Enzyme Immunoassay for Detection of Immunoglobulin G (IgG), IgM, and IgA Antibodies against Type 6B Pneumococcal Capsular Polysaccharide and Cell Wall C Polysaccharide in Chinchilla Serum

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Conjugation of the capsular polysaccharides of *Streptococcus pneumoniae* to protein carriers has introduced a new generation of pneumococcal vaccines which may be efficacious in preventing pneumococcal otitis media during infancy. The chinchilla model has been used extensively for studying the pathogenesis of pneumococcal otitis media and for testing the efficacy of early pneumococcal capsular polysaccharide (PCP) vaccines, but immunologic studies in the chinchilla have been limited by the lack of antibodies against specific immunoglobulin isotypes. By using affinity-purified rabbit immunoglobulin G (IgG) anti-chinchilla IgG, IgM, and IgA, we developed a sensitive enzyme immunoassay that is highly specific for IgG, IgM, and IgA antibodies against type 6B PCP (anti-6B) and against C polysaccharide in chinchilla serum. Antibody titers increased in serum from five chinchillas immunized with a type 6B outer membrane protein complex vaccine. Increases of anti-6B IgG and IgM antibody titers were more striking than increases of anti-6B IgA or anti-C polysaccharide IgG, IgM, or IgA titers were.

Acute otitis media is a very common infectious disease in infants and children (29, 34), and *Streptococcus pneumoniae* is the leading cause of acute otitis media (14). The purified capsular polysaccharides (PCPs) from the most common pneumococcal serotypes that cause disease have been formulated into polyvalent vaccines, and children immunized with the vaccines develop specific antibodies against some of the serotypes (3, 7, 10, 11, 22-24, 33). However, the antibody responses in children younger than 2 years of age have been poor, especially against the most common serotypes, such as 6A and 6B. It is suspected that the high frequency of disease caused by these serotypes is, therefore, related to the poor immunogenicities of these capsular polysaccharides.

Conjugation of the capsular polysaccharides of *S. pneumoniae* to protein carriers has introduced a second generation of pneumococcal vaccines (30). Type 6B PCP has been conjugated with *Neisseria meningitidis* group B outer membrane protein complex (OMPC) by a methodology similar to that used in conjugating the polyribosyl ribitol phosphate capsular polysaccharide of *Haemophilus influenzae* type b to OMPC (25). Since the conjugated polyribosyl ribitol phosphate-OMPC vaccine elicits a protective antibody response in infants as young as 2 months of age (31), it is reasonable to expect that the PCP-OMPC conjugates will have equally good immunogenicities. Several PCP-protein conjugates have been demonstrated in recently reported studies (1, 5, 12, 13, 32) to have enhanced immunogenicities.

The chinchilla species has become one of the most suitable laboratory animals for experimental otitis media research (17). Antibodies against PCP have been measured by radioimmunoassay (RIA) in chinchilla serum and middle ear fluid on the basis of Farr's technique (18), but this assay reflects only total anti-PCP antibody concentrations, not isotype-

specific antibodies. A sensitive, isotype-specific anti-PCP assay is required to explore further the immunogenicity of the new PCP-OMPC conjugate vaccines and to study the immunopathogenesis of pneumococcal otitis media in the chinchilla model. We describe here a sensitive enzyme immunoassay (EIA) that is highly specific for measuring immunoglobulin G (IgG), IgM, and IgA antibodies against type 6B PCP (anti-6B) in chinchilla serum.

MATERIALS AND METHODS

Chinchilla serum specimens. Five healthy chinchillas (age, 1 to 2 years; weight, 400 to 600 g) were used in this study. They received food and water ad libitum and were under the care of the University of Minnesota Research Animal Resources staff. Serum was obtained from chinchillas by cardiac paracentesis every 2 weeks after immunization. The use of laboratory animals in this research conformed with the University of Minnesota Regents' Policies and Procedures for Animal Care and Usage, the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the Animal Welfare Act (Public Law 89-144 as amended).

The type 6B-OMPC vaccine (lot 1193), provided by Merck Sharp & Dohme Research Laboratories (West Point, Pa.), was composed of serotype-specific PCP (10 µg/ml) conjugated to an OMPC from group B *N. meningitidis* and contained aluminum hydroxide, as an adjuvant, and 1:20,000 thimerosal. A dose of 8 µg of PCP per kg of body weight was administered by intramuscular injection into the proximal thigh.

Reference sera. Preliminary screening of chinchilla sera for the presence of anti-6B antibodies by the EIA was initially performed by using a normal chinchilla serum pool as a negative reference and serum obtained from a chinchilla 4 weeks after administering intraperitoneally 500 µg of type 6A PCP mixed with incomplete Freund's adjuvant as a positive reference. In the final anti-6B antibody measure-

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ments, a pool of nine serum samples from six different chinchillas immunized with the 6B-OMPC vaccine resulted in a high antibody titer in the preliminary EIA, and this pool was used as a positive reference serum on each microplate in subsequent assays for anti-6B antibodies.

RIA for anti-PCP antibodies. Total anti-6B type-specific antibody in serum was measured by RIA at Merck Sharp & Dohme Research Laboratories through the courtesy of Philip Vella. Results were reported as micrograms of antibody protein per milliliter of serum.

EIA for pneumococcal antibodies. IgG, IgM, and IgA antibodies in chinchilla serum against type 6B PCP and against pneumococcal cell wall C polysaccharide (C-ps) were measured by EIA by modifying the EIA method described by Koskela (20) for the corresponding antibodies in human serum. Odd-numbered wells of polystyrene microplates (Immuno Plate PolySorp; Nunc Products, Roskilde, Denmark) were coated with 6B PCP (provided by Merck Sharp & Dohme Research Laboratories) or C-ps by incubating 100 μ l of the appropriate antigen solution (20 μ g/ml in 0.01 M phosphate-buffered saline [PBS; pH 7.2]) per well for 5 h at 37°C and then overnight at 4°C. The C-ps was isolated by the method of Pedersen et al. (28) from a pneumococcal strain with a polysaccharide capsule that consists only of C-ps (C mutant CSR, SCS-2, clone 1); the pneumococcal strain was kindly provided by Jorgen Henriksen (Statens Serum Institut, Copenhagen, Denmark). Even-numbered wells were incubated only with PBS to control for nonspecific binding of antibodies during the subsequent EIA steps. After incubation, plates were coated with 1% skim milk (Difco, Detroit, Mich.) for 1 h at 37°C.

Before assaying serum for anti-6B antibodies, anti-C-ps activity was neutralized by incubating equal volumes of chinchilla serum and a C-ps solution (1 mg/ml in PBS) in a glass tube for 2 h at 37°C and then overnight at 4°C. This was necessary because the 6B PCP used to coat the plates also contained 13% (wt/vol) of C-ps (described below) and because healthy, nonimmunized chinchillas had activity against C-ps in their sera. The serum sample was further diluted 1:40 with PBS containing 1% skim milk and 1% Tween 20. This diluent was used in all subsequent steps of the EIA procedure. The elimination of anti-C-ps activity in serum diluted for anti-PCP type 6B antibody measurement was controlled by EIA on a separate plate coated with C-ps.

Plates coated with the appropriate antigen were washed eight times with PBS containing 0.05% Tween 20 (model 1550 Micro Plate Washer; Bio-Rad, Richmond, Calif.). The same washings were performed after each subsequent antibody incubation. Chinchilla serum samples diluted to either 1:40 or 1:200, as determined by screening tests, were added to wells of the paired (antigen-coated and antigenfree) columns and were serially diluted twofold down the columns and incubated for 2 h at 37°C. After washing, 100 μ l of affinity-purified rabbit IgG specific for chinchilla IgG, IgM, or IgA (19) (5 μ g of antibody protein per ml) was incubated in each well for 2 h at 37°C. After washing, 100 μ l of horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin conjugate (Zymed Laboratories, Inc., San Francisco, Calif.) diluted 1:2,000 was incubated in each well overnight at room temperature. After washing, 200 μ l of substrate solution containing 0.4 mg of *o*-phenylenediamine dihydrochloride (Sigma Chemical Co., St. Louis, Mo.) and 0.24 μ l of 30% H₂O₂ per ml of 60 mM citrate buffer (pH 5.0) was incubated in each well for exactly 30 min. The enzyme reaction was stopped by adding 50 μ l of 2.5 N H₂SO₄ per well. The optical density (ODs) of the wells were measured

at 492 and 630 nm (reference wavelength) in a Titertek Multiscan MCC/340 spectrophotometer (Flow Laboratories, Inc., McLean, Va.). Results were recorded in the Skansoft program (Flow Laboratories, Inc.). The net OD for each serum dilution was determined by subtracting the OD value of the antigenfree well from the OD value of the paired antigen-coated well. The net serum antibody titer was interpolated from the intersection of the net OD-versus-serum dilution linear regression with 0.3 OD unit, and this titer was corrected with the antibody titer of the corresponding reference serum, which was run on each plate.

EIA for C-ps antigen. The C-ps concentration in the type 6B PCP used as the EIA capture antigen for anti-6B antibodies was measured by an EIA modified from the method described above for the serum antibodies. Odd-numbered wells of microplates were coated with a mouse monoclonal IgM anti-pneumococcal C-ps antibody (10 μ g/ml of PBS; provided by Jorgen Henriksen), and even-numbered wells were coated with PBS for 2 h at 37°C. After incubation, all wells were coated with 1% normal goat serum diluted with PBS for 1 h at 4°C. After washing, reference C-ps and 6B PCP solutions diluted serially twofold in PBS containing 1% normal goat serum and 0.1% Tween 20 (diluent) were incubated in the wells for 2 h at 37°C. After washing, 100 μ l of rabbit immunoglobulin against pneumococcal C-ps (5 μ g of diluent per ml; provided by Jorgen Henriksen) was incubated in the wells for 2 h at 37°C. After washing, HRP-labeled goat anti-rabbit IgG conjugate diluted 1:4,000 was incubated in each well overnight at room temperature; this was followed by washing and substrate incubation as described above. After the OD values were read, the proportion of C-ps in 6B PCP antigen was calculated on the basis of the standard curve obtained with the reference C-ps antigen.

RESULTS

Each antibody measurement was performed in paired microplate columns, the first of which was coated with 6B-PCP antigen and the second of which was coated with PBS not containing PCP. This specimen-specific blanking was essential because nonspecific binding of antibodies to the plastic microplate varied among different animal sera. Coating the wells with 1% skim milk and dilution of all antibody-containing reagents thereafter in PBS containing 1% skim milk and 1% Tween 20 was more effective in minimizing the nonspecific background than was the use of bovine albumin or gelatin instead of skim milk (data not shown).

Maximal binding of 6B PCP and C-ps on the PolySorp microplates was achieved with 10 μ g of PCP per ml; to ensure antigen excess, 20 μ g of each antigen per ml was used in subsequent assays. Working concentrations of affinity-purified rabbit IgG anti-chinchilla IgG, IgM, and IgA and HRP-labeled goat anti-rabbit IgG were established by checkerboard titration; the optimum concentration of affinity-purified rabbit IgG reagents was 5 μ g/ml, and the optimum concentration of the HRP conjugates was 1:2,000 for IgG antibodies and 1:1,000 for IgM and IgA antibodies. Interassay variation was calculated from the titers of the reference serum run on each plate; the between-day coefficients of variation for the six antibody assays ranged between 0.36 and 0.50.

Anti-6B antibody by RIA. Four chinchillas produced significant concentrations of anti-6B antibodies in serum, as detected by RIA (Fig. 1). The geometric mean concentration

FIG. 1. Concentrations of total anti-6B antibodies in serum measured by RIA in five chinchillas immunized with the 6B-OMPC vaccine. Geometric mean titers are shown by the heavy line and closed squares.

increased from 0.28 $\mu\text{g/ml}$ preimmunization to 1.24 $\mu\text{g/ml}$ 56 days later. The ratios of the 8-week postimmunization concentrations to the preimmunization concentrations ranged between 1.8 and 14.7 (median, 4.8).

Anti-6B antibodies by EIA. Two animals had high preimmunization anti-6B IgG antibody titers; for one of these animals, there was only a modest increase in this antibody isotype after immunization, and for the other animal the antibody isotype increased 7.5 times (Fig. 2A). Two of three animals with low preexisting titers had a large increase in this antibody type after immunization (75 and 145 times).

Two animals had high preimmunization anti-6B IgM antibody titers, and both had significant IgM responses 2 weeks after immunization (6.5 and 10.0 times); titers returned

nearly to preimmunization levels by 8 weeks postimmunization (Fig. 2B). For the other three animals with low preimmunization titers, there was only a modest increase in this antibody isotype after immunization.

Anti-6B IgA antibody response patterns after immunization were quite variable, and only a slight rise in the geometric mean titer was observed (Fig. 2C). Three of five animals had higher IgA titers 8 weeks after immunization than they did before immunization.

Anti-C-*ps* antibodies by EIA. All five animals had appreciable IgG antibodies against C-*ps* before immunization, but only one animal showed a transient increase in antibody after immunization (Fig. 3A). Only one animal had appreciable IgM anti-C-*ps* antibody before immunization (Fig. 3B), and this animal also had preexisting IgA anti-C-*ps* antibody (Fig. 3C). None of the animals showed an increase in IgM or IgA anti-C-*ps* antibodies after immunization (Fig. 3B and C).

The slopes of the OD-versus-serum dilution curves for sera from individual animals from which samples were obtained before and after immunization were similar.

Relationships between preimmunization and postimmunization titers were explored by calculating correlation coefficients. Only IgM anti-6B pre- and postimmunization antibody titers were significantly correlated ($r = 0.65$; $P = 0.028$). However, there was a significant indirect correlation between preimmunization antibody titers and the postimmunization/preimmunization antibody ratios for four antibodies: IgG anti-6B ($r = -0.578$; $P = 0.05$), IgM anti-6B ($r = -0.42$; $P = 0.13$), IgG anti-C-*ps* ($r = -0.70$; $P = 0.02$), and IgM anti-C-*ps* ($r = -0.50$; $P = 0.09$).

Relationships among the several antibodies produced by 6B-OMPC immunization were also explored by calculating linear regression correlation coefficients. Total antibodies detected by RIA were significantly correlated with anti-6B IgG ($r = 0.66$; $P < 0.01$) and IgM antibodies ($r = 0.63$; $P < 0.01$), but not with anti-C-*ps* antibodies. IgG anti-6B and IgG anti-C-*ps* antibodies were weakly correlated ($r = 0.37$; $P <$

FIG. 2. Concentrations of anti-6B antibodies in serum measured by EIA in five chinchillas immunized with the 6B-OMPC vaccine. Geometric mean titers are shown by the heavy lines and closed squares. (A) IgG antibodies; (B) IgM antibodies; (C) IgA antibodies.

FIG. 3. Concentrations of anti-C-ps antibodies in serum measured by EIA in five chinchillas immunized with the 6B-OMPC vaccine. Geometric mean titers are shown by the heavy lines and closed squares. (A) IgG antibodies; (B) IgM antibodies; (C) IgA antibodies.

0.01). IgG and IgM antibodies against 6B PCP were significantly correlated ($r = 0.64$; $P < 0.01$), but IgG and IgM antibodies against C-ps were not correlated ($r = 0.23$; $P > 0.05$).

DISCUSSION

We described the first EIA for chinchilla IgG, IgM, and IgA antibodies in which affinity-purified rabbit IgG antibodies against chinchilla immunoglobulin heavy chains were used (19). IgG and IgM anti-6B antibodies measured by this EIA were significantly correlated with total anti-6B antibodies measured by RIA. Koskela and Leinonen (21) also found strong correlations between RIA and the sum of EIA titers in human serum (correlation coefficients of between 0.47 and 0.81 for types 3, 6A, 14, 18C, 19F, and 23F). Callahan et al. (8) found poor agreement between EIA and RIA for each of 14 PCPs, but she did not neutralize C-ps activity and her EIA was not isotype specific. Bocktor et al. (6) reported RIA versus EIA correlation coefficients that were nearly unity, an observation that is difficult to explain for an assay that was not isotype specific and that did not use anti-C-ps neutralization.

Measurement of anti-C-ps activity as anti-PCP antibody has been the major cause of anti-PCP antibody assay nonspecificity. Purified PCPs, when they are used as EIA antigens (20) or as vaccines (35), contain variable concentrations of C-ps. There was 13% (wt/vol) C-ps in the 6B PCP used as an antigen in our EIA. Anti-C-ps activity is commonly present in human sera (20), and its activity can increase in response to pneumococcal otitis media (20) and immunization (20, 28). Neutralization of anti-C-ps activity in the serum sample with an excess of soluble C-ps before the measurement of anti-PCP antibody by EIA has been a reliable way of eliminating the effect of anti-C-ps on anti-PCP findings (22, 27).

Direct binding of serum antibodies to EIA microtiter plates also yields nonspecific antibody findings, and this

nonspecific activity varies among individual serum specimens. Therefore, in our EIA the net OD values for each serum sample were calculated by subtracting the OD values in wells not containing antigen (background wells) from the OD values in paired antigen-coated wells. None of the common blocking reagents significantly influenced this background activity, although it was lowest with skim milk blocking.

Variable anti-PCP and anti-C-ps antibody affinities did not appear to influence our EIA results, since there was not a change in the slope of the OD-versus-serum dilution curves among different serum samples. Koskela and Leinonen (21) made similar observations for serum from children.

In the EIA described here, the coefficients of variation for between-day runs ranged between 0.36 and 0.50 and were comparable to those reported by Koskela and Leinonen (21) for human serum. Within-plate coefficients of variation were considerably lower (range, 0.05 to 0.15). Between-day variation was therefore minimized during testing of serum samples by including on each plate a reference serum sample and by correcting the test sample titers with the ratio of the plate reference titer divided by the mean reference titer. Bernstson et al. (4) and Melville-Smith and Sheffield (26) also bound PCP directly to plates, but they did not report assay variability and did not neutralize anti-C-ps activity before measuring anti-PCP antibodies. Barrett et al. (2) reported a coefficient of variation of 0.24 by using an EIA in which PCP was captured on the microplate by serum from rabbits immunized with the pneumococcal serotype being tested. Carlson et al. (9) improved the coefficient of variation of this assay to between 0.03 and 0.11 by using rabbit IgG Fab₂ fragments to capture PCP antigen. Bocktor et al. (6) reported coefficients of variation of between 0.05 and 0.19 by using tyraminated polysaccharide bound directly to microplates; these low coefficients of variation were remarkable, considering that anti-C-ps activity was not neutralized, the assay

was not isotype specific, and background activity in serum was not measured.

The ability of our EIA to detect increases in concentrations of anti-PCP IgG, IgM, and IgA antibodies in serum after immunization with a PCP-protein conjugate vaccine suggests that the assay will be a valuable tool for analyzing chinchilla antibodies. A more detailed analysis of antibody responses to the type 6B-OMPC vaccine is in press (15); and an analysis of responses to types 14, 19F, and 23F conjugate vaccines, as well as their activities in preventing vaccine-type pneumococcal otitis media, has been submitted for publication (16).

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