Quantitation of IgG Subclass Antibodies to Pneumococcal Capsular Polysaccharides by ELISA, Using Pneumovax®-Specific Antibodies as a Reference

KIMIKAZU KOJIMA, AKIHITO ISHIZAKA, EIKI OSHIKA, YUICHI TAGUCHI, KAZUHIRO TOMIZAWA, MASANORI NAKANISHI, YUKIO SAKIYAMA and SHUZO MATSUMOTO

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KOJIMA, K., ISHIZAKA, A., OSHIKA, E., TAGUCHI, Y., TOMIZAWA, K., NAKANISHI, M., SAKIYAMA, Y. and MATSUMOTO, S. Quantitation of IgG Subclass Antibodies to Pneumococcal Capsular Polysaccharides by ELISA, Using Pneumovax®-Specific Antibodies as a Reference. Tohoku J. Exp. Med., 1990, 161 (3), 209-215 — A quantitative enzyme-linked immunosorbent assay (ELISA) method has been developed to assay the levels of IgG subclasses to pneumococcal capsular polysaccharides (PCP) by using a reference standard. This standard solution containing specific antibodies to a polyvalent pneumococcal vaccine (Pneumovax®) was purified from the serum of an immunized healthy adult by affinity chromatography. In order to determine the predominant response to Pneumovax in the four IgG subclasses, specific IgG subclasses in preimmune and postimmune sera from six healthy adults were assessed quantitatively by the ELISA. With regard to peak concentrations after immunization, there was a marked increase in the IgG2 subclass, compared with those of IgG1 and IgG3. Such a quantitative assay of Pneumovax-specific IgG subclass antibodies is useful for the direct evaluation of immune responses to immunization with a polyvalent pneumococcal vaccine, and at the same time, for estimating the IgG2 response to PCP antigens in individuals. ——— ELISA; polyvalent pneumococcal vaccine; specific IgG subclass antibodies

Streptococcus pneumoniae (pneumococcus) is a common pathogenic organism causing respiratory and systemic infections in adults and children. To prevent pneumococcal infections, purified polyvalent pneumococcal vaccines have been extensively investigated and their prophylactic efficacy has been suggested, especially in adults (Austrian 1977; Bolan et al. 1986).

In order to assess specific antibody responses to pneumococcal capsular
polysaccharides (PCP), a sensitive enzyme-linked immunosorbent assay (ELISA) has been developed by a number of investigators. ELISA can also be used to determine the levels of specific IgG subclass antibodies to PCP (Barrett and Ayoub 1986; Chudwin et al. 1987; Schatz and Barrett 1987).

Recently, polyvalent pneumococcal vaccine has been used as a coating antigen in ELISA and such assays are successful in evaluating specific antibodies to PCP antigens (Windebank et al. 1987; Snijder et al. 1988). However, it is considered more important to measure the specific IgG2 subclass level in the evaluation of antibody responses to pneumococcal antigens, since IgG antibody to PCP is largely restricted to the IgG2 subclass (Yount et al. 1968; Riesen et al. 1976; Barrett and Ayoub 1986; Schatz and Barrett 1987).

This article describes a simple ELISA method for quantitating specific IgG subclasses to PCP by using a Pneumovax-specific IgG antibody purified by affinity chromatography as a reference standard and pneumococcal vaccine as a coating antigen.

**MATERIALS AND METHODS**

**Pneumococcal vaccine.** A polyvalent pneumococcal vaccine (Pneumovax®; Merck Sharp & Dohme, West Point, PA, USA) containing 23 serotypes: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F (expressed in Danish nomenclature) was used as coating antigen in ELISA as well as in immunization. Each 0.5 ml dose contained 25 μg of polysaccharide from each capsular type of pneumococcus.

**Sera.** Six healthy adult volunteers were immunized subcutaneously with 0.5 ml of Pneumovax. Sera were collected before immunization, once a week for 4 weeks and at 6 months postimmunization. All samples were stored at -20°C before measurement.

**Purification of Pneumovax-specific antibodies.** In order to obtain Pneumovax-specific antibodies as a reference standard, affinity chromatography was employed. CNBr-activated Sepharose® 4B (Pharacia LKB Biotechnology AB, Uppsala, Sweden) was swollen and washed with 1 mM HCl on a sintered glass filter. Pneumovax dialysed overnight at 4°C against coupling buffer (0.1 M NaHCO3, pH 8.3, containing 0.5 M NaCl), was mixed with the gel, and rotated end-over-end for 2 hr at room temperature. After washing away the excess ligand with coupling buffer, the remaining active sites of the gel were blocked with 1 M ethanolamine, pH 9, for 2 hr room temperature. After washing away the excess ligand with coupling buffer, the remaining active sites of the gel were blocked with 1 M ethanolamine, pH 9, for 2 hr room temperature. The product was washed alternately with three cycles of 0.1 M acetate buffer, pH 4, containing 0.5 M NaCl and 0.1 M Tris-HCl buffer, pH 8, containing 0.5 M NaCl.

Serum from a single healthy donor collected one month after immunization with Pneumovax was precipitated with the same volume of ammonium sulfate. After centrifugation, the precipitate was resuspended in 0.01 M phosphate-buffered saline (PBS), pH 7.4, to the same volume as that of the original serum, and dialysed against PBS overnight at 4°C. This ammonium sulfate-precipitated serum was passed slowly through the gel coupled with Pneumovax collected in a plastic tube, and Pneumovax-specific antibodies were then eluted with 3 M NaSCN, pH 7.4.

**Monoclonal antibodies to human IgG subclasses.** Mouse monoclonal antibodies to human IgG1 (HP6069), IgG2 (HP6014), IgG3 (HP6050) and IgG4 (HP6011) were kindly donated by the World Health Organization (WHO). In the present study, anti-human IgG1, IgG2 and IgG4 were diluted 1:1000 and IgG3 was diluted 1:500 in 0.01 M PBS containing 0.05% Tween-20 (PBS-Tween).

**ELISA.** Before quantitating Pneumovax-specific IgG subclass antibodies, each IgG
subclass level in the purified solution containing Pneumovax-specific antibodies was measured quantitatively by the previously described ELISA technique (Ishizaka et al. 1989). Briefly, 96-well microtiter plates (Costar, Cambridge, MA, USA) were coated with 90 μl of goat anti-human κ- and λ-chains (Tago Inc., Burlingame, CA, USA) diluted to 10 μg/ml with 0.05 M carbonate buffer, pH 9.6, overnight at 4°C. The plates were washed twice with PBS and supplemented with 200 μl of 0.25% gelatin in PBS to block nonspecific sites. After blocking for 4 hr at room temperature, the plates were washed twice with PBS-Tween. Then, a standard serum (WHO-REF-SERA 67/97) and the purified serum diluted serially with PBS-Tween were added to each well. Following a 2-hr incubation at room temperature and four washings, each mouse anti-human monoclonal antibody described above was added, and the plates were left for 1.5 hr at room temperature followed by four washings. A 1:2,000 dilution of peroxidase-conjugated goat anti-mouse IgG (Tago Inc.) was added and the solution was incubated for 1.5 hr at room temperature. After washing four times with PBS-Tween and once with 0.15 M citrate-phosphate buffer, pH 5, 100 μl of o-phenylenediamine substrate solution (final concentration 0.34 mg/ml) in citrate-phosphate buffer containing 0.0015% H2O2 was added to the wells. The reaction was stopped by adding 50 μl of 12.5% H2SO4. The absorbance was measured at 492 nm in a multichannelled photometer (Titertek Multiskan; Flow Laboratories, McLean, VA, USA). The antibody content of the purified serum was determined by comparing it to the standard curve of each IgG subclass.

When measuring Pneumovax-specific IgG1 and IgG2 concentrations in each test serum, the plates were coated with a 1:10 dilution of Pneumovax, and the purified solution containing Pneumovax-specific antibodies was used as a reference standard in this ELISA method. All sera were measured twice and values were averaged.

**Results**

**Purified Pneumovax-specific antibodies.** Pneumovax-specific antibodies were purified by means of affinity chromatography. Serum was collected from a single healthy adult one month after immunization with Pneumovax, and was precipitated using ammonium sulfate. Then, the precipitate diluted in PBS was passed through an affinity column containing CNBr-activated Sepharose 4B coupled with Pneumovax (effluent). Pneumovax-specific antibodies were

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<th>Table 1. Immunoglobulin class and subclass levels during purification of Pneumovax-specific antibodies</th>
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*Total protein.

*Serum was precipitated with the same volume of ammonium sulfate.

Before passing through the affinity column, the precipitated serum was redissolved in PBS to the same volume as that of the original serum.

*Not detected.
obtained by elution (*eluate*). In these four samples (the original serum, the precipitated serum, the effluent and the eluate), the concentrations of immunoglobulin classes and IgG subclasses were measured by ELISA (Table 1). The results showed an extreme change in the constituent ratio of IgG subclasses in the eluate. The three other samples had almost the same constituent ratio, that is, the IgG1 subclass antibody was predominant. In contrast, the ratio of IgG1/IgG2 was clearly inverted in the eluate, which contained IgG1: 39 \( \mu g/ml \), IgG2: 193 \( \mu g/ml \) and IgG3: 119 ng/ml. IgG4 was not detected by this ELISA method.

In order to rule out the possibility of contamination with nonspecific antibodies in the eluate, ammonium sulfate-precipitated serum was passed through the other affinity column containing CNBr-activated Sepharose 4B not coupled with ligands. The eluate obtained from this affinity column contained no antibodies.

**Quantitation of IgG subclasses to Pneumovax by ELISA.** Using the purified specific antibodies to Pneumovax as a reference standard, Pneumovax-specific IgG subclasses in the pre- and post-immunized sera were measured quantitatively by ELISA. Fig. 1 shows the standard curves of IgG1 and IgG2 created from serial dilutions of the purified specific antibodies. Correlation coefficients between the absorbances and the antibody concentration in the linear portion were 0.995 and 0.998, respectively.

Before immunization, the mean concentrations of IgG1-3 subclass antibodies were 2.6 \( \mu g/ml \) (ranging from 1.9 \( \mu g/ml \) to 5.1 \( \mu g/ml \)), 53.0 \( \mu g/ml \) (17.7–94.2 \( \mu g/ml \)) and 1.7ng/ml (1.2–2.6 ng/ml), respectively. Peak levels were observed 2 to 4 weeks after immunization. There was a remarkable increase in the mean concentration of IgG2 (478.7 \( \mu g/ml \); ranging from 340.7 \( \mu g/ml \) to 709.0 \( \mu g/ml \)) except in one volunteer (78.6 \( \mu g/ml \)), in contrast to those of IgG1 (23.8 \( \mu g/ml \); 4.2–40.2

![Fig. 1](image-url) **Fig. 1.** The standard curves of IgG1 (open circles) and IgG2 subclass (closed circles) created from serial dilutions of the purified Pneumovax-specific antibodies. Correlation coefficients between the absorbance and the antibody concentration in the linear portion were 0.995 and 0.998, respectively.
IgG2 Subclasses Specific for Pneumococcal Polysaccharides

As seen in Fig. 2, the antibody response of the IgG2 subclass to Pneumovax was extremely remarkable in comparison with that of IgG1. Pneumovax-specific IgG1 and IgG2 levels in sera six months post-immunization were higher than those of before immunization.

DISCUSSION

Following the availability of prophylactic vaccination against pneumococcal infections, several methods have been developed to assess antibody responses to pneumococcal capsular polysaccharides (PCP). Of those methods, the radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) are currently available, and estimated to be highly specific and sensitive. Immunoglobulin classes and subclasses directed to PCP have been measured quantitatively by RIA, and expressed as antibody nitrogen per ml (Schiffman et al. 1980; Chudwin et al. 1987). However, the RIA procedure is unable to be applied in every laboratory because of the need for use of a radioactive isotope. Recently ELISA has been applied to measure the levels of specific antibodies against PCP because this procedure is simple, rapid and in addition, suitable for many laboratories.

In order to measure quantitatively immunoglobulin classes and subclass antibodies against PCP, several modifications of the ELISA method have been attempted. However, technical problems in the procedure seem to make it difficult to quantitate specific antibodies to PCP. In our present study, IgG subclass antibody responses to a polyvalent pneumococcal vaccine (Pneumovax) were assessed by quantitative ELISA, using purified Pneumovax-specific antibodies as a reference standard. Comparisons of direct optical density (OD)
readings within a single assay are practical but not on different days. Moreover, it is technically difficult to evaluate differences of each IgG subclass response to PCP on a quantitative basis even within a single assay. In our study, the mean peak level of IgG2 in the post-immunization serum was less than twice as much as that of IgG1, when expressed as raw OD values (data not shown). However, the results from quantitative ELISA showed a twenty-fold difference between the mean peak levels of IgG1 and IgG2 in post-immunization sera except in one low responder (Fig. 2).

We have developed an easier method for quantitative analysis of Pneumovax-specific IgG subclasses by using purified IgG antibodies to Pneumovax as a reference standard. When measured by this ELISA method, all healthy volunteers except one showed a good response to immunization with Pneumovax, and IgG subclass antibody directed against Pneumovax was predominantly IgG2. On the other hand, using human myelomas of a defined IgG subclass as a reference serum standard, Schatz and Barrett (1987) measured IgG subclasses to pneumococcal polysaccharide type 3 after immunization in children, expressing values quantitatively in ng/ml. In their study, there was no significant difference in the level of IgG1 antibody to type 3 between age groups, but profound age-dependent changes in IgG2 antibody concentration were noted. These results indicate that IgG antibody to PCP is largely restricted to the IgG2 subclass, and are in agreement with previous studies (Yount et al. 1968; Riesen et al. 1976; Siber et al. 1980; Barrett and Ayoub 1986).

In one exceptional case in our study the peak concentration of specific IgG2 subclass was below 100 µg/ml. This suggests that there are poor responders to PCP antigens. Sarvas et al. (1989) demonstrated that after vaccination with a 23-valent pneumococcal polysaccharide vaccine, allotype G2m(n)-positive homozygotes had about four times more IgG2 antibodies (anti-14 and anti-18C) than did G2m(n)-negative vaccinated individuals, and heterozygotes occupied an intermediate position, which suggested codominant inheritance of the G2m(n)-associated strong responsiveness. If their hypothesis is accepted, our poor responder may be a G2m(n)-negative homozygote or heterozygote. To clarify this, however, would require more investigation.

Acknowledgments

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References
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