively, for IgG1 and 10.3 and 11.3%, respectively, for IgG2.

For the IgG subclasses, the minimal amount of antibody detectable by these assays was 0.200 A₄₅₀ unit above the value for the blank. The limit of detection calculated on the basis of the mean plus 3 standard deviations of the value for the blank also performed for 20 determinations. Within-run and day-to-day coefficients of variation were 6.5 and 10.6%, respectively, for IgG1 and 10.3 and 11.3%, respectively, for IgG2.

**Specificity of the antibodies to vaccine.**

(i) Inhibition experiments. Increasing concentrations (range, 0 to 80 µg/ml) of pneumococcal vaccine were incubated with 1 ml of a 1:50 dilution of two pools of sera with specific IgG concentrations of 9,250 and 150 U/ml, respectively, for 2 h at 37°C and were separated by centrifugation at 3,500 rpm before the addition of serum to vaccine-coated plates. The procedure was carried out three times.

(ii) Cross-reactivity experiment between anti-*S. pneumoniae* and anti-Hib antibodies. Because the capsular antigens of *S. pneumoniae* and *Haemophilus influenzae* are both polysaccharide antigens, we tested to see whether binding in the anti-*S. pneumoniae* ELISA of any serum could be inhibited by *H. influenzae* type b (Hib) polysaccharide.

The same two pools of sera used in the inhibition experiments were incubated with increasing concentrations of *H. influenzae* polysaccharide (range, 0.05 to 100 µg/ml) for 2 h at 37°C and the contents were separated by centrifugation at 3,500 rpm. The amount of specific IgG to *S. pneumoniae* in the supernatant was measured by the same ELISA method.

(iii) Purified IgG antibodies. To obtain IgG-enriched antibody preparations, affinity chromatography with protein A-Sepharose CL-4B (Pharmacia Diagnostics, Uppsala, Sweden) was performed (13). The column (5 ml) was equilibrated with 20 mM phosphate buffer (pH 7.2). A postvaccination serum pool (IgG, 226 mg/dl; IgM, 312 mg/dl; IgG1, 1,190 mg/dl; IgG1, 717 mg/dl; IgG2, 493 mg/dl; IgG3, 76 mg/dl; IgG4, 33 mg/dl; and specific IgG to *S. pneumoniae*, 3,300 U/ml) was loaded in the column and the column was washed with the equilibrated buffer. Bound proteins were removed with 100 mM glycine buffer (pH 3). The fractions from the second peak (maxima higher than 0.200 A₄₅₀ units) of the two eluates obtained were collected. The immunoglobulins, including IgG subclasses and IgG specific to *S. pneumoniae*, were determined in each eluate.

**Data analysis.** The results were expressed as the mean and 95% confidence interval of the post- and preimmunization antibody titers; the same results were calculated for differences and ratios of antibody titers. For calculations, antibody titers, as well as changes in titers, were transformed to their natural logarithms to meet the assumption of normality. Because of the characteristics of this transformation, whenever the difference between post- and preimmunization titers was negative or the ratio was <1, these values were assigned the lowest value of the variable.

In these calculations, samples with undetectable levels were also assigned the value of the lower limit of detection. An arbitrary value was defined as the minimum significant increase to define the response to the vaccine. This value corresponds to the lower limit of the 90% probability interval (one-tailed) of the variables comprising the amounts of specific IgG, IgG1, and IgG2 postimmunization. All subjects showing an increase in specific antibody titers equal to or greater than this value were considered responders.

**RESULTS**

**Linearity of the ELISA method.** Each serum sample was tested at different dilutions to create a curve. Figure 1 shows the regression lines that were generated. The slope of each curve was similar to that for the reference serum, and thus we may assume that the behaviors of sera and the reference serum do not differ. In all, eight different dilutions of each serum sample were tested, and the regression lines were created with four points.

**FIG. 1.** Regression lines for samples with different antipneumococcal antibody titers and the reference serum sample. Titers of antibodies and slopes are presented. ▼, 2,500 U/ml (SI, −4.6 × 10⁻⁹; reference serum); ○, 600 U/ml (SI, −3.5 × 10⁻⁹); □, 300 U/ml (SI, −3 × 10⁻⁹); ○, 150 U/ml (SI, −2.7 × 10⁻⁹); ▲, 2,050 U/ml (SI, −5 × 10⁻⁹); □, 2,250 U/ml (SI, −4.9 × 10⁻⁹).

**Specificity of the antibodies to the vaccine.**

(i) Effect of adsorption with the pneumococcal vaccine. Results of inhibition experiments in which the pneumococcal vaccine was added to the sera showed decreases in the mean A₄₅₀ of from 2.5 to 0.6 and from 0.4 to <0.05, indicating effective competition with the coated pneumococcal polysaccharides for antibody binding (Fig. 2).

(ii) Cross-reactivity experiment. As expected, soluble Hib polysaccharide added to the sera did not decrease the absorbance of specific IgG to *S. pneumoniae* before or after incubation with Hib polysaccharide (Fig. 2).

(iii) Purified IgG antibodies. Figure 3 shows the elution patterns obtained from affinity chromatography with protein A-Sepharose. In the first peak, containing fractions with elution volumes of between 4 and 10 ml, the following results were obtained: IgG, 20 mg/dl; IgA, 97 mg/dl; IgM, 30 mg/dl; IgG1, <5 mg/dl; IgG2, <5 mg/dl; IgG3, 20 mg/dl; IgG4, <5 mg/dl; and IgG specific to *S. pneumoniae*, 17 U/ml.

The results for the second peak, containing fractions with elution volumes of between 14 and 20 ml, were as follows: IgG, 716 mg/dl; IgA, 19 mg/dl; IgM, 16 mg/dl; IgG1, 400 mg/dl; IgG2, 320 mg/dl; IgG3, <5 mg/dl; IgG4, 16 mg/dl; and IgG specific to *S. pneumoniae*, 3,200 U/ml.

**Antibody response to the vaccine.** Table 1 presents the values for specific antipneumococcal IgG, IgG1, and IgG2 pre- and postimmunization in our study population.

To define response in our population, we calculated the 90% CI (one-tailed) of the log-transformed titers of total IgG, IgG1, and IgG2 in postimmunization sera. The lower limits of the CI were 395 U/ml for IgG1, 0.350 A₄₅₀ units for IgG1, and 0.314 A₄₅₀ units for IgG2. We took these values to be the minimum significant increase and defined a responder as an individual who has an increase in antibody titers greater than these values.

Figure 4 presents the distribution of responses of specific IgG and its subtypes (IgG1 and IgG2). Of the 40 subjects studied, 32 (80%) met the response criteria for specific total
IgG, 27 (67.5%) met the response criteria for IgG1, and 29 (72.5%) met the response criteria for IgG2. Of the eight who failed to respond adequately with total IgG, four responded with IgG1 and four presented no response with any of the subclasses.

To establish response reference values for each of the subclasses, the means (95% CIs) for postvaccine values of responders were used for IgG1 and IgG2 and were 1.118 (0.949 to 1.318; n = 27) and 1.376 (1.192 to 1.559; n = 29) A450 units, respectively.

**DISCUSSION**

In this study, an ELISA method was used to assess specific IgG and its subclasses against pneumococcal capsular polysaccharide antigens. Like Siber et al. (23) and Mascart-Lemone et al. (14), we used the polyvalent pneumococcal vaccine coupled to the polystyrene plate as the antigen. The inconvenience of using the vaccine is that polysaccharides bind poorly to plastic surfaces, and high concentrations must be used with extended coating times (2); in contrast, it possesses the advantage that it is not conjugated with any protein, and therefore, the response detected is specific to polysaccharides. The specificity of this method is demonstrated by (i) the decrease in specific anti-pneumococcal IgG titers obtained in postvaccination samples prior to adsorption of the same sample with polysaccharide antigens (Fig. 2), (ii) the lack of cross-reactions with Hib (Fig. 2), and (iii) the results obtained after IgG purification by affinity chromatography with protein A (Fig. 3).

Our ELISA method differs in some aspects from the preliminary standard pneumococcal ELISA protocol proposed by the World Health Organization (10), because our study was initiated in 1993 before the World Health Organization project started. In any event, the standard protocol is directed mainly to the use of a standard method of evaluating the immunological efficacies of the new conjugated vaccines and uses a reference serum composed of 10 serotypes, the total antibody values of which are still considered provisional. Our aim was to define the criteria for the antibody response to pneumococcal polysaccharides for use in the diagnosis of the lack of response to polysaccharide antigen syndrome in patients with recurrent respiratory infections. For this purpose, the use of particular

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**FIG. 2.** Effect of absorption with pneumococcal vaccine and cross-reactivity between anti-*S. pneumoniae* and anti-Hib antibodies. (A) Serum with specific IgG to *S. pneumoniae* of 9,250 U/ml (2.5 A450 units). (B) Serum with specific IgG to *S. pneumoniae* of 150 U/ml (0.4 A450 units). ■, adsorption with pneumococcal vaccine; □, adsorption with Hib vaccine.

**FIG. 3.** Elution patterns of a pool of sera from individuals postvaccination obtained by protein A-Sepharose CL-4B affinity chromatography. □, phosphate, 20 mM (pH 7.2); ⨰, glycine, 100 mM (pH 3); ★, units.
serotypes as antigens might lead to misdiagnosis, since, owing to different previous exposures, different geographical distributions of serotypes, or simply chance, a lack of response to these serotypes does not rule out an appropriate response to others.

By our ELISA method we observed that 80% (32 of 40) of our population responded with a significant increase in their specific total IgG titers and 10% (4 of 40) responded with an increase exclusively in IgG1, but with no significant modification in their total IgG values. In view of this, we propose that the response of the different IgG subclasses together with total IgG be studied before classifying an individual as a nonresponder. Since a lack of response to any particular IgG subclass may be normal, the concentrations of different specific IgG subclasses should be compared with the range of concentrations of the controls who responded with this particular subclass. The postimmunization titers of responders for both IgG1 and IgG2 are presented in the Results section.

There is no uniform criterion for the expression of a specific antibody response. Thus, some studies express response as the fold increase. Wong et al. (29) found a mean increase in specific IgG of 7.3 times in healthy volunteers, and Cryz et al. (4) considered a response to exist when the increase was equal to or higher than four times the basal value and found responses in 77% of white Anglo-Saxon individuals and 45% Hispanics. In contrast, Kroon et al. (11) considered an increase of more than twice the basal value to be positive, and in a study with 10 healthy subjects, all of whom were responders, they found a mean increase of 5.8, a little higher than the 4.2 found in our study. Musher et al. (16), using an ELISA method and the same criterion as before, found 93 to 100% responders to five pneumococcal polysaccharides among 15 healthy individuals. Those same investigators, in a later work (19) with 25 individuals, found a lower percentage (between 60 and 84%) of responders, similar to the 80% found in our population for total IgG by using our response criterion. In a recent study, Weiss et al. (28) obtained results similar to ours for a control group of 15 healthy individuals with a mean age similar to that of our group (30.5 years): a mean fold rise in antibody titers for five separate polysaccharides of from 3.1 to 13.5 and a proportion of responders (those with at least a twofold increase) to the different polysaccharides of from 40 to 87%. However, other investigators define response as obtaining some postimmunization values considered to be protective. In this respect, some investigators have suggested a protective pneumococcal antibody level of 300 ng of N/ml, as measured by radioimmunoassay (12). This approach may give rise to error, since a normal immune response and protective levels are not synonymous. Moreover, protection against pneumococcal infection depends on many other immunologic as well as nonimmunologic factors (9). The use of fold increases to assess response offers the advantage of permitting comparisons with other laboratories since they are not influenced by the units used in the expression of the results. Nevertheless, it must be borne in mind that the use of fold increases may prove to be deceptive, because it is not the same to duplicate from low values or high values (27). Thus, for correct response evaluation, we believe that it would be useful to use a yardstick not influenced by preimmunization titers. In this respect, we propose the use of the lower limit of the 90% probability interval of postimmunization titers; i.e., if our population represents the general population, a normal subject has a 95% probability of presenting a postimmunization titer above this value. We have termed this value the minimum significant increase (MSI) and define a responder as an individual who suffers an increase greater than this value (final value – initial value > MSI). The MSI obtained for total IgG was 395 arbitrary units/ml, that for IgG1 was 0.350 A450 units, and that for IgG2 was 0.314 A450 units.

With respect to the specific IgG isotype response, it has classically been accepted that the response of antibodies to protein antigens is predominantly of the IgG1 and IgG3 types, whereas the response to polysaccharide antigens in adults is mainly of the IgG2 type (3, 24). In our study, we found that 67.5% (27 of 40) of individuals responded with a significant increase in IgG1. Of these, four responded only with this subclass. In contrast, the greatest increases were observed for IgG2, and the number of responders was slightly higher, 72.5% (29 of 40), although none responded with IgG2 alone. These results are similar to those obtained in other studies, which show a mixed response of IgG1 and IgG2 (5, 17, 20). It must be emphasized that in our population four of the nonresponders with total IgG responded with some of the subclasses, which would justify study of the same subclasses.

We believe that establishment of defined criteria for the specific total IgG, IgG1, and IgG2 response to pneumococci is a step forward in research on the efficacy of the vaccine in risk groups and will permit in-depth studies of the immunological response in patients with known humoral immunodeficiencies or suspected selective response deficiencies to polysaccharide antigens.

![FIG. 4. Distribution of the specific antibody response against S. pneumoniae.](http://cvi.asm.org/ on February 11, 2021 by guest)
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REFERENCES


