Serum IgM Antibodies Contribute to High Levels of Opsonophagocytic Activities in Toddlers Immunized with a Single Dose of the 9-Valent Pneumococcal Conjugate Vaccine

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In immunogenicity trials of pneumococcal conjugate vaccines (PCVs), only IgG antibody concentrations to pneumococcal capsular polysaccharides (PPSs) are usually determined, along with the opsonophagocytic activity (OPA) of antipneumococcal antibodies. We aimed to determine the role of both IgG and IgM in OPA in toddlers receiving one dose of 9-valent PCV (PCV9). The IgG and IgM antibody concentrations to PPSs of serotypes 6A, 9V, 14, 19F, and 23F were measured by enzyme immunoassay in sera from toddlers (ages 18 to 35 months) 1 month after a single PCV9 dose. The OPA for the same serotypes was measured by multiplexed opsonophagocytosis assay (MOPA). Further, IgG and IgM concentrations and MOPA were measured to PPSs of serotypes 6A, 14, and 19F in sera collected 12 months after vaccination. The detected MOPA titers were high in comparison to the IgG concentrations 1 month after immunization. The IgM concentrations were higher than IgG concentrations for serotypes 6A, 14, and 19F (P < 0.001) and as high as IgG for serotypes 9V, 19F, and 23F. Correlation of the IgM antibody concentrations with MOPA (r = 0.35 to 0.65) was stronger compared to that of the IgG antibodies (r = 0.07 to 0.41). The depletion of IgG antibodies in three sets of pooled sera only slightly decreased the OPA activity against serotype 14. At 12 months after immunization, 50 to 100% of serum samples still showed detectable MOPA activity against serotypes 6A, 14, and 19F. Our results suggest that IgM contributes to OPA 1 month after a single PCV9 vaccination in toddlers and that functionally active IgM and IgG antibodies persist for at least a year.

Streptococcus pneumoniae (pneumococcus) is a leading cause of morbidity and mortality worldwide. The dramatic success with Haemophilus influenzae type b (Hib) conjugate vaccines in reducing invasive Hib disease and carriage (4, 14) encouraged the development and use of protein conjugate vaccines (PCVs). At present there are three licensed PCVs (1, 11), which are recommended generally to be used in infants and toddlers. Recently, the use of 13-valent PCV was extended for prevention of pneumococcal pneumonia in adults 50 years and older (15). Vaccination schedules containing usually two or three PCV doses in early infancy and a booster dose at the second year of life are widely used (1). To get the greatest effect when adopting PCV to a national immunization program, some countries use catch-up campaigns of one or two doses of PCV for children under 2 years. The ability of new PCVs and new vaccination schedules to induce protective immunity and the effect of simultaneously given vaccines on immune response to PCVs is evaluated in immunogenicity trials. The immunogenicity of PCVs is primarily assessed by measurement of serum serotype-specific IgG antibody concentrations and secondarily by serotype-specific functional antibody titers, as stated in the World Health Organization (WHO) recommendations to assure the quality, safety, and efficacy of PCVs (26).

The primary mechanism to eliminate pneumococci from the immunocompetent host is opsonophagocytosis. The bacteria are opsonized with anti-capsular antibodies, followed by activation of the complement system, resulting in receptor-mediated uptake and killing of pneumococci by the host phagocytic cells (10, 17, 25). The first line of antibodies produced in the humoral response are of IgM class, which can be expressed without isotype switching (12). Some IgM is also produced in secondary and subsequent responses, although other isotypes (mainly IgG) dominate the later phases of the antibody response. IgM molecules form pentamers whose 10 antigen-binding sites can simultaneously bind to multivalent antigens, such as bacterial capsular polysaccharides. By conferring high avidity, this multipoint-binding compensates for the relatively low affinity of the IgM monomers. The pentameric structure of IgM makes it especially effective in activating the complement system. IgM antibody concentrations are expected to decline rapidly after immunization, and thus the protection achieved by highly functional IgM can be short-lived. In previous studies, a single dose of PCV was able to induce an IgM response (6, 13, 20), while the role of IgM anti-PPS antibodies in the opsonophagocytosis of pneumococci has not been elucidated earlier.

The objective of the present study was to determine the role of both IgG and IgM in opsonophagocytosis against pneumococci in toddlers who have received a single dose of 9-valent PCV (PCV9) (8). We show that anti-PPS IgM contributes to the opsonophago-
cytosis against pneumococci in toddlers immunized with a single dose of PCV.

(These data were presented in part on 14 to 18 March 2010 at the 7th International Symposium on Pneumococci and Pneumococcal Diseases, Tel Aviv, Israel.)

**MATERIALS AND METHODS**

**Study subjects and serum samples.** The serum samples were obtained from a previous, randomized study (8) on the effect of a PCV9 on pneumococcal nasopharyngeal carriage in healthy Israeli toddlers attending to day care centers. The subjects of the present study include toddlers aged 18 to 35 months, who were immunized with a single dose of PCV9. The blood samples for serological assays were obtained 1 month (n = 71) and 12 months (n = 54) after immunization.

**Vaccine.** The PCV9 vaccine was used; in this vaccine, 2 μg each of pneumococcal serotype 1, 4, 5, 9V, 14, 18C, 19F, and 23F carbohydrates and 4 μg of serotype 6B carbohydrate were coupled to a nontoxic variant of diphtheria toxin, CRM197, and are presented as lyophilized preparations (Wyeth-Lederle Vaccines [now Pfizer]).

**EIA.** The concentrations of anti-PPS IgG antibodies in sera collected 1 month after immunization have been analyzed in an earlier study by non-22F pneumococcal enzyme immunoassay (EIA) (7). In the present study, a modification of 22F inhibition EIA method (22) applied by the WHO reference laboratory at the Institute of Child Health (London, United Kingdom) was used to measure the concentrations of serum anti-PPS IgG and IgM against pneumococcal serotypes 6A, 9V, 14, 19F, and 23F 1 month after immunization. In addition, The IgG and IgM concentrations against serotypes 6A, 14, and 19F were measured in sera collected 12 months after immunization. Serotypes 9V, 14, 19F, and 23F belonged to the most frequently carried serotypes in the study population. Serotype 6A was selected in order to analyze the cross-protection by a PCV that did not contain 6A conjugate. If the antibody concentration was less than the limit of quantitation, the concentration was assigned a value half of the quantitation limit. The reproducibility of the EIA was confirmed by using two control sera on each plate. Depending on the PPS antigen used, the interassay coefficients of variation (CVs) of the control sera for anti-PPS IgG and IgM ranged from 14 to 27% and from 10 to 27%, respectively.

**MOPA.** The opsonic activities of sera collected 1 month after immunization were measured against pneumococcal serotypes 6A, 9V, 14, 19F, and 23F by a 4-fold multiplexed opsonophagocytosis assay (MOPA) as previously described (3), with minor modifications (23). In addition, the opsonic activities in sera collected 12 months after immunization against serotypes 6A, 14, and 19F were measured in a further set of analyses. Serotypes to the latter analyses were selected on the basis of MOPA method available at that time. Due to the long calendar time elapsed (i.e., several years) between the MOPA analyses of sera collected after at 1 and 12 months after immunization, the results cannot be directly compared due to changes in the bacterial and reagent batches in the course of time. Pneumococcal strains of serotypes 6A, 9V, 14, 19F, and 23F made resistant to either optochin, spectinomycin, streptomycin, or trimethoprim (3) were received from Moon Nahm (University of Alabama at Birmingham). Differentiated HL-60 cells (American Type Culture Collection, Manassas, VA) were allowed to phagocytose pneumococci in the presence of serum antibodies and baby rabbit complement (Dynal Invitrogen Corp., Bromborough, United Kingdom). An aliquot of the reaction mixture was plated onto four different Todd-Hewitt–0.5% yeast extract agar plates containing appropriate antibiotics. The bacterial colonies were enumerated after overnight incubation at 37°C with 5% CO2. The opsonic titers (OPT) were defined as the reciprocal of serum dilution with 50% killing compared to the bacterial growth in control wells without serum. For sera with OPT ≤ 4, a value of 2 was assigned. The reproducibility was followed by including a control serum into each plate. In the former analyses (1-month sera), the CVs of the control sera for serotypes 6A, 9V, 14, 19F, and 23F were 32, 35, 32, 22, and 35%, respectively. In the latter analyses (12-month sera), the CVs of the control sera for serotypes 6A, 14, 19F, and 19 were 35, 33, and 25%, respectively.

**IgG depletion.** To assess the possible role of IgM in opsonophagocytosis, we depleted the IgG antibodies from selected serum samples collected 1 month after immunization and measured the IgG and IgM concentrations, as well as MOPA against serotype 14 (14MOPA), before and after IgG depletion. Due to the small volumes of the sera available, we combined the selected sera into three distinct serum pools. The selection criteria were as follows: (i) pool 1 consisted of four sera with high 14MOPA activity and low anti-IgG but low IgM antibody concentration compared to PPS 14; (ii) pool 2 consisted of five sera with high 14MOPA activity and comparable IgG and IgM concentrations to PPS 14; and (iii) pool 3 consisted of five sera with high 14MOPA and low IgG and high IgM concentrations to PPS 14. IgG depletion was performed by affinity chromatography with AlbuMin & IgG Depletion SpinTrap (GE Healthcare Europe GmbH, Freiburg, Germany). Briefly, serum pools were diluted 1:2 with binding buffer containing 20 mM NaPO4 and 0.15 M NaCl (pH 7.4) and applied to the columns. The columns were incubated for 5 min without mixing at room temperature. The columns were then centrifuged for 30 s at 800 × g, and the eluate containing the depleted sample was collected. Then, 100 μl of binding buffer was added to the columns, the columns were centrifuged for 30 s at 800 × g, and the eluate was collected. The preceding step was performed twice.

**Statistical methods.** Antibody concentrations are given as geometric mean concentrations (GMC), and opsonophagocytic activities are given as geometric mean opsonic titers (GMOPT), along with the 95% confidence intervals. Comparisons between groups were performed by using the Student t test. The Pearson correlation coefficient was used to evaluate the correlation between GMOPT and antibody concentrations. In all statistical comparisons, log-transformed data were used, and P values of <0.05 were considered statistically significant.

**RESULTS**

Anti-PPS IgG antibody concentrations and opsonophagocytic activity of antipneumococcal antibodies at 1 month after immunization. We first measured the IgG antibody concentrations to PPSs 6A, 9V, 14, 19F, and 23F and the opsonophagocytic activities of serum antipneumococcal antibodies against the same serotypes in sera collected 1 month after immunization. The IgG concentrations for 9V, 14, 19F, and 23F measured in the present study with 22F inhibition EIA correlated significantly with the previous results obtained with non-22F EIA (r = 0.83 to 0.90, P < 0.01) (7), while the antibody concentrations tended to be slightly lower with 22F EIA compared to non-22F EIA. Antibodies to serotype 6A were not determined in the previous study. The opsonophagocytic activity of antipneumococcal antibodies to all serotypes seemed to be surprisingly high compared to the detected anti-PPS IgG concentrations (Table 1), particularly to serotypes 9V and 14.

![Table 1](https://example.com/table1.png)
The correlations between the IgG concentrations and MOPA were poor (Fig. 1, IgG compared to MOPA).

Anti-PPS IgM antibody concentrations and opsonophagocytic activity of antipneumococcal antibodies at 1 month after immunization. As a possible explanation for our findings, the IgM anti-PPS antibodies were speculated to contribute to the high opsonophagocytic activities detected 1 month after immunization. Thus, we measured the concentrations of IgM anti-PPS in the same sera to the same serotypes. The IgM concentrations 1 month after PCV9 immunization were found to be significantly higher compared to IgG to serotypes 6A and 14 (P < 0.001) and as high as IgG to serotypes 9V, 19F, and 23F (Table 1). Correlation of IgM anti-PPS concentrations with MOPA was statistically significant and for all serotypes stronger than that of the IgG antibodies (Fig. 1, IgM compared to MOPA). The correlation of sum of IgG and IgM (IgG+M) concentrations with MOPA was similar to that of IgM alone (Fig. 1, IgG+M compared to MOPA).

Effect of IgG depletion on opsonophagocytic activity against serotype 14 at 1 month after immunization. In order to further clarify the possible role of IgM in opsonophagocytosis, we next depleted the IgG antibodies in three sets of pooled sera. The anti-PPS14 IgG and IgM antibody concentrations and MOPA for se-

FIG 1 Correlation of IgG, IgM, and IgG+IgM antibody concentrations to PPSs of serotypes 6A, 9V, 14, 19F, and 23F with opsonophagocytic activities of antipneumococcal antibodies (MOPA) against these serotypes in sera collected 1 month after PCV9 immunization. *, P < 0.05; **, P < 0.01.
IgG depletion was successful: the IgG anti-PPS14 concentrations were undetectable after the depletion of IgG antibodies in all three sets of pooled sera (Fig. 2). The depletion process also had an effect on detected IgM anti-PPS14 concentrations, which were reduced after IgG depletion, particularly in pool 3 (Fig. 2). GMOPT measured after IgG depletion showed that depletion of IgG antibodies did not abolish 14MOPA activity. In contrast, a large degree of MOPA activity still remained after IgG depletion. The 14MOPA was related to the IgM anti-PPS14 concentration both before and after the IgG depletion (Fig. 3).

Persistence of anti-PPS IgG and IgM antibody concentrations and MOPA against serotypes 6A, 14, and 19F at 12 months after immunization. The persistence of IgG and IgM antibody concentrations against serotypes 6A, 14, and 19F was evaluated in sera collected 12 months after PCV9 immunization (Table 2). The GMC of anti-14 IgG was significantly higher in sera collected 12 months after immunization compared to the previous measurement of sera collected 1 month after immunization (P < 0.05), whereas the GMC of anti-14 IgM was significantly lower at 12 months compared to the measurement 1 month after immunization (P < 0.001). The GMC of both anti-6A and anti-19F IgG and IgM were comparable in sample sets collected at 1 month and 12 months after immunization. More than half of the tested serum samples collected 12 months after immunization (n = 54) showed detectable MOPA against serotypes 6A (50%), 14 (100%), and 19F (81%). Due to methodological constraints, we were not able to directly compare the MOPA titers in sera collected at 1 month and 12 months after vaccination.

DISCUSSION

This study originated from a discrepancy detected between the IgG concentrations and the high functional activity of antipneumococcal antibodies measured in sera collected from toddlers 1 month after immunization with a single dose of PCV9. A single dose of PCV is able to induce a significant IgM response measurable 1 month after immunization (6, 13). Our results suggest that IgM antibodies contribute to the high opsonophagocytic activities measured 1 month after a single dose of PCV9 in toddlers and that IgM and IgG antibodies as well as functional antibody activity are sustained for at least a year.

The 7-valent PCV (PCV7) was the first PCV introduced into the routine infant and toddler immunization programs (5). WHO has established recommendations to assure the quality, safety, and efficacy of PCVs in infants (26). These recommendations state that the primary comparison of PCV-induced immune responses should be based on serotype-specific IgG concentrations measured by EIA. In addition, determination of serotype-specific functional antibody titers measured by OPA for a subset of vaccinated study subjects has been recommended. Thus, the concentrations of IgM anti-PPS antibodies are not routinely analyzed in PCV immunogenicity studies.

In a recent study by Clutterbuck et al. (6), IgM was shown to dominate the antibody responses after first dose of PCV in 12-month-old toddlers. In another study by Nieminen et al. (13), antibody responses to four 4-valent PCVs were analyzed by non-22F inhibition EIA, and a >2-fold increase in both anti-PPS IgG and IgM concentrations was detected in at least 25 of 40 children aged 24 months. Also, comparison of the anti-PPS14 IgM concentrations in pre- and postimmunization sera (unpublished data) from a previous study of 11-valent PCV conducted among Israeli toddlers (28) showed low prevaccination anti-PPS14 IgM concentrations and a >2-fold increase in postvaccination anti-PPS14 IgM concentrations in 15 of 23 toddlers, suggesting vaccine-induced IgM responses (unpublished data).

In the present study there were no preimmunization sera avail-
able, and we were thus not able to measure the baseline anti-PPS antibody concentrations or MOPA prior to immunization. It is therefore essential to note that the IgG and IgM concentrations measured in the present study may also be affected by the natural exposure to pneumococci prior to immunization and cannot thereby be interpreted as purely vaccine induced (i.e., the postvaccination IgM response may partly represent a secondary response to previous pneumococcal exposure). A kind of indicator for this is serotype 6A, which was not included in the vaccine formulation tested. Although the high postimmunization OPA titers against serotype 6A may at least partly represent vaccine-induced cross-reactive antibodies to serotype 6B, they point toward preexisting anti-PPS6A due to exposure rather than vaccination.

The role of IgM antibodies in opsonophagocytosis of pneumococci has not been extensively studied and, accordingly, studies reporting both IgM and IgG anti-PPS antibody concentrations in comparison to the functional antibody activity are scarce. A similar observation of high MOPA versus low IgG concentrations was recently made in another study among children who received a single dose of 10-valent PCV (21). In that study, the postimmunization OPA of sera from 3-year-old control subjects receiving their first PCV dose was high compared to the low IgG concentrations for certain serotypes (particularly 7F, 9V, 14, and 23F), whereas the anti-PPS IgM concentrations were not analyzed. In a study by Vidarsson et al. (27), infants and adults were vaccinated with PPS of serotype 6B conjugated to tetanus toxoid and post-vaccination serum IgG, IgM, and IgA antibody levels, as well as opsonic activities, were compared. In an infant group injected at 7, 9, and 18 months of age, the opsonic activity correlated well with IgG but also with IgM. Furthermore, in the same group of infants the IgM concentrations were slightly higher compared to IgG after the first injection. Studies conducted among the elderly population suggest that the lower opsonization titers than expected by the IgG concentration (16, 19) might at least partly be explained by the lower IgM antibody concentration in the elderly (18, 22).

In regard to the meningococcal conjugate vaccines, indirect evidence for the possible role of IgM in the functional activity of serum antibodies has been reported by Tsai et al. (24): low IgG antibody concentrations but high serum bactericidal antibody activity was detected 4 days after immunization with meningococcal group C polysaccharide-conjugate vaccine, and a contribution of IgM to the serum bactericidal antibody activity was suggested. There are also two studies that have described an association of IgM with functional antibody activity against Hib in older children and adults (2, 9).

The successful depletion of IgG antibodies from three sets of pooled sera did not abolish the opsonophagocytic activity of antipneumococcal antibodies against serotype 14, which speaks for other factors than IgG behind the high MOPA. The high IgM anti-PPS concentrations and the stronger correlation of IgM antibodies with serotype-specific MOPA compared to IgG suggest that IgM has a role in opsonophagocytosis and consequently in the protection of toddlers after immunization with PCV. The possible role of IgM antibodies in high opsonophagocytic activities could have been clarified by further depletion rounds of either IgM and/or both IgG and IgM anti-PPS14 antibodies but, unfortunately, the sample volumes were inadequate for these further analyses.

Several questions remain to be answered. For example, are the high IgM concentrations also detected after more than one dose of PCV and/or in other age groups (e.g., in adults)? Are there other factors besides IgM that contribute to the high opsonophagocytic activities detected in toddlers? How does prior exposure with pneumococci affect the postimmunization IgM concentrations, and finally, are the high IgM concentrations connected with only short-lasting opsonophagocytic activity and protection after vaccination? To elucidate this last question (i.e., the persistence of functional antibodies), we tested a set (n = 54) of sera collected at 12 months after immunization. Although the anti-14 IgM concentrations had significantly declined (Table 2), the anti-6A and anti-19F IgM concentrations at 12 months were as high as at 1 month after immunization, suggesting natural boosting (PCV was not in the national immunization program in Israel at the time of this PCV9 trial). Depending on the serotype, 50 to 100% of the serum samples collected 12 months after immunization still showed detectable MOPA activity against serotypes 6A, 14, and 19F. The long period of time that elapsed between the MOPA analyses of 1-month and 12-month sera, however, made direct comparison of the results not feasible due to methodological constraints (e.g., different batches of reagents and bacteria).

Based on our findings, it seems likely that in toddlers immunized for the first time with a single dose of PCV, IgG measurement might not reveal the entire picture in regard to the immunogenicity of the vaccine and the level of protection. Our data speak for the potentially important additional role of IgM in the opsonophagocytosis of pneumococci. This is an issue that should be taken into account when designing PCV immunogenicity studies in toddlers or older children for whom a single PCV dose is often recommended.

ACKNOWLEDGMENTS

This study was conducted as part of the research of the PneumoCarr Consortium funded by a grant from the Bill and Melinda Gates Foundation through the Grand Challenges in Global Health Initiative.

We thank Kaisa Jousimies and Sinikka Grönholm for skilled technical assistance. We thank Mika Lahdenkari for help with the statistical analyses.

R.D. has received, in the last 5 years, grants and research support from Berna/Crucell, Wyeth/Pfizer, MSD, and Protea, has been a scientific consultant for Berna/Crucell, GSK Bio, Novartis, Wyeth/Pfizer, Protea, and MSD, has been a speaker for Berna/Crucell, GSK Bio, Wyeth/Pfizer, and is a shareholder of Protea. H.K. has provided consultancies on advisory boards for GSK Bio, has had travel expenses paid by GSK Bio and Novartis as a scientific consultant, invited speaker, or expert at symposia, and has received honoraria from GSK Bio.

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