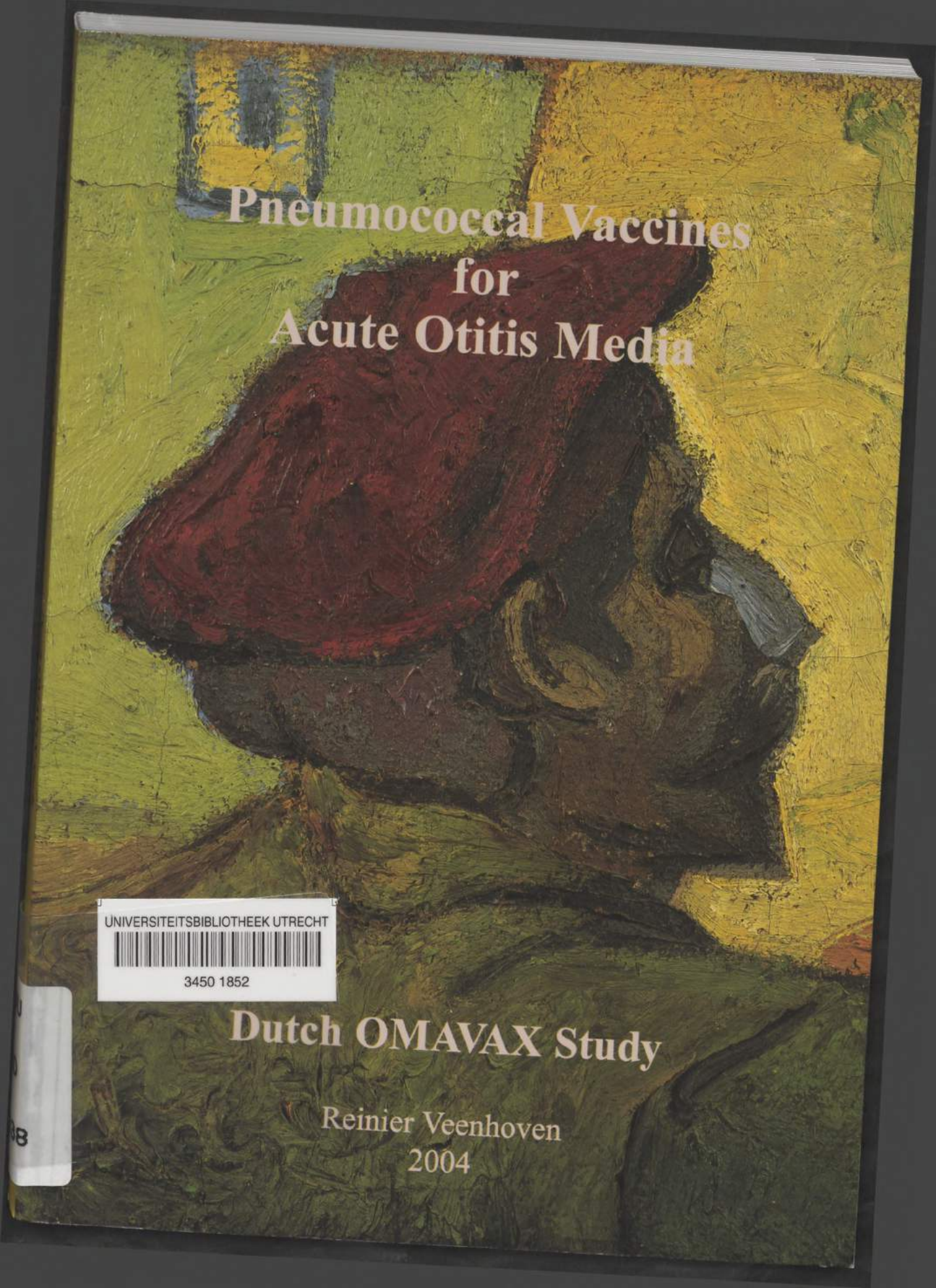
Pneumococcal Vaccines for Acute Otitis Media



Dutch OMAVAX Study

Reinier Veenhoven

2004

Pneumococcal Vaccines for Acute Otitis Media

Pneumococcal Vaccines for Acute Otitis Media

(met een samenvatting in het Nederlands)

Proefschrift

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voor Saskia,
Diederik, Jorien en Annemiek

ter herinnering aan mijn lieve ouders
en broer Wim

Pneumococcal Vaccines for Acute Otitis Media

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Chapter 1

General introduction

Acute otitis media (AOM) is the most frequently diagnosed bacterial infection in children. Its peak incidence is between 6 and 18 months of age;[1,2] by the age of 3 years, 50-85% of all children has suffered from at least one episode of AOM.[1,3-5] In most children the natural course of AOM is favourable, although up to 15% of children is prone to recurrent episodes of AOM.[6-11] Various factors contribute to the susceptibility to recurrent AOM, including dysfunction of the Eustachian tube, immature mucosal or systemic immunity, and potentially unfavourable genetic polymorphisms.[12,13]

Currently, recurrent AOM is managed by antibiotics or surgery. A systematic review of the effects of antibiotic prophylaxis in children with recurrent AOM has shown that antibiotic prophylaxis results in an average decrease of 0.09 (95% CI,-.12,-.05) episodes per child-month, or about 1 episode of AOM per year.[14] Similarly, tympanostomy tubes have been shown to reduce the AOM incidence by a mean of 1 episode per child-year (95% CI, 0.40-1.66); a relative decrease of 56%.[15] Adenoidectomy reduces AOM incidence by 0.3 episodes (95% CI, 0.03-0.61) per child-year in children who have previously been treated with tympanostomy tubes (26% relative decrease).[16] In children without prior tubes no beneficial effect of adenoidectomy has been demonstrated.[17] If insertion of tympanostomy is combined with adenoidectomy in children with recurrent AOM, the likelihood of reinsertion of tubes is reduced by 50% (RR 0.5; 95% CI 0.5-0.6).[18] Taken together, the benefits of surgery or antibiotic prophylaxis in children with recurrent AOM are modest. The question is whether the benefits of these therapies outweigh the disadvantages.[19-21] Increased resistance of bacteria to common antibiotics is a particular cause for concern.[22-24] This has prompted the search for other strategies to manage recurrent AOM, such as by vaccination. Since pneumococcus is the most frequent bacterial cause of AOM, research over the last decade's has focused on pneumococcal vaccination.

Streptococcus pneumoniae

S. pneumoniae is a gram-positive coccus in a diplococcal form surrounded by a polysaccharide capsule. Currently more than 90 serotypes have been described,[25] distinguished by chemical differences in their polysaccharides, and by the ability of the immune system to recognize these differences and to respond with specific antibodies against the antigens of each different type.

In children only a small fraction of all capsular types (types 4, 6, 9, 14, 18, 19, and 23) cause the greater part (50-80%) of pneumococcal infections, ranging from mucosal infections such as AOM and community-acquired bacterial pneumonia to life-threatening invasive infections as sepsis and meningitis.[26] Relative contributions of pneumococcal AOM, pneumonia and invasive disease to overall pneumococcal infections and derivative mortality worldwide are given in figure 1.

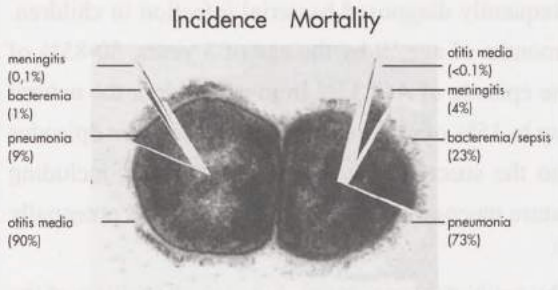

Figure 1. Relative contribution of pneumococcal AOM, pneumonia and invasive disease to overall pneumococcal infections and derivative mortality worldwide.

Nasopharyngeal carriage of S. pneumoniae

S. pneumoniae is a common component of the nasopharyngeal flora in healthy persons; there is little doubt that *S. pneumoniae* isolated from the middle ear during an episode of AOM originates from the nasopharynx.

Pneumococcal colonisation is a dynamic process, which begins soon after birth. The duration of colonisation depends on pneumococcal serotype and age:[27] in children younger than 1 year of age median nasopharyngeal carriage lasts 30 days, in adults 14 days.[28] The prevalence of pneumococcal carriage increases during the first year of life. At the age of one year, about half of children in developed countries have been colonised with *S. pneumoniae* at least once.[29] Pneumococcal carriage rates are highest in toddlers: 40-50% on average.[30] From age 3-5 years, carriage declines to a rate of 20-30% in adults. Environmental factors play an important role in carriage; children attending day care may have pneumococcal carriage rates up to 80%.[31,32] Furthermore, viral respiratory tract infections or AOM irrespective of cause may be accompanied by pneumococcal carriage rates higher than in "healthy" episodes.[31,33] Whether pneumococcal carriage during clinically infection-free intervals in children with recurrent AOM differs from that in healthy children is still a matter of controversy.[34]

About 15% of children who acquire a new pneumococcal serotype in their nasopharynx develop AOM. This occurs usually within 1 month.[27] Viral respiratory tract infections predispose to the development of pneumococcal AOM[35,36] by causing Eustachian tube dysfunction,[37] or enhancement of adherence of *S. pneumoniae* to human respiratory tract epithelium by up-regulation of epithelial cell receptors.[38]

S. pneumoniae and acute otitis media

Pneumococcus is the most common bacterium involved in AOM. It is cultured from middle ear fluid in 25-50% of all AOM episodes;[39-41] in nearly 60% of middle ear samples pneumococcal antigens can be demonstrated with counterimmunoelectrophoresis, latex

agglutination or pneumococcal polymerase chain reaction.[42-44] Of the 90 pneumococcal serotypes, only a limited number of serotypes contribute to AOM in infancy. A recent review based on nine datasets of different parts of the world, including 3,232 children with AOM, shows that the major serotypes involved are 19F and 23F (each 13-25% of pneumococcal middle ear fluid isolates), 14 and 6B (each 6-18%), and 6A, 19A and 9V (each 5-10%).[45] In children with recurrent AOM the relative contribution of *S. pneumoniae* to AOM appears to be similar as compared to children without recurrences.[41] The serotypes involved in AOM also account for the majority of penicillin non-susceptible and multi-drug resistant pneumococci.[46,47] In the Netherlands, no information on the relative contribution of *S. pneumoniae* and its serotypes to AOM is available. In contrast to other countries in Europe, however, the prevalence of penicillin-resistant *S. pneumoniae* in the Netherlands is known to be very low (<1%) [EARSS System 2002, www.rivm.nl/infectieziektenbulletin/bul101/earss.html].

Anti-pneumococcal antibody response

Host defences against pneumococcal infections depend on opsonization of the bacteria by type-specific serum antibodies and on complement, followed by phagocytosis and killing by polymorphonuclear leukocytes and macrophages (figure 2). Type specific antibodies directed to carbohydrates in the capsular polysaccharides play a crucial role in this process. Polysaccharides are able to induce antibody responses in absence of T-cells (T-cell independent antigens). This distinguishes them from proteins (T-cell dependent antigens), the antibody responses of which are dependent on interaction with T-cells via CD40-CD40L. Responsiveness to polysaccharides developes relatively late in ontogeny (18-24 months) compared with that to proteins (2 weeks to 2 months). In contrast to proteins, polysaccharides fail to induce immunological memory; in consequence no booster responses and no avidity maturation are seen after repeated immunisation.[48] The human response upon immunisation with polysaccharides involves IgM, IgG1, IgG2, and IgA isotypes. In infants and children, the IgG anti-pneumococcal polysaccharide antibodies are predominantly of the IgG1 subclass. Only later in childhood do IgG2 responses become more apparent.[49]

Effective phagocytosis of pneumococci by polymorphonuclear neutrophils depends primarily on IgG2-anti-pneumococcal antibodies in several in vitro studies.[50-53] Therefore, late onset of IgG2 antibody responsiveness may attribute to the enhanced susceptibility to pneumococcal infections like AOM in infants and toddlers. With respect to prevention of invasive infections, however, IgG1 antibodies have also proven to be effective.[54]

Anti-pneumococcal antibody response in otitis-prone children

In children with recurrent AOM both increased as well as normal serum IgA, IgM, IgG, and IgG1 levels have been found;[55,56] for IgG2, both low and normal values.[55-58] Regarding

specific anticapsular antibody activity against *S. pneumoniae*, children with recurrent AOM seem to have lower IgG or IgG2 anti-pneumococcal antibodies than do healthy controls.[59-61] One studie, however, showed specific IgG1 anti-pneumococcal antibodies even to be higher in otitis-prone children as compared to healthy controls.[59] The response upon vaccination with the pneumococcal polysaccharide vaccine is also different in children with recurrent respiratory tract infections aged 2 years and older: IgG2 and IgA anti-pneumococcal antibodies are often low to absent.[62,63] Furthermore, the genetically determined FcγRIIaH131 recepter on phagocytic cells that binds IgG2 effectively has been found to be decreased in children with recurrent mucosal infections.[12] These findings underline the importance of IgG2 levels in the defence against pneumococcal mucosal disease.

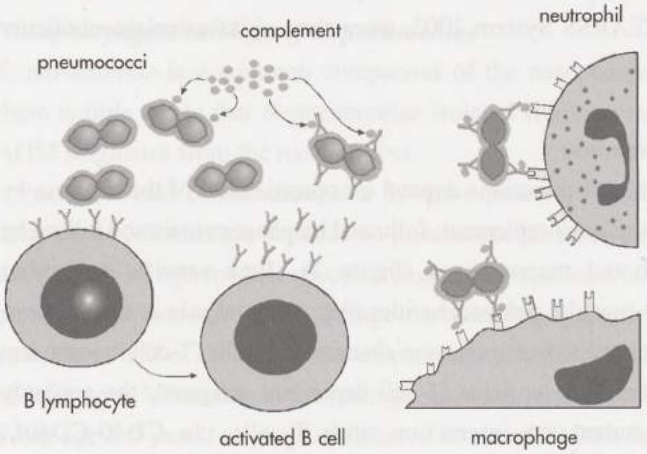

Figure 2. Host defense against *S. pneumoniae*

Pneumococcal vaccines

At present, 2 different pneumococcal vaccines are licensed: the 23-valent pneumococcal polysaccharide vaccine (Pneumovax® [APMSD], Pneumo 23® [APMSD]) and a 7-valent pneumococcal conjugate vaccine (Prevnar® [Wyeth]).

Pneumococcal polysaccharide vaccine

Development. The anti-capsular antibody protection against pneumococcal disease is serotype specific. By using purified capsular polysaccharides of different pneumococcal serotypes as a vaccine, type-specific anticapsular antibodies can be induced. A first pneumococcal polysaccharide vaccine for prevention of invasive pneumococcal infections was marketed as early as 1946. In the 1950s this vaccine was taken off the market because newly

developed antibiotics were believed to solve the problem of pneumococcal infections. This later proved not to be the case, prompting the reintroduction of polyvalent pneumococcal polysaccharide vaccines in 1977. The currently available 23-valent pneumococcal polysaccharide vaccines (PPSV23) contain 25 μg of capsular polysaccharide of 23 different serotypes of *Streptococcus pneumoniae* (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F). These 23 pneumococcal serotypes account for 85-90% of invasive pneumococcal disease among children and adults worldwide.[64]

Immunogenicity. Immunocompetent healthy adults produce protective anti-pneumococcal antibody titers two to three weeks after vaccination with the polysaccharide vaccine. Although mean titers of IgG directed against each capsular polysaccharide increase significantly after vaccination, some individuals fail to show any response to certain serotypes and would not, therefore, be expected to be protected by the vaccine. In this regard, after PPSV23 53% of healthy adults showed measurable levels of IgG antibody to all of 10 pneumococcal capsular polysaccharides (PPS) studied, 36% had IgG to 6-9 PPS, and 11% had IgG to ≤ 5 PPS.[65] With respect to the individual serotypes, the number of subjects with antibody response ≥ 1 mg/L or with at least a twofold increase after vaccination varied from 40% for serotype 4 to 80% for serotype 3.[66] Nearly all IgG reactive with pneumococcal antigens in adults appeared to be of the IgG2 subclass.[67] In children below 2 years of age - the group with the highest incidence of pneumococcal infections - the immunogenicity of polysaccharide vaccines is poor because of the T-cell independent character of polysaccharide antigens.[68] Infants and toddlers may respond to strong immunogenic pneumococcal serotypes in the vaccine, such as types 3 and 8, but this response is unpredictable and usually short-lived. For other serotypes, including types 14, 19F and especially type 6B, the response remains poor up till the age of 5 years.[69-72] However, the majority of pneumococcal infections occur in patients that are immunocompromised, and that at all ages. In these groups as well, the polysaccharide vaccine is poorly immunogenic.

Clinical efficacy against AOM episodes. Clinical effectiveness of 8- and 14-valent pneumococcal polysaccharide vaccines in children has been evaluated in several randomised trials published in the early 1980s.[69,73-79] Pneumococcal nasopharyngeal carriage was found to be unaffected by pneumococcal polysaccharide vaccination.[80,81] The efficacy of the vaccine against AOM, recently evaluated in a systematic review of 8 randomised controlled trials,[82] was at best moderate in children older than 24 months (RR 0.78; 95% CI 0.63 to 0.97) and only marginal in children younger than 24 months. These results do not call for large-scale pneumococcal polysaccharide vaccination to prevent AOM in childhood.

Pneumococcal conjugate vaccine

Development. Conjugation of capsular polysaccharides to proteins alters its immunogenic

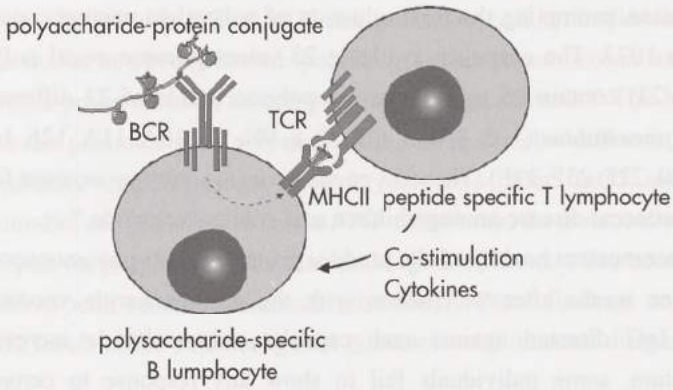

Figure 3. Recruiting T-cell help for polysaccharide antigens. B-cells with surface immunoglobulin specific for the polysaccharide bind the polysaccharide-protein complex, internalise it, and process the protein antigen into peptides. These peptides are then presented in the context of class II major histocompatibility complex (MHCII) molecules to T helper cells bearing specific T-cell receptors (TCR). The activated T-cells in turn stimulate directly as well as via cytokines B-cell proliferation and differentiation into antibody-secreting cells.

properties and changes the antibody response from a T-cell independent to T-cell dependent type (figure 3). Therefore, conjugate vaccines induce production of antibodies and immunologic memory even in young infants. In 1929 Goebel *et al.* found that a conjugate of type 3 pneumococcal polysaccharide and horse serum globulin elicited high antibody titers as well as booster responses upon repeated vaccination in rabbits.[83] The *Haemophilus influenzae* type B (Hib) conjugate vaccine became the first to be applied in children on a large scale in the 1980s, after it had been proven to be immunogenic and highly protective against invasive Hib infections in infants and toddlers.[84-86] This success has led to the development of pneumococcal conjugate vaccines.

The 7-valent pneumococcal conjugate vaccine (PCV7, Prevnar®, Wyeth Lederle Vaccines) has become the first pneumococcal conjugate vaccine to be licensed for prevention of invasive pneumococcal disease in children below the age of 2 years. This vaccine consists of 2μg each of capsular polysaccharides of pneumococcal serotypes 4, 9V, 14, 19F, and 23F, 4μg of the weak immunogenic serotype 6B polysaccharide, and 2μg of serotype 18C oligosaccharide, conjugated to 20μg of a mutant non-toxic diphtheria toxin (CRM_{197}). Regarding AOM, serotypes included in the PCV7 account for 60-70% of all pneumococci isolated in middle ear fluid of children aged 6-59 months (figure 4) and for 40-50% in that of children younger than 6 months or older than 6 years of age.[45] Irrespective of age, vaccine-related serotypes (mainly 6A and 19A) comprise an additional 10-15% of all

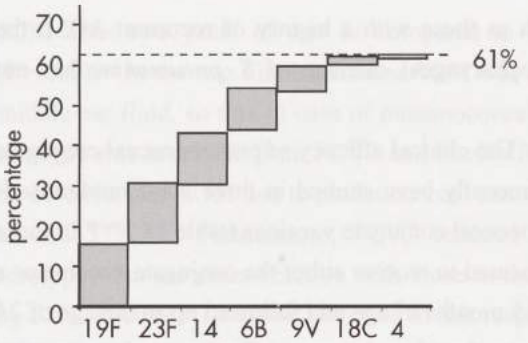

Figure 4. Relative contribution of most frequently isolated pneumococcal serotypes to overall pneumococcal AOM

pneumococcal isolates. Importantly, four serotypes included in PCV7 (23F, 19F, 14 and 6B) account for 83% of all penicillin-resistant pneumococci.[45]

Immunogenicity. Pneumococcal conjugate vaccines are safe and well tolerated in infants and children.[54, 87-93] They are immunogenic not only in healthy infants but also in children at increased risk for pneumococcal infections, such as those infected with the human immunodeficiency virus.[94] In children with severe or recurrent AOM, including those who have failed to produce anti-pneumococcal antibodies after vaccination with the polysaccharide vaccine, the conjugate vaccine also induces good antibody responses.[95-98] Anamnestic responses can be elicited both by repeated PCV7,[54,88,90,91,99-101] and by PPSV23 booster.[96,99,102-104] In fact, booster vaccination with PPSV23 upon priming with a conjugate vaccine induces even higher responses than upon booster vaccination with the conjugate.[105] This could be due to the higher doses of each of the polysaccharides (25 μg) in the polysaccharide vaccine as compared to the conjugate vaccine (2-4 μg). In comparison with the conjugate vaccine, booster vaccination with PPSV23 also induces higher IgG2 anti-pneumococcal antibody responses both in healthy as well as in otitis-prone children.[96,102] Since these IgG2 antibodies may play an important role in the defence against mucosal pneumococcal infections like AOM, it could be favourable to booster with PPSV23.[57,62] The PPSV23 also has the potential advantage of broader serotype coverage above 2 years of age.

Effect on colonisation with S. pneumoniae. In contrast to pneumococcal polysaccharide vaccines, conjugate vaccines have been shown to decrease nasopharyngeal carriage of vaccine type *S. pneumoniae* in healthy infants and toddlers.[106-110] However, along with decreased carriage of vaccine type *S. pneumoniae* an increase of non-conjugate vaccine serotypes has been observed.[107,109,110] Most likely, this shift from conjugate vaccine to non-vaccine *S. pneumoniae* is due to serotype replacement.[110-112] Alternatively, observed increases may be due to "unmasking", in which non-vaccine serotypes are more readily detected among vaccinees than among controls because vaccine serotypes are no longer present, or they may be caused by serotype switch within a specific genotype.[113,114] In children

at risk for pneumococcal infections, such as those with a history of recurrent AOM, the influence of conjugate vaccines on nasopharyngeal carriage of *S. pneumoniae* has not previously been studied.

Clinical efficacy against AOM episodes. The clinical efficacy of pneumococcal conjugate vaccines in the prevention of AOM has recently been studied in three large randomised-controlled trials with two 7-valent pneumococcal conjugate vaccines (table 1).[54,92,105] In these studies healthy infants were randomly allocated to receive either the conjugate vaccine or a control vaccine at 2, 4, 6 months and 12-15 months of age and followed up to the age of 24 months. PCV7 (Prevnar®) vaccination was found to be very effective against invasive disease (bacteraemia and meningitis).[54] Against AOM, however, the vaccine was much less effective: a reduction of 6-7%.[54,92] In the third trial conducted in Finland, with a 7-valent conjugate vaccine composed of pneumococcal antigens linked to a meningococcal outer membrane protein (PncOMPC, Merck®), no reduction of overall AOM episodes was found.[105]

Table 1. Clinical efficacy of 7-valent pneumococcal conugate vaccines against AOM in infants.

Outcome	Northern California Kaiser Permanente study (n=37,868)	Finnish PCV7 study (n=1,662)	Finnish PncOMPC study (n=1,666)
Episodes of AOM	7 (4 to 9)	6 (-4 to 16)	-1 (-12 to 10)
Episodes of Pneumococcal AOM	NA	34 (21 to 45)	25 (11 to 37)
Pneumococcal AOM due to vaccine serotypes	67 *	57 (44 to 76)	56 (44 to 66)
Pneumococcal AOM due to cross-reactive serotypes	NA	51 (27 to 67)	-5 (-47 to 25)
Pneumococcal AOM due to other serotypes	NA	-33 (-80 to 1)	-27 (-70 to 6)
Recurrent AOM (≥ 4 episodes/year)	9 (3 to 15)	16 (-6 to 35)	NA
Tympanostomy tube placement	20 (2 to 35)	39 **	NA

Results are expressed as the percentage reduction in incidence of each outcome in the per-protocol analysis, with 95% confidence intervals noted in parentheses, when available.
Abbreviations: AOM, acute otitis media; NA, not assessed
* Vaccine type *S. pneumococci* were assessed in only 16 cases of pneumococcal AOM.
** estimated vaccine efficacy[115]

Children prone to develop recurrent AOM episodes seem to benefit most from the conjugate vaccine with nearly 23% fewer children experiencing frequent recurrent AOM as the frequency of AOM episodes increased to five episodes within 6-month period or six episodes per year.[54] This is also reflected by a 20% reduction in the number of children receiving tympanostomy tubes.[54] The efficacy of PCV7 seems to diminish with increasing age. In children vaccinated at infancy the reduction of physician office visits for AOM was

3.7% after two years of age as compared to 7.8% during the first two years of life (P=0.19).[116] In the two Finnish studies AOM was diagnosed by myringotomy and culture of middle ear fluid, so that in case of pneumococcal AOM it could also be established which serotype was involved. Both PCV7 and PncOMPC reduced AOM caused by pneumococci by 25-34%, and AOM due to pneumococcal serotypes included in the conjugate vaccine by around 57%.[92,105] Furthermore, PCV7 reduced the number of AOM episodes due to serotypes that are cross-reactive with those in the vaccine (types 6A, 9N, 18B, 19A, 23A) by 51%. PncOMPC was not effective in this respect.

Up to now the efficacy of a pneumococcal conjugate vaccine administered at a later age has been studied only once in a group of healthy children aged 12 to 35 months attending day care centres.[117] A single dose of a 9-valent pneumococcal conjugate vaccine was found to reduce AOM episodes (diagnosed by parents, not validated by physicians) by 17% (P=0.08) during a 2-year follow-up. In addition, a 20% reduction in antibiotic use for AOM was observed (P<0.001).

Scope of this thesis

Based on the results of the trials with PCV7 (Prevnar®) conducted in the US and Finland, the American Academy of Paediatrics and Advisory Committee on Immunization Practices has recommended immunisation with PCV7 in all children below the age of 6 years with a history of recurrent or severe acute AOM, as well as in children with tympanostomy tubes because of recurrent AOM.[118,119] The question is, however, whether results obtained in healthy infants may be extrapolated to older children who have had episodes of AOM before vaccination. Such children may have subtle immunedeficiencies that alter the vaccine's immunogenicity;[62,120,121] genetically determined factors in innate and adaptive immunity may also affect the effectiveness of the vaccine.[12,13] Further, vaccine effectiveness in older children might differ from that in infants due to differences in pneumococcal serotype coverage and environmental factors.[41] Hence, clinical efficacy of PCV7 in older children at risk for pneumococcal infections should be assessed before implementation of this vaccine in these groups of children. To this end, a clinical efficacy trial was designed with combined vaccination with PCV7 followed by PPSV23 in children aged 1-7 years, with two or more documented episodes of AOM before vaccination (figure 5). The combination of conjugate and polysaccharide vaccines was chosen because of the booster effect of PPSV23,[96] and potential broader pneumococcal serotype coverage by PPSV23, of which especially children over the age of 2 years might benefit.[82]

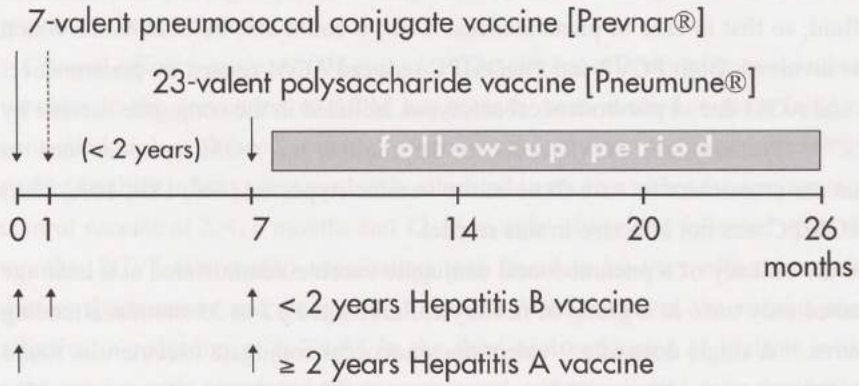

Figure 5. Vaccination scheme in Dutch trial

The results of this trial, regarding the clinical efficacy of combined pneumococcal conjugate and polysaccharide vaccinations against recurrent AOM and the impact of vaccination on culture-confirmed pneumococcal AOM and nasopharyngeal carriage rates will be discussed in chapter 2. Chapter 3 provides detailed information on the impact of the different pneumococcal vaccination schemes in children aged 12-24 months and those of 25 months and older on nasopharyngeal carriage. In chapter 4 the influence of pneumococcal vaccinations on the genetic population structure and resistance profiles of pneumococcal strains isolated from nasopharyngeal swabs is assessed. A newly developed colony blot assay, allowing the detection of multiple pneumococcal serotypes within clinical specimens will be presented in chapter 5. This assay may have important applications with respect to monitoring of pneumococcal vaccination programs. With respect to humoral immunology, chapter 6 describes serum immunoglobulin levels in our large group of children with recurrent AOM as compared to normal values for age. The influence of previous number of AOM episodes is discussed. Chapter 7 describes serum antibody responses to the seven conjugate pneumococcal serotypes after combined pneumococcal conjugate and polysaccharide vaccination, in particular, the impact of the different vaccination schemes and previous history of recurrent AOM on those responses. Finally, in chapter 8 results of all chapters will be summarized and discussed and recommendations will be given for further research in the management of children with recurrent AOM.

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Chapter 2

Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study

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Summary

Background. Pneumococcal conjugate vaccine prevents recurrent acute otitis media (AOM) in infants immunised at 2, 4, 6 and 12-15 months of age. We aimed to find out whether this vaccine also prevents AOM in older children who have had previous episodes of AOM.

Methods. In this double-blind, randomised study, we enrolled 383 patients aged 1-7 years who had had two or more episodes of AOM in the year before entry. Randomisation was stratified in 4 groups according to age (12-24 months *vs* 25-84 months) and the number of previous AOM episodes (two or three episodes *vs* four or more episodes). Children received either 7-valent pneumococcal conjugate vaccine followed by 23-valent pneumococcal polysaccharide vaccine, or hepatitis A or B vaccines. They were followed up for 18 months for recurrence of AOM. We also cultured samples of middle-ear fluid and nasopharyngeal swabs to assess association of pneumococcal serotypes with AOM after vaccination.

Findings. We noted no reduction of AOM episodes in the pneumococcal vaccine group compared with controls (intention-to-treat analysis: rate ratio 1·25, 95% CI 0·99-1·57). Although nasopharyngeal carriage of pneumococci of serotypes included in the conjugate vaccine was greatly reduced after pneumococcal vaccinations, immediate and complete replacement by non-vaccine pneumococcal serotypes took place.

Interpretation. These data do not lend support to the use of pneumococcal conjugate vaccine to prevent otitis media in previously unvaccinated toddlers and children with a history of recurrent AOM.

Introduction

The American Academy of Pediatrics has recommended immunisation with 7-valent pneumococcal conjugate vaccine (PCV7) for children with recurrent or severe acute otitis media (AOM) and children who have tympanostomy tubes because of recurrent AOM.[1] This advice was based on the results of two clinical trials with PCV7. The trials included almost 40.000 healthy infants, who were immunised at 2, 4, and 6 months of age, and had booster vaccinations at 12-15 months of age.[2,3] These children were followed up for the occurrence of AOM up to their second birthday. The pneumococcal vaccine reduced the number of infants with recurrent episodes of AOM by 9%. The largest effect was a reduction of 23% in the number of children developing a severely otitis-prone condition (five episodes in 6 months or six episodes per year).[2] Furthermore, the number of children receiving tympanostomy tubes was reduced by 20%.[2]

However, the benefits of pneumococcal conjugate vaccine have not been investigated in previously unvaccinated toddlers and older children who have documented episodes of AOM before vaccination. Assessment of the vaccine's effectiveness is especially important in this group, since children with recurrent AOM can have subtle immunodeficiencies that alter the vaccine's immunogenicity.[4-6] Genetically determined factors in innate and adaptive immunity may also affect the effectiveness of the vaccine.[7,8] Furthermore, vaccine effectiveness in older children might differ from that in infants due to differences in pneumococcal serotype coverage and environmental factors.[9] Therefore, the efficacy of pneumococcal conjugate vaccine needs to be assessed in randomised trials to support recommendations that these children should also be immunised.

We investigated whether combined vaccination with PCV7 followed by 23-valent pneumococcal polysaccharide vaccine (PPSV23) could prevent AOM in children aged 1-7 years, with two or more documented episodes of AOM before vaccination. This combination was chosen because of the booster effect of the polysaccharide vaccine after priming with conjugate vaccine both in infants and in children prone to otitis.[10,11] Furthermore, the broad pneumococcal serotype coverage by the 23-valent vaccine could benefit children older than two years of age. We assessed the protective efficacy of pneumococcal vaccination against recurrent AOM, and the effect of vaccination on culture-confirmed pneumococcal AOM and nasopharyngeal carriage.

Methods

We did a randomised, double-blind trial between April, 1998, and January, 2002, at a general hospital (Spaarne Hospital, Haarlem) and a tertiary care hospital (Wilhelmina Children's Hospital of the University Medical Center Utrecht) in the Netherlands. Parents were informed about the study by primary care physicians, paediatricians, and otolaryn-

gologists from across the Netherlands. Parents who were willing to participate signed a consent form to enrol their child in the study.

Inclusion criteria for the study were two or more episodes of AOM in the year before study entry, and age 1-7 years. The number of previous AOM episodes was based both on parental report - with AOM defined as having one or more of the symptoms: acute earache, new-onset otorrhea, irritability and fever - and on clinical confirmation of the diagnosis by a physician. Exclusion criteria were primary or secondary immunodeficiency, cystic fibrosis, immotile cilia syndrome, craniofacial abnormalities such as cleft palate, chromosomal abnormalities such as Down's syndrome, and severe adverse events during previous vaccinations.

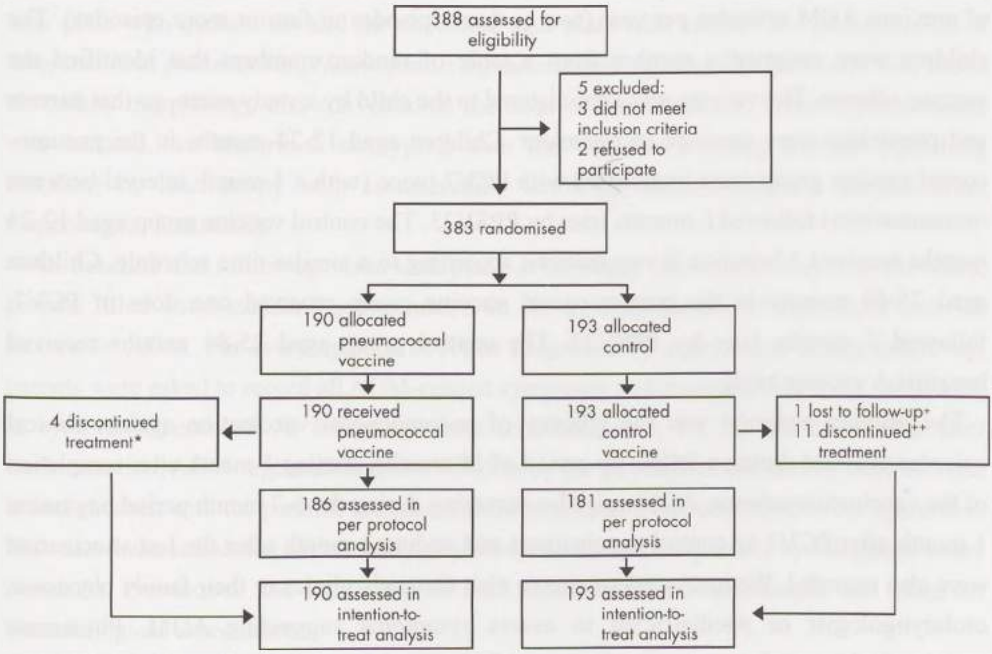


Figure 1: Trial profile

*One child discontinued treatment because of gastroenteritis directly after first vaccination (link with vaccination questionable); three discontinued because parents were not motivated. + The patient moved and we did not know their new address, ++ Four discontinued because of fears about vaccination and venous sampling; two because of disappointment of parents about efficacy of vaccine; two because parents were not motivated; one because the patient's mother was disappointed about communication with study physicians; one because common variable immune deficiency was diagnosed immediately after first vaccination; one for unknown reasons.

The children were randomised to receive either PCV7 followed by PPSV23, or hepatitis A or B vaccines. PCV7 (Prevnar®, Wyeth, Rochester, NY, USA) consisted of 2 μg each of capsular polysaccharides of pneumococcal serotypes 4, 9V, 14, 19F, and 23F, 4 μg of serotype 6B polysaccharide, and 2 μg of serotype 18C oligosaccharide, each conjugated individually to the CRM197 protein. PPSV23 (Pneumune®, Wyeth) consisted of 25 μg of capsular polysaccharides of each of the pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. Control vaccines were recombinant hepatitis B vaccine (Engerix-B=AE Junior®, GlaxoSmithkline, Rixensart, Belgium) and hepatitis A vaccine (Havrix=AE Junior®, GlaxoSmithkline).

Since we expected that age at baseline and the number of episodes of AOM in the year before study entry would be important prognostic indicators for AOM, we randomised the children within four groups according to age (12-24 months *vs* 25-84 months) and number of previous AOM episodes per year (two or three episodes *vs* four or more episodes). The children were assigned a number from a table of random numbers that identified the vaccine scheme. The vaccine was administered to the child by a study nurse, so that parents and physicians were unaware of treatment. Children aged 12-24 months in the pneumococcal vaccine group were immunised with PCV7 twice (with a 1-month interval between immunisations) followed 6 months later by PPSV23. The control vaccine group aged 12-24 months received 3 hepatitis B vaccinations according to a similar time schedule. Children aged 25-84 months in the pneumococcal vaccine group received one dose of PCV7, followed 7 months later by PPSV23. The control group aged 25-84 months received hepatitis A vaccine twice.

The primary endpoint was the efficacy of pneumococcal vaccination against clinical episodes of AOM during a follow-up period of 18 months, starting 1 month after completion of the vaccination scheme. AOM episodes occurring during the 6-7 month period beginning 1 month after PCV7 or control vaccinations and ending 1 month after the last vaccination were also recorded. We instructed parents to visit the study clinics or their family physician, otolaryngologist or paediatrician to assess symptoms suggesting AOM. Physicians registered signs and symptoms of every AOM episode on standard registration forms. Guidelines issued by the Dutch College of General Practitioners define AOM as the presence of an abnormal tympanic membrane on otoscopy (red, dull, or bulging), or otorrhea and at least one of these signs or symptoms of acute infection: acute earache, new-onset otorrhea, irritability, or fever greater than 38·5°C rectally or 38·0°C axillary.[12] New episodes of AOM were recorded after a minimum 7-day interval free of AOM-related symptoms and treatment.

Additional outcomes in our study included number of AOM episodes due to the seven pneumococcal serotypes included in the conjugate vaccine and nasopharyngeal carriage of conjugate vaccine serotypes. Bacterial cultures from middle-ear fluid were obtained only

once in every child, at the time of the first AOM episode arising at least 1 month after the last vaccination. Parents had been asked to bring their child to the study clinic within 24 h after the onset of symptoms suggesting AOM. After clinical confirmation of the diagnosis of AOM, middle-ear fluid was collected by myringotomy or by spontaneous drainage near the perforation site with an aspirator (Juhn Tym-Tap collector, Xomed, Jacksonville, USA) or sterile dry cotton-wool swab (Copan Italia, Transwab, Medical Wire and Equipment Company, Corsham, England). At study entry and follow-up visits, we took nasopharyngeal samples transnasally with a flexible, sterile, dry cotton-wool swab. After sampling, we immediately placed swabs in Stuart's transport medium. Samples of middle-ear fluid and nasopharyngeal swabs were plated within 6 h onto two 5% sheep blood agar plates, a 5% sheep blood agar plate with 5 mg/L gentamicin, and a chocolate agar plate. Agar plates were incubated at 37°C for 48 h; the blood agar plates aerobically and anaerobically, the blood agar plate with gentamicin and the chocolate agar plate with raised CO_2. Identification of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* was based on colony morphology and conventional methods of determination. When *S pneumoniae* was isolated, we undertook serotyping with the capsular swelling method (Quellung reaction) by microscopy with commercially available antisera (Statens Seruminstitut, Copenhagen, Denmark).

All baseline and follow-up visits took place at the study clinics. At entry into the study, parents filled out a standard questionnaire on the medical history of their child and risk factors for AOM. For every episode of AOM diagnosed by a physician during follow-up, parents were asked to record all AOM-related symptoms and treatment in a diary for as long as symptoms persisted. At follow-up visits scheduled at 7, 14, 20 and 26 months after randomisation, AOM registration forms filled in by the physicians and diaries were brought in and checked with the parents. We recorded information on ear, nose, and throat operations. Between these scheduled visits, study physicians contacted the parents by telephone every 3 months. Previous to and 1 month after each vaccination, a blood sample was taken for immunological assessment.

Concentrations of IgG to the seven pneumococcal serotypes in the conjugate vaccine were measured in serum by ELISA.[13] All laboratory work was done by individuals who were unaware of treatment allocation.

The study was undertaken in accordance with the European statements for good clinical practice, which includes the provisions of the Declaration of Helsinki of 1989. The medical ethics committees of both participating hospitals approved the study protocol.

Statistical analysis

On the basis of data from previous studies in the Netherlands, we estimated that 55% of our high-risk patients in the control group would have at least one episode of AOM during the

18 months of follow-up after completion of vaccinations. In view of the multifactorial causes of AOM and comparison of the expected benefit of vaccinations to that of antibiotic prophylaxis and tympanostomy tubes, we judged a reduction of at least 25% to a recurrence rate of 40% of one or more AOM episodes in the pneumococcal vaccine group to be clinically relevant. In order to detect such a reduction, with α (2-sided) 0·05 and power 80%, 176 patients would have to be included in each group. To compensate for an estimated dropout of about 10%, 388 patients would have to be randomised.

Vaccine efficacy was assessed with Cox-type proportional hazards regression models, including a frailty term allowing for differences between individuals in numbers of recurrent AOM episodes. We undertook this analysis in S-plus, version 2000; all other analyses were done with SPSS 10·1. Results are presented as rate ratios with 95% CI; we judged significance to be reached when CI did not include 1. We did both intention-to-treat and per-protocol analyses.

The differences in conjugate and non-conjugate nasopharyngeal pneumococcal carriage between the treatment groups were assessed as follows: children were classified as having had a positive culture for any pneumococcal serotype included in PCV7 or any pneumococcal serotype not included in PCV7 if they had such a positive culture at any of the scheduled follow-up visits after complete vaccination. Proportional differences in pneumococcal carriage and pathogens causing AOM were analysed with χ^2 tests or Fisher's exact tests when appropriate. We judged $p<0·05$ to be significant. Differences in diary data between groups were assessed with the Mann-Whitney U test.

Results

We enrolled 383 children between April, 1998, and January, 2001; 190 children were randomised to receive pneumococcal vaccinations and 193 to receive control hepatitis vaccinations (figure 1). Age, sex, number of previous AOM episodes and other risk factors for AOM did not differ between the groups (table 1). In the pneumococcal vaccine group, 186 of 190 children (98%) completed the vaccination scheme, as did 181 of 193 controls (94%). The median follow-up after complete vaccination was similar in the pneumococcal vaccine group (18·1 months, range 2·4-23·0) and control group (18·0 months, range 0·5-23·0). One patient was lost to follow-up immediately after the first vaccination. No serious adverse events were noted after pneumococcal or hepatitis vaccinations.

Of the 475 AOM episodes diagnosed during follow-up after the final vaccination, 275 episodes were recorded in 107 of 186 children (58%) in the pneumococcal vaccine group who completed all vaccinations (recurrence rate 1·1 episodes per person-year) and 200 episodes in 101 of 181 controls (56%; recurrence rate 0·83 episodes per person-year). In this per-protocol analysis after complete vaccination, the rate ratio of recurrence of AOM for the pneumococcal vaccine group versus controls was 1·29 (95% CI 1·02-1·62). The results of

Table 1. Baseline characteristics, ear, nose, and throat history, and risk factors for AOM.

Variable	Pneumococcal vaccine group (n=190)		Control vaccine group (n=193)	
Male sex	118	(62%)	119	(62%)
Median age, years (range)	2·09	(1-6·86)	2·36	(1-6·99)
Age				
12-24 months	83	(44%)	79	(41%)
25-84 months	107	(56%)	114	(59%)
Number of AOM episodes in preceding year (%)				
2-3	72	(38%)	69	(36%)
4-5	55	(29%)	63	(33%)
6 or more	63	(33%)	61	(32%)
Ventilation tube placement				
None	90	(47%)	96	(50%)
Once	63	(33%)	63	(33%)
Twice or more	37	(20%)	34	(18%)
Adenoidectomy	90	(48%)	89	(46%)
Mean gestational age in weeks	39·3	(SD 2·1)	39·4	(SD 2·1)
Mean birthweight (g)	3358·4	(SD 603·5)	3334·8	(SD 637·1)
Day care (%)				
At age 12-24 months	38 of 83	(46%)	35 of 79	(44%)
At age 25-48 months	53 of 66	(80%)	55 of 65	(85%)
Mean number of siblings	1.05	(SD 0·81)	1.11	(SD 0·93)
Median age at first AOM episode, months (range)	8.0	(1-54)	9.0	(1-48)
Breastfeeding > 3 months	83	(44%)	85	(44%)
Atopy*				
Patient history	94	(50%)	100	(52%)
Family history	110	(58%)	115	(60%)
Family history of recurrent AOM				
Parents	107	(56%)	117	(61%)
Siblings	83	(44%)	72	(37%)
Tobacco smoke exposure indoors	58	(31%)	63	(33%)

*Atopy defined as having eczema, hay fever, or recurrent wheezing or asthma.

the intention-to-treat analysis did not differ from those of the per-protocol analysis over the same period (rate ratio 1·25, 95% CI 0·99-1·57). The cumulative hazard function for AOM of the fully vaccinated pneumococcal vaccine group and controls is shown in figure 2.

Subgroup analysis suggested a slightly higher rate ratio of recurrence of AOM in the pneumococcal vaccine group than in controls in children older than 2 years at the time of first vaccination (rate ratio 1·45, 95% CI 1·09-1·94), compared with the group aged 1-2 years (rate ratio 1·07, 95% CI 0·72-1·60). The rate ratio also seemed higher in children who had two or three episodes of AOM in the year preceding the study (rate ratio 1·66; 95% CI 1·11-2·49) compared with those who had four or more episodes (rate ratio 1·20, 95% CI 0·92-1·56). However, since neither of the interactions between age and treatment effect (1·37, 95% CI 0·87-2·14) and between previous AOM episodes and treatment effect (0·74, 95% CI 0·45-1·22) was significant, we were not able to conclude that rate ratios differed across subgroups. Excluding the severely otitis-prone children with six or more AOM episodes in the year before study entry from the analyses did not change the outcome of the study (rate ratio 1·30, 95% CI 0·83-2·06).

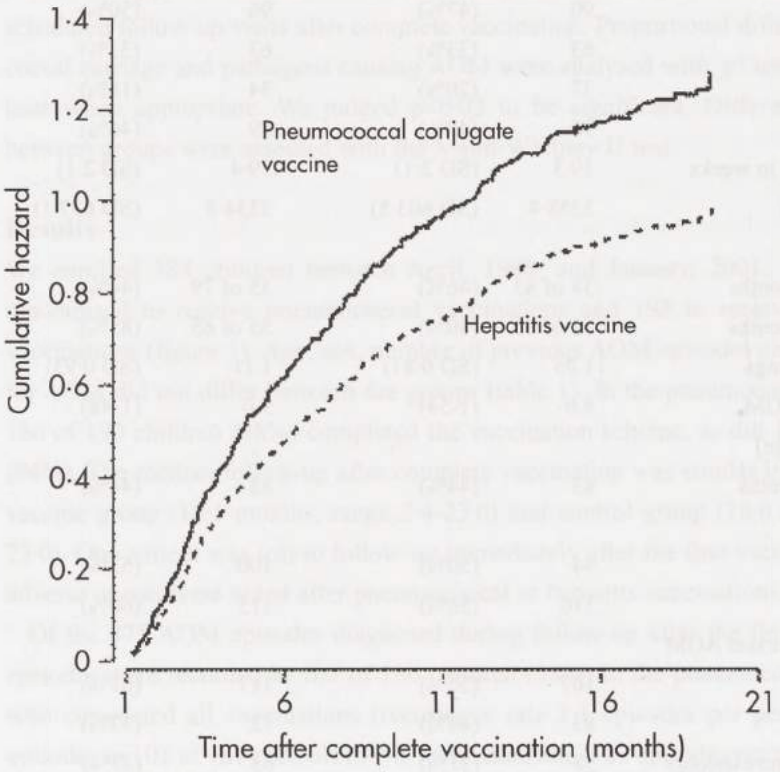


Figure 2: Cumulative hazard function for risk of AOM.

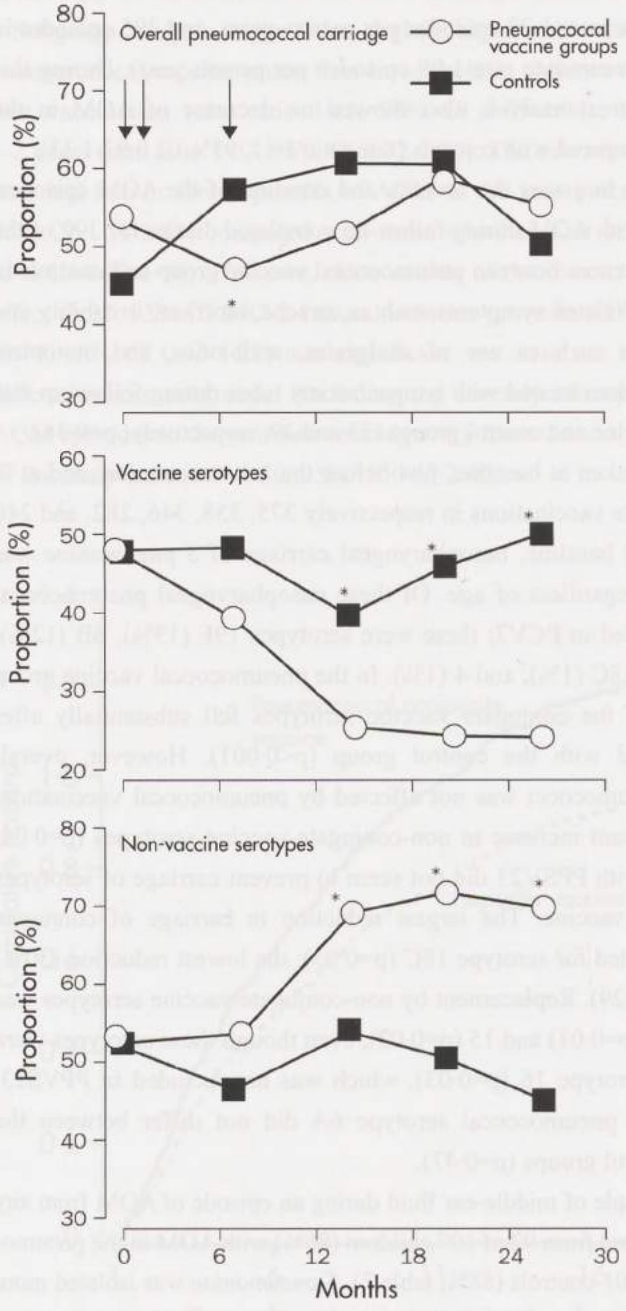
We recorded a total of 840 episodes of AOM during the investigation, including those that arose in the period of 6-7 months between first study vaccinations and 1 month after the last vaccination. 445 AOM episodes were in 135 of the 190 children (71%) in the pneumococcal vaccine group (recurrence rate: 1·23 episodes per person-year), and 395 episodes in 139 of the 192 controls (72%; recurrence rate 1·08 episodes per person-year). During this whole period, the intention-to-treat analysis also showed no decrease of AOM in the pneumococcal vaccine group compared with controls (rate ratio 1·11, 95% CI 0·92-1·33).

We used data from the diaries to assess the severity and duration of the AOM episodes. Parents of 179 of 208 children with AOM during follow-up completed diaries for 399 of the 475 episodes. We noted no differences between pneumococcal vaccine group and controls in median days per episode for ear-related symptoms such as earache, otorrhea, irritability and fever, and ear-related treatment such as use of analgesics, antibiotics, and ototopical medications. The number of children treated with tympanostomy tubes during follow-up was similar in the pneumococcal vaccine and control groups (33 and 39, respectively; p=0·36).

Nasopharyngeal swabs were taken at baseline, just before the last vaccination, and at 7, 13, and 19 months after complete vaccinations in respectively 375, 358, 346, 282, and 240 of the children, respectively. At baseline, nasopharyngeal carriage of *S pneumoniae* was found in 49% of all children, regardless of age. Of these nasopharyngeal pneumococcal serotypes, 53% had been included in PCV7; these were serotypes 19F (13%), 6B (12%), 23F (11%), 14 (9%), 9V (5%), 18C (1%), and 4 (1%). In the pneumococcal vaccine group the nasopharyngeal carriage of the conjugate vaccine serotypes fell substantially after complete vaccination compared with the control group (p<0·001). However, overall nasopharyngeal carriage of pneumococci was not affected by pneumococcal vaccination, because of a concurrent significant increase in non-conjugate-vaccine serotypes (p=0·04; figure 3). Booster vaccination with PPSV23 did not seem to prevent carriage of serotypes not included in the conjugate vaccine. The largest reduction in carriage of conjugate vaccine serotypes (69%) was noted for serotype 18C (p=0·03); the lowest reduction (30%) was found for serotype 6B (p=0·29). Replacement by non-conjugate vaccine serotypes was mainly caused by serotypes 11 (p=0·01) and 15 (p=0·02), even though these serotypes were included in PPSV23, and by serotype 16 (p=0·03), which was not included in PPVS23. Carriage rate of cross-reacting pneumococcal serotype 6A did not differ between the pneumococcal vaccine and control groups (p=0·47).

We took no more than one sample of middle-ear fluid during an episode of AOM from any child. Middle-ear fluid was obtained from 92 of 107 children (86%) with AOM in the pneumococcal vaccine group and 89 of 101 controls (88%; table 2). *S pneumoniae* was isolated more often in middle-ear fluid samples in controls (21%) than in the pneumococcal vaccine group (14%). 4% of middle-ear fluid samples from the pneumococcal vaccine group were positive for pneumococcal serotypes included in PCV7, compared with 9% of controls.

Figure 3. Nasopharyngeal carriage of pneumococci



*Differences in nasopharyngeal carriage of conjugate vaccine and non-conjugate vaccine pneumococcal serotypes were significant between the two treatment groups ($p<0.05$, see results section).

These numbers were too small for meaningful statistical analysis. 30% of our bacterial cultures were negative. Numbers of middle-ear fluid cultures of untypable *H influenzae, M catharralis*, Group A streptococci, and *P aeruginosa* did not differ between the groups. However, we isolated *S aureus* more often in the pneumococcal vaccine group than in the control group (26 *vs* nine children, p=0·002). All *S aureus* cultures and *P aeruginosa* cultures were derived from spontaneous drainage of ears; 75% of the children had ventilation tubes.

Table 2. Pathogens cultured at the first AOM episode after completion of the vaccination scheme

	Pneumococcal vaccination	**Control vaccination**	**p**
Number of children with at least one AOM episode	107	101	
Number of AOM episodes at which MEF obtained	92	89	
MEF obtained by:			
Spontaneous drainage	71	66	
Myringotomy	21	23	
Culture confirmed as			
S pneumoniae	13	19	0·22
PCV7 pneumococcal serotypes	4	8	0·21
Other pneumococcal serotypes	9	11	0·44
H influenzae	21	23	0·64
M cattharralis	8	6	0·62
Group A streptococcus	6	4	0·75
Negative cultures	32	35	0.53
Others (all from spontaneously draining ears)			
P aerigunosa	9	6	0·46
S aureus	26	9	0·002

MEF=midlle-ear fluid

IgG anti-pneumococcal antibody concentrations were measured for 126 randomly selected children, 24 from each of the four randomisation groups who received pneumococcal vaccines and 30 controls. Geometric mean concentrations of these antibodies were consistently higher in the pneumococcal vaccine group than in controls, and reached values far

above 1·5 mg/L, apart from concentrations of serotype 6B, which remained below 0·2 mg/L (table 3).

Table 3. Geometric mean concentrations (mg/L) of IgG anti-pneumococcal antibodies against conjugate vaccine pneumococcal serotypes.

Pneumococcal Serotype	Pre-vaccination		1 month after PCV7 vaccination		1 month after PPSV23 vaccination	
	PV	Controls	PV	Controls	PV	Controls
4	0·04	0·05	0·38	0·11	2·48	0·11
6B	0·04	0·04	0·06	0·02	0·19	0·05
9V	0·16	0·16	0·51	0·32	4·94	0·30
14	1·44	1·85	6·24	4·02	30·44	8·01
18C	0·17	0·16	6·61	0·29	10·62	0·27
19F	0·25	0·27	1·25	0·37	8·49	0·40
23F	0·47	0·45	5·67	0·50	24·32	0·65

PV=pneumococcal vaccine group.

Discussion

Our results show that combined pneumococcal conjugate and polysaccharide vaccination is not effective in prevention of AOM in children older than 1 year of age with recurrent AOM. Exclusion of children who were severely prone to otitis from the analysis did not change the outcome of the investigation.

During the trial we saw a marked reduction in AOM episodes both in the pneumococcal vaccine and control groups to an average of one episode per child per year. This decrease could be the result of overestimation of the number of AOM episodes by parents before study entry; such overestimation has been reported previously in studies of children with recurrent AOM.[14] Furthermore, spontaneous recovery of recurrent AOM with increasing age would have had a role in our investigation,[15] since the recurrence rate of AOM episodes per person-year decreased in the total group of patients from 1·63 in the interval between first and last vaccination to 0·97 between the last vaccination and the end of the study. Finally, evidence suggests that medical outcomes can improve substantially due to trial participation itself, which is assumed to be related to expectation of future benefit, improved clinical follow-up, and other aspects of management of the condition.[16,17]

In accord with our assumptions, 101 of 181 (56%) children in the control group had at least one episode of AOM during follow-up. On the basis of results from previous trials with PCV7 in healthy infants,[2,3] we assumed the efficacy of the vaccine to be higher in children with increased baseline risk of AOM. The children in our study already had already had recurrent episodes of AOM and were followed up for a sufficiently long period to detect the reduction of AOM episodes by PCV7 that we intended. Our results do not show any beneficial effect of this vaccination scheme in terms of reduction of AOM. Since randomisation was successful, loss to follow-up was very low, and AOM episodes were meticulously recorded, we believe that this outcome is valid and that a further increase of precision (more included children) would be unlikely to change these estimates.

We noted very good IgG antibody responses to pneumococcal vaccination in our group of children with recurrent otitis. These responses were significantly higher than those reported in the California and Finnish infant studies,[2,3] except for those to serotype 6B. Recent data from the Finnish otitis media study group also show higher concentrations of antibody in infants after booster vaccination with the polysaccharide vaccine at 14 months of age, compared with PCV7 booster vaccination, which was associated with a better clinical protection against AOM caused by serotype 19F.[18] The deficient response to serotype 6B in our study might be due to a subtle immune deficiency, which is characteristic of children who are prone to otitis.[19] Results of other studies have shown that when healthy infants and toddlers were vaccinated with PCV, they were less likely to carry serotype 6B and cross-reactive serotype 6A or have AOM caused by these pathogens.[3,20] By contrast, we found a low effect of pneumococcal vaccination against carriage of serotype 6B and no effect against 6A. This finding is probably the result of the low titres of antibody against serotype 6B, and might have influenced the outcome of our study, since serotypes 6B and 6A are among the most common AOM serotypes.[3]

Our findings of no beneficial effect of pneumococcal vaccinations contrast with those of the two landmark studies on prevention of AOM by PCV7 in infants.[2,3] These investigations both showed a small but beneficial effect on AOM and improved results in prevention of frequent recurrent AOM. Apart from the booster vaccination with PPSV23, the most important difference between these two studies and ours is that the former studies include healthy infants, who were vaccinated as early as 2 months of age. At this age the child has not yet developed AOM and does not have fully established nasopharyngeal pneumococcal carriage.[21] *S pneumoniae* is a frequent pathogen in early AOM.[9] Because of inflammation and subsequent damage to the middle-ear mucosa and eustachian tube, early pneumococcal AOM could predispose infants to recurrent AOM caused by other pathogens such as *H influenzae*, which was shown to become increasingly important in recurrent AOM episodes.[9] Arguably, conjugate vaccination at infant age might prohibit or delay nasopharyngeal acquisition of the most frequent pneumococcal serotypes, preventing or

delaying pneumococcal AOM until a later age, at which time the child is immunologically and anatomically more mature and more capable of handling an AOM infection than in infancy. Thus, prevention of early pneumococcal AOM could be especially important for the prevention of the otitis-prone condition.

In our study, pneumococcal carriage was noted in 50% of children at study entry. This proportion remained constant throughout follow-up, both in the pneumococcal vaccine group and in controls. Although pneumococcal vaccinations did reduce nasopharyngeal carriage of the seven conjugate vaccine serotypes, including serotype 6B, this reduction was accompanied by an increase in pneumococcal serotypes not included in the conjugate vaccine. This shift in nasopharyngeal pneumococcal carriage after conjugate vaccination is consistent with observations in other studies[22,23] and is most probably the result of replacement.[24,25] The finding that replacement by serotypes 11 and 15 cannot be prevented by PPSV23, which includes these serotypes, lends support to previous results showing that polysaccharide vaccine did not affect nasopharyngeal carriage.[26,27] Although children aged 2-7 years showed better responses to the polysaccharides 11 and 15 compared with the younger group, nasopharyngeal carriage was still unaffected by vaccination (data not shown). By induction of nasopharyngeal replacement with non-conjugate pneumococcal serotypes, PCV could even induce recurrence of AOM, because newly acquired carriage is associated with an increased risk for AOM compared with the risk associated with established carriage.[28] This risk might account for the increased number of AOM episodes in the pneumococcal vaccine group in our study. The potentially pathogenic capacity of non-conjugate-vaccine pneumococcal serotypes was previously shown in the Finnish infant study on AOM;[3] the conjugate vaccine reduced AOM caused by conjugate vaccine type pneumococci by 57%, but AOM caused by non-conjugate-vaccine pneumococcal serotypes was increased by 34%.

We were not able to confirm that replacement took place in middle-ear fluid. For ethics reasons, we obtained middle-ear fluid only in the first episode of AOM after vaccination. Therefore, the number of middle-ear fluid cultures investigated was small. We noted a 51% reduction in AOM caused by conjugate vaccine serotype pneumococci, and overall pneumococcal AOM was reduced by 34%; this finding was similar to that of the Finnish study.[3] We noted no difference between the groups in presence of other middle ear pathogens, apart from *S aureus*. This species was noted more often in middle-ear fluid cultures from the pneumococcal vaccine group, although only in samples taken from spontaneously draining ears. Whether *S aureus* is a true AOM pathogen or is the result of contamination from the external ear canal is uncertain,[29,30] but the double-blind nature of our study suggests that pneumococcal vaccination has an effect on the isolation of *S aureus* in samples from spontaneously draining ears.

To summarise, we found that pneumococcal conjugate vaccination combined with

pneumococcal polysaccharide vaccination does not prevent AOM in children older than 1 year who have had recurrent episodes of AOM before vaccination. Therefore, pneumococcal vaccinations are not indicated in the management of recurrent AOM in toddlers and older children with recurrent AOM. In view of the results of other studies, we might conclude that to prevent pneumococcal AOM in general, and to protect children from developing the otitis-prone condition, pneumococcal vaccinations should be given early in life, at least before 12 months of age and preferably before two or more episodes of AOM have occurred.

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Chapter 3

Nasopharyngeal pneumococcal carriage after combined pneumococcal conjugate and polysaccharide vaccination in children with a history of recurrent acute otitis media

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Abstract

Background. Recently we showed that vaccination with a 7-valent pneumococcal conjugate vaccine (PCV7) followed by a 23-valent pneumococcal polysaccharide vaccine (PPSV23) failed to prevent new acute otitis media episodes in previously unvaccinated toddlers and children with a history of recurrent AOM. We now describe in detail the impact of pneumococcal vaccinations on nasopharyngeal carriage of *S. pneumoniae* in this study population.

Methods. The impact of PCV7 followed by PPSV23 on pneumococcal nasopharyngeal carriage was studied in a prospective, randomised trial, including 383 children with previous acute otitis media, aged 1-7 years. Nasopharyngeal swabs were collected at baseline and at 6-7 months intervals during 26 months follow-up.

Results. Overall pneumococcal carriage rates did not diminish, remaining around 50% in both pneumococcal and control vaccinees. A significant shift from conjugate vaccine to non-conjugate vaccine type pneumococci was observed in children aged 1-2 years, who received twice the conjugate vaccine before polysaccharide vaccination. Conjugate vaccine serotype carriage was not influenced in older children, who received the conjugate vaccine once before the polysaccharide booster.

Conclusions. Conjugate vaccinations at least twice also after 2 years of age may be mandatory for carriage reduction of conjugate vaccine serotypes in children with recurrent otitis media. Polysaccharide booster vaccination did not affect nasopharyngeal colonization of serotypes not included in the conjugate vaccine.

Introduction

Streptococcus pneumoniae is one of the leading causes of bacterial infections worldwide, mucosal disease like acute otitis media (AOM) being a thousand times more frequent as life-threatening invasive disease like meningitis. *S. pneumoniae* commonly colonizes the nasopharynx with carriage being highest in infants and toddlers.[1-3] From ages 3 to 5 years carriage rates decrease, coinciding with a natural increase of antibodies against the capsular polysaccharide antigens of pneumococci and natural decrease of pneumococcal diseases.[4]

Pneumococcal conjugate vaccines have been shown to reduce nasopharyngeal (NP) carriage of conjugate vaccine type (CVT) *S. pneumoniae* in healthy infants and toddlers.[5-8] This may partly explain the observed reduction of upper and lower respiratory tract infections and AOM.[9-12] In several studies, however, decreased carriage of CVT *S. pneumoniae* after vaccination coincided with an increase of non-conjugate vaccine serotypes (NCVT).[5,6,8] Most likely, this shift from CVT to NCVT *S. pneumoniae* is due to replacement.[13,14] So far, no significant increase in NVCT *S. pneumoniae* in invasive disease has been reported.[15] For mucosal infections like acute otitis media however, infants vaccinated with heptavalent pneumococcal conjugate vaccine showed a 27-33% higher rate of AOM caused by NCVT *S. pneumoniae* than controls, alongside a significant decrease of AOM caused by CVT *S. pneumoniae*.[10,16] Serotype replacement at the NP level therefore may have a larger impact on mucosal infections as compared to invasive disease.[17]

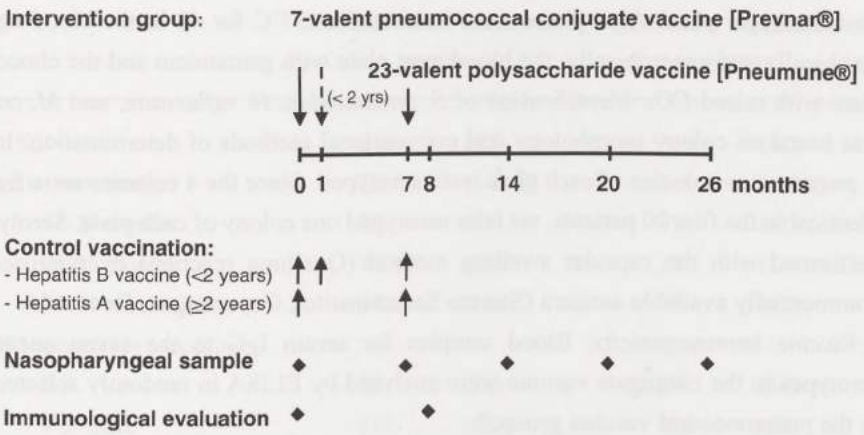
Sofar, reported clinical efficacy on AOM and carriage studies of pneumococcal conjugate vaccines mainly focused on healthy infants and toddlers.[9-11,16] One study published data of the clinical efficacy of a 7-valent pneumococcal conjugate vaccine (PCV7, Prevnar®, Wyeth) in children with recurrent AOM.[18] In this study combined vaccination with PCV7 and 23-valent pneumococcal polysaccharide vaccine (PPSV23) had no effect on the clinical recurrence rate of AOM. Possibly, particularly in children prone to recurrent respiratory tract infections, phenomena like pneumococcal replacement at the NP level after pneumococcal vaccination strongly influence the clinical outcome in the prevention of mucosal infections.

We now describe in detail the impact of vaccination with PCV7 followed by a booster PPSV23 on NP carriage of *S. pneumoniae* in children aged 1 to 7 years with a history of recurrent AOM with respect to the different pneumococcal vaccination schemes used in the study.

Subjects, Materials, and Methods

Nasopharyngeal carriage of *S. pneumoniae* was studied in a double-blind, randomised, controlled trial, conducted between April 1998 and January 2002. This study was part of a larger study on the clinical efficacy of pneumococcal vaccination on AOM in children with recurrent AOM. Overall results of this study have recently been published.[18] Subjects, materials and methods of the current study were also described.

Figure 1. Study Schedule



Vaccination scheme and time points at which nasopharyngeal sampling and immunological evaluation (anti-pneumococcal antibodies against PCV7 serotypes) were performed. Children aged 12-24 months received PCV7 twice and children aged 25-84 months once before the booster PPSV23. The controls received hepatitis vaccines in a similar time-schedule.

Inclusion criteria for the study were two or more AOM episodes in the year before study entry, and age 1-7 years.[18] Exclusion criteria for the study were primary or secondary immunodeficiency, cystic fibrosis, immotile cilia syndrome, craniofacial malformation such as cleft palate, chromosomal abnormalities such as Down syndrome, and severe adverse events during previous vaccinations. Vaccination schemes and timing of NP sampling and anti-pneumococcal antibody evaluation are shown in figure 1.

Vaccinations. The children were randomised to receive either PCV7 followed by PPSV23, or hepatitis A or B vaccines. PCV7 (Prevnar®, Wyeth) contained 2 μg each of saccharides of pneumococcal serotypes 4, 9V, 14, 18C, 19F and 23F and 4 μg of 6B, coupled to the protein carrier CRM197. PPSV23 (Pneumune®, Wyeth) consisted of 25 μg each of polysaccharides of pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. Control vaccines were recombinant hepatitis B vaccine (Engerix®-B=AE Junior, GlaxoSmithkline) and hepatitis A vaccine (Havrix®=AE Junior, GlaxoSmithkline).

Nasopharyngeal swabs. NP samples were cultured for *S. pneumoniae, H. influenzae*, and *M. catarrhalis*. NP samples were obtained by the study physicians by using a flexible sterile dry cotton-wool swab (Copan Italia, Transwab, Medical Wire & Equipment Co. Ltd., Corsham, England) induced into the nostrils and advanced until resistance was found. The

swabs were inoculated directly in Stuart's transport medium and plated within 6 h onto two 5% sheep blood agar plates, a 5% sheep blood agar plate with 5 mg/L gentamicin, and a chocolate agar plate. Agar plates were incubated at 37°C for 48 h; the blood agar plates aerobically and anaerobically, the blood agar plate with gentamicin and the chocolate agar plate with raised CO_2. Identification of *S. pneumoniae, H. influenzae*, and *M. catarrhalis* was based on colony morphology and conventional methods of determination. Initially, 4 *S. pneumoniae* colonies of each plate were serotyped. Since the 4 colonies were found to be identical in the first 20 patients, we later serotyped one colony of each plate. Serotyping was performed with the capsular swelling method (Quellung reaction) by microscopy with commercially available antisera (Statens Seruminstitut, Copenhagen, Denmark).

Vaccine immunogenicity. Blood samples for serum IgG to the seven pneumococcal serotypes in the conjugate vaccine were analysed by ELISA in randomly selected patients in the pneumococcal vaccine group.[19]

Statistical analysis. In children with a positive culture for *S. pneumoniae*, carriage of CVT and NCVT *S. pneumoniae* and individual serotypes were analysed both at 7 months after conjugate/control vaccination(s) and after booster polysaccharide/control vaccination. After this last vaccination, for each individual only the first pneumococcal culture occurring at 14, 20 or 26 months was included in the analysis.

Proportional differences in carriage of *S. pneumoniae, H. influenzae*, and *M. catarrhalis* at enrolment, after conjugate vaccination alone and after booster with the polysaccharide vaccine were analysed using Chi-square tests or Fisher's exact tests when appropriate. Differences between IgG anti-pneumococcal antibodies between children aged 12-24 months and children aged 25-84 months were analysed with Mann-Whitney U test. We judged $P<0.05$ to be significant.

The study was undertaken in accordance with the ethical standards of the responsible committees of both participating hospitals and with the Helsinki Declaration of 1975, as revised in 1983.

Results

A total of 383 children were enrolled; 190 children received pneumococcal vaccinations and 193 control hepatitis vaccinations. Both groups were comparable for age, sex, number of previous AOM episodes and environmental risk factors for AOM, like out-of home care (table 1). More than 95% of all children completed the vaccination scheme and the median follow-up in both groups was similar.[18]

Nasopharyngeal swabs were obtained in a similar number of children of the pneumococcal vaccine and control group (respectively 185, 182, 175, 141, and 121 children *vs.* 190, 174, 169, 140, and 118 children) at study entry and 7, 14, 20, and 26 months after the first vaccine dose.

Table 1. Baseline and clinical characteristics

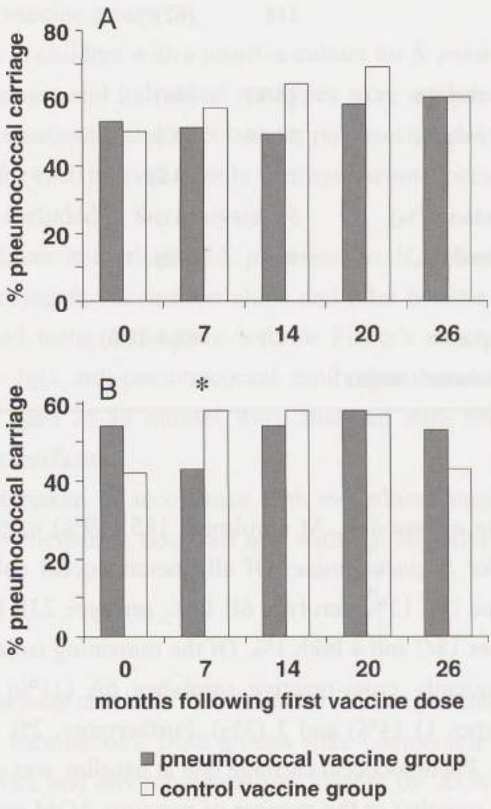
Variable		Pneumococcal vaccinees n=190		Control vaccinees n=193	
Male sex (%)		118	(62)	119	(62)
Median age in years (range)		2.1	(1-6.9)	2.4	(1-7.0)
Age (%)	12-24 months	83	(44)	79	(41)
	25-36 months	42	(22)	35	(18)
	37-84 months	65	(34)	79	(41)
No. of AOM episodes in preceding year (%)	2-3	72	(38)	69	(36)
	4 or more	118	(62)	124	(64)
Day care (%)					
	At age 12-24 months	38/83	(46)	35/79	(44)
	At age 25-48 months	53/66	(80)	55/65	(85)
Mean no. of siblings (SD)		1.1	(0.8)	1.1	(0.9)
Tobacco smoke exposure indoors (%)		58	(31)	63	(33)
No. of children who completed the vaccination scheme (%)		186	(98)	181	(94)
Median months of follow-up after completion of vaccination scheme (range)		18.1	(2.4-23.0)	18.0	(0.5-23.0)

Pneumococcal carriage at baseline. At enrolment 185 (49%) of the 375 NP swabs of children were positive for *S. pneumoniae*. Of all pneumococcal cultures obtained, 53% belonged to CVT: serotype 19F 13%; serotype 6B 12%; serotype 23F 11%; serotype 14 9%; serotype 9V 6%; serotypes 18C and 4 both 1%. Of the remaining isolates, 45% were found to be NCVT, most frequently cross-reactive serotypes 6A (11%) and 19A (3%) and non-cross-reactive serotypes 11 (4%) and 3 (3%). Furthermore, 2% of all pneumococcal isolates were not typable. Pneumococcal carriage rate at baseline was not influenced by age (12-24 months *vs.* 25-84 months) or the number of previous AOM episodes (2-3 AOM *vs.* 4 or more) in the year prior to first vaccination (results not shown). However, the relative contribution of CVT pneumococci tended to be less in children 25 months and older (49/102 pneumococcal isolates; 48%) compared with the younger group aged 1-2 years (49/83 pneumococcal isolates; 59%, P=0.14). Even after the age of 36 months still 25 of the 60 isolated pneumococci (42%) belonged to the CVT. Regardless of age, the relative

contribution of CVT and NCVT *S. pneumoniae* was not influenced by the number of previous AOM episodes (results not shown).

Impact of vaccination on pneumococcal carriage. Seven months after the first PCV7, the number of children carrying *S. pneumoniae* (85 of 182 children; 47%) was found to be decreased compared with controls (101 of 174 children; 58%, P=0.04). With respect to age, this difference was found to be significant only in children older than 2 years of age at the time of the first vaccination (47% vs. 57%; P=0.04). After PPSV23 overall pneumococcal carriage was not reduced compared with controls, remaining around 50% in both age groups during follow-up (figure 2).

Figure 2. Overall pneumococcal carriage in pneumococcal and control vaccinees



Overall pneumococcal carriage percentages at study entry and at 7, 14, 20 and 26 months after the first vaccination in children according to age 12-24 months (A) and 25-84 months (B) at the time of first vaccination. For the number of vaccinees at various time points see results section. Differences in carriage of *S. pneumoniae* were analysed using Chi-square tests.

* Statistical significance was considered reached at P<0.05.

The relative contribution of CVT *S. pneumoniae* to all pneumococcal isolates 7 months after the first vaccination was lower in pneumococcal vaccinees (33/85 pneumococcal isolates; 39%) compared with controls (49/101; 49%). This difference was not significant (P=0.19) and not influenced by age (table 2). After PPSV23 however, the contribution CVT *S. pneumoniae* to all pneumococcal cultures was now markedly decreased (35/141 pneumococcal isolates; 25%) compared with control vaccinees (64/144; 44%, P=0.001). This difference was most pronounced in those children first vaccinated at age 12-24 months (25% *vs.* 52%; P=0.002) (table 2). Among children enrolled between 2-7 years, CVT carriage rates fell over time among both treatment groups and less difference in CVT contribution was observed between pneumococcal vaccinees and controls (25% *vs.* 38%; P=0.06).

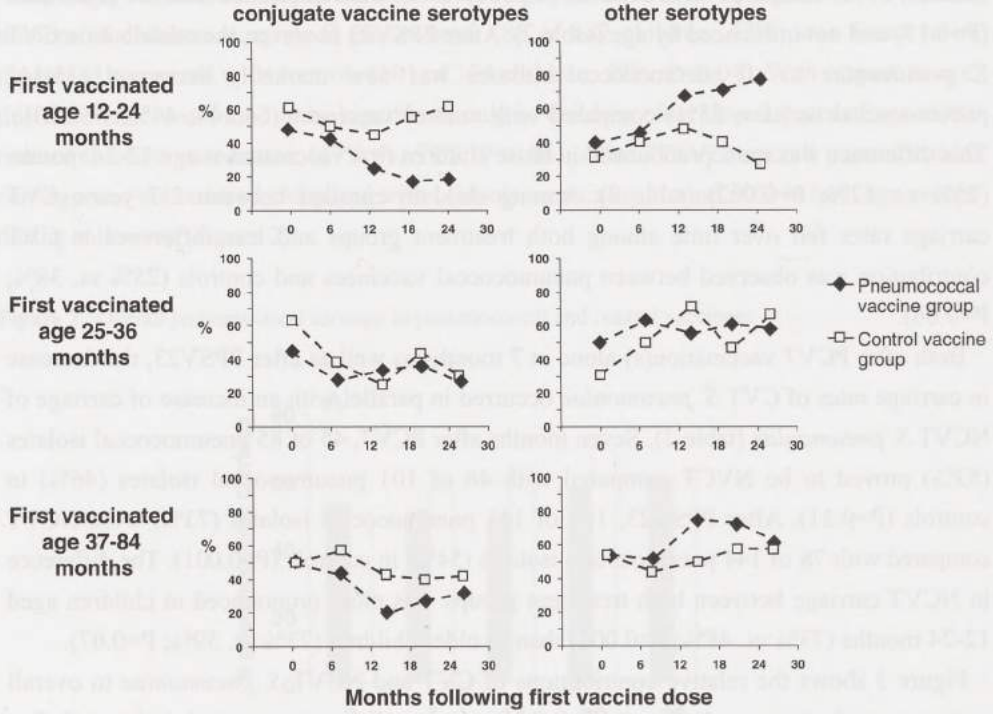
Both after PCV7 vaccination(s) alone at 7 months as well as after PPSV23, the decrease in carriage rates of CVT *S. pneumoniae* occurred in parallel with an increase of carriage of NCVT *S. pneumoniae* (table 2). Seven months after PCV7, 45 of 85 pneumococcal isolates (53%) proved to be NVCT compared with 46 of 101 pneumococcal isolates (46%) in controls (P=0.31). After PPSV23, 103 of 141 pneumococcal isolates (73%) were NCVT compared with 78 of 144 pneumococcal isolates (54%) in controls (P=0.001). The difference in NCVT carriage between both treatment groups was more pronounced in children aged 12-24 months (73% *vs.* 48%; P=0.004) than in older children (73% *vs.* 59%; P=0.07).

Figure 3 shows the relative contributions of CVT and NCVT *S. pneumoniae* to overall pneumococcal carriage during total follow-up according to age at the time of first vaccination; the lower impact of pneumococcal vaccination on pneumococcal NP carriage among children enrolled between 2-7 years of age held true when this group was stratified further into those 25 to 36 months and those 37 to 84 months at the time of first vaccination.

With respect to the impact of pneumococcal vaccinations on the individual CVT pneumococci in children carrying *S. pneumoniae* 7 months after the first dose of PCV7, only the reduction of the conjugate vaccine serotype 14 was found to be significant (P=0.04) in children aged 25-84 months at the time of first vaccination (table 2). After PPSV23 the reduction of serotype 14 and other individual CVT serotypes did not reach significance. The increase of NCVT *S. pneumoniae* was significant for serotype 11 (P=0.007) only after PPSV23 in the youngest children (table 2). With respect to possible cross-protection between different serotypes of the same serogroup, no protective effect of pneumococcal vaccination for serotype 6A was observed. Serotypes 19A, 23A and 23B were only rarely cultured.

Vaccine immunogenicity. After complete vaccination, geometric mean IgG concentrations reached values far above 1.0 mg/L and were roughly similar in children first vaccinated at age 12-24 months and older children for all CVT serotypes with the exception of 6B, which remained below 1.0 mg/L in the youngest group (table 3).

Figure 3. Carriage of CVT and NCVT *S. pneumoniae* in pneumococcal and control vaccinees.



Relative contribution of CVT and NCVT *S. pneumoniae* to overall pneumococcal carriage according to age at first vaccination. Percentages of CVT and NCVT at study entry and at 7, 14, 20 and 26 months after the first vaccine dose are shown for pneumococcal vaccinees (solid squares) and control vaccinees (open squares).

Nasopharyngeal carriage of H. influenzae and M. catarrhalis. At study entry *H. influenzae* and *M. catarrhalis* were found in the nasopharynx of 58% and 50% of children, respectively. With respect to age and history of AOM, only carriage of *M. catarrhalis* was influenced by age; 12-24 months 57% and 25-84 months 44% (P=0.02). Pneumococcal vaccinations did not affect carriage of *H. influenzae* or *M. catarrhalis* during follow-up as compared with control vaccinations (data not shown).

Table 2. Relative contribution of CVT and NCVT *S. pneumoniae*

	7 months after first vaccination				Follow-up after last vaccination			
	Age first vaccination				Age first vaccination			
	12-24 months		25-84 months		12-24 months		25-84 months	
	PV	CV	PV	CV	PV	CV	PV	CV
	PI, n=41	PI, n= 41	PI, n=44	PI, n=60	PI, n=60	PI, n=63	PI, n=81	PI, n=81
All PCV7 serotypes	**18*(44%)***	**21*(51%)***	**15*(34%)***	**28*(47%)***	**15*(25%)****	**33*(52%)***	**20*(25%)***	**31*(38%)***
4	0	0	1	0	0	1	1	0
6B	5	7	2	6	4	9	5	5
9V	0	2	2	2	1	3	0	2
14	0	4	0	6*	1	3	3	6
18C	0	1	1	1	0	5	1	3
19F	8	3	3	7	3	4	6	9
23F	5	4	6	6	6	8	4	6
Other serotypes	**19*(46%)***	**17*(41%)***	**26*(59%)***	**29*(48%)***	**44*(73%)****	**30*(48%)***	**59*(73%)***	**48*(59%)***
6A **	4	8	5	3	10	10	10	7
19A**	0	1	1	2	0	2	2	3
23A**	1	1	3	0	1	2	2	1
23B**	1	0	2	1	3	0	4	4
3	1	0	1	1	1	2	3	4
10	1	0	1	1	1	4	2	3
11	3	1	3	4	10*	4	10	6
15	3	2	3	4	9	4	5	3
16	0	1	0	1	2	2	6	3
21	3	0	0	0	1	0	2	2
Various	2	3	7	12	6	3	13	12
Non-Typable	***4(10%)***	***3(8%)***	***3(7%)***	***3(5%)***	***1(2%)***	***0(0%)***	***2(2%)***	***2(3%)***

In children positive for pneumococcal isolates (PI, n) the number of children with a conjugate vaccine serotype (CVT) or non-conjugate vaccine serotype (NCVT) are shown for pneumococcal vaccinees (PV) and control vaccinees (CV). Children are stratified according to age at the time of the first vaccination. Results are shown for time points 7 months after the first PCV7/control vaccination and after booster PPSV23/control vaccination. Data of results at 14, 20 and 26 months are pooled, including only the first pneumococcal isolate in a child analysis. Differences in carriage of the individual CVT and NCVT *S. pneumoniae* between both vaccine groups were analysed using Chi-square tests or Fisher's exact tests when appropriate. * $P<0.05$ was considered significant. ** Cross-reactive serotypes with PCV7.

Table 3. Anti-pneumococcal antibodies

PCV7 serotypes	Pre-vaccination		p-value	After last vaccination		p-value
	12-24 months	≥25 months		12-24 months	≥ 25 months	
4	0.05	0.04	0.02 *	5.85	3.33	0.02 *
6B	0.04	0.04	0.70	0.56	1.52	0.01 *
9V	0.18	0.20	0.61	28.40	24.94	0.64
14	1.03	2.01	0.09	78.06	75.89	0.86
18C	0.15	0.29	0.003 *	9.63	9.01	0.97
19F	0.22	0.36	0.04 *	12.34	13.05	0.98
23F	0.42	0.60	0.02 *	1.71	3.69	0.05

Geometric mean concentrations (mg/L) of IgG antibodies against the 7 conjugate vaccine pneumococcal serotypes at baseline and one month after booster PPSV23 are shown separately for children first vaccinated at age 12-24 months (n=42) and age 25-84 months (n=51). Statistical differences between the youngest and older children were analysed with Mann-Whitney U test. * We judged $P<0.05$ to be significant.

Discussion

We describe for the first time the impact of 7-valent pneumococcal conjugate followed by 23-valent polysaccharide vaccination on NP carriage of *S. pneumoniae* in a group of toddlers and children aged 1-7 years with a history of recurrent AOM. Overall pneumococcal NP carriage rates in these children did not diminish after pneumococcal vaccinations during 2 years of follow-up. However, in particular in children first vaccinated at age 1-2 years who received PCV7 twice before PPSV23, a shift was induced from CVT to NCVT *S. pneumoniae*. In older children, who received PCV7 only once before PPSV23, the impact of pneumococcal vaccinations on pneumococcal carriage was far less pronounced. Since both age groups showed a similar contribution of CVT *S. pneumoniae* to NP carriage at start of the study, this does not explain the lower vaccine influence after 2 years of age. Also both age groups achieved roughly similar IgG serum levels against pneumococcal serotypes by pneumococcal vaccinations except for IgG titers against serotype 6B, which remained below 1 mg/L in the youngest group. We therefore assume that the greater shift from CVT to NCVT in children aged 1-2 years is primarily explained by the difference in vaccine schemes; the younger patients receiving two and the older patients one PCV7. This is underlined by the fact that

vaccination influence was already nearly absent in pneumococcal vaccinees aged 25 to 36 months.

The T-cell dependent characteristics of a conjugate vaccine result in recruitment of new memory B-cells, which increase after each vaccination whereas a polysaccharide vaccine may only trigger pre-existing memory cells.[20] At the mucosal level, the extra B-cell recruitment may result in better opportunity to boost mucosal immunity by transient natural contacts with conjugate vaccine serotypes. Nurkka *et al.* previously described an increase in salivary IgG several months after a last vaccination with PCV7 in several children whereas only one child showed a late increase in salivary IgG after a booster polysaccharide vaccine.[21] Thus, although both PCV7 and PPSV23 booster vaccinations significantly increase serum quantitative IgG responses, the conjugate vaccine may be superior in inducing mucosal memory for the conjugate vaccine serotypes. Also other qualities like antibody affinity may be superior after extra conjugate vaccinations as compared to the polysaccharide booster, which may also lead to better mucosal immunity.[22] Pneumococcal polysaccharide vaccine is known to have little influence on NP pneumococcal colonization.[23,24] Our study shows it also fails to prohibit the increase of nasopharyngeal carriage of NCVT pneumococci such as serotype 11, despite induction of good serum IgG levels[18] and apparently does not affect the NP flora of those serotypes included in the PPSV23 but not in PCV7. Hypothetically, a polysaccharide booster might even frustrate future antibody responses at the mucosal site on subsequent natural challenge with polysaccharides.[25] After the conjugate vaccinations, we saw an initial decrease of pneumococcal carriage just before booster vaccination with the 23-valent polysaccharide vaccine, particularly in children over 2 years of age. After the polysaccharide booster however, pneumococcal carriage rates subsequently increased again to levels similar to before vaccinations and identical to controls. We do not think however, that this failure to bring down pneumococcal carriage is due to the polysaccharide booster because after pneumococcal vaccination in healthy infants and toddlers overall pneumococcal carriage also was not affected.[6-8]

In our risk group for AOM, baseline NP pneumococcal carriage rate was around 50%, which is in line with average carriage rates for similar age groups worldwide.[1] PCV7 serotypes comprised 53% of the pneumococcal strains isolated from the nasopharynx, which is similar to recent Finnish data on NP pneumococcal carriage in children.[26] Whether otitis-prone children do have higher pneumococcal carriage rates during health as compared to non otitis-prone children is still a matter of debate.[27] However, in contrast to a recent report in the Netherlands,[28] in our study older children aged 3-7 years did not show decreasing overall pneumococcal colonization rates even after 2 years of follow-up. It may be speculated that older children suffering from recurrent otitis still have high pneumococcal carriage rates because they are unable to eradicate pneumococci from their

nasopharynx or prevent new acquisition due to minor immunodeficiency.[18,29-33]

Despite low mean serum IgG concentrations for serotype 6B (< 1 mg/L), a significant decrease in carriage of serotype 6B by 50% was noted in the youngest age group. We found a lack of influence of pneumococcal vaccinations on carriage of the cross-reactive serotype 6A, most probably related to the low anti-6B antibody response. Väkeväinen *et al.* showed that on average, 2-6 times more anti-6B antibodies were needed for 50% opsonophagocytic killing of the type 6A than the type 6B strain.[34] A low IgG response against serotype 6B upon PCV7 as in both younger and older patients of our study group was also reported in other small groups of infection-prone patients.[35,36] A third study in otitis-prone children could not confirm this observation.[37]

The observed nasopharyngeal replacement from CVT to NCVT pneumococci after pneumococcal vaccination in our study population seems to be due largely to replacement [Bogaert *et al.* submitted]. Apart from replacement by NVCT pneumococci, pneumococcal vaccinations did not influence carriage of other in potential AOM pathogens, like *H. influenzae* and *M. Catarrhalis*.

Clinically, we previously reported that no protective effect of combined pneumococcal conjugate and polysaccharide vaccination was found for the prevention of AOM after 1 year of age in children with a history of AOM.[18] Particularly in the youngest children this may be due to the replacement of CVT by NCVT *S. pneumoniae*. After two years of age, pneumococcal carriage was less influenced by vaccination and therefore may not have affected the incidence of AOM.

The results of our study show that in high risk groups not only vaccine immunogenicity studies should be performed but also clinical efficacy studies combined with the evaluation of the bacterial changes in nasopharyngeal colonization to provide insight in the impact of vaccinations. Despite adequate quantitative serum IgG levels, at least two conjugate vaccinations seem to be mandatory for reduction of nasopharyngeal carriage of CVT serotypes. To obtain higher serum IgG levels for serotype 6B, a third or perhaps fourth conjugate vaccination may be required at all ages, which may only then result in reduction of carriage of serotype 6A. Booster vaccination with a polysaccharide vaccine does not seem to enhance mucosal immunity. The results of this study should be kept in mind when recommending pneumococcal vaccinations for mucosal disease like AOM but also for lower respiratory tract infections like pneumonia in children after 1 year of age, particularly for risk groups.

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Chapter 4

Molecular epidemiology of pneumococcal colonization in response to pneumococcal conjugate vaccination in children with recurrent acute otitis media

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Summary

Introduction. A randomised double-blind trial with a 7-valent pneumococcal conjugate vaccine was conducted in The Netherlands among 383 children, aged 1-7 years, with a history of recurrent acute otitis media. No effect of vaccination was found on the pneumococcal colonization rate. However, a shift in serotype distribution was clearly observed (Veenhoven, 2003. Lancet:361:2189-95).

Methods. We investigated the molecular epidemiology of 921 pneumococcal isolates retrieved from both the pneumococcal vaccine (PV) and control vaccine (CV) group during the vaccination study.

Results. Within individuals a high turnover rate of pneumococcal genotypes was observed, which was unaffected by vaccination. Comparison of the genetic structure before and after completion of the vaccination scheme revealed, despite a shift in serotypes, clustering between 70% of the pneumococcal populations. The remaining isolates (30%) were equally observed in the PV and the CV group. In addition, the degree of genetic clustering was unaffected by vaccination. However, within the population genetic structure, non-vaccine serotype clusters with the serotypes 11, 15 and 23B became predominant over vaccine-type clusters after vaccination. Finally, overall pneumococcal resistance was low (14%), and, albeit not significant, a reduction in pneumococcal resistance as a result of pneumococcal vaccination was observed.

Conclusion. Molecular surveillance of colonization in Dutch children shows no effect of pneumococcal conjugate vaccination on the degree of genetic clustering, and the genetic structure of the pneumococcal population. However, within the genetic pneumococcal population structure, a clear shift was observed towards non-vaccine serotype clusters.

Introduction

Streptococcus pneumoniae is worldwide one of the major bacterial causes of invasive disease and respiratory tract infections in children. Risk groups for pneumococcal infections are young children, elderly and immunodeficient patients. Despite adequate antibiotic treatment, morbidity and mortality due to pneumococcal disease remains high.[6] Moreover, the increasing (multi) drug resistance among pneumococcal isolates hampers adequate treatment.[1,11,21,30] New pneumococcal conjugate vaccines, have shown to be effective against invasive diseases in young children.[4] Furthermore, a protective effect against respiratory tract infections such as (recurrent) otitis media has been observed.[4,15] Thus far, the 7-valent pneumococcal conjugate vaccine Prevnar® (Wyeth, USA) has been approved by the Food and Drug Administration (USA), and the Committee on Proprietary Medicinal Products (Europe), and is recommended by the Advisory Committee on Immunization Practices (USA) for the prevention of invasive diseases in children under 2 years of age. Recommendations are also made for older children at increased risk for invasive disease, like those with HIV and asplenia, and those with increased risk for pneumococcal mucosal disease, such as children with recurrent acute otitis media.[2] We recently studied the effect of a 7-valent pneumococcal conjugate vaccine followed by a 23-valent polysaccharide vaccine in children aged 1-7 years with a history of recurrent acute otitis media.[32] Clinically, no protective effect of the pneumococcal vaccines on recurrence of acute otitis media was found. At the nasopharyngeal level, however, a significant reduction of colonization with vaccine-type pneumococci was found after vaccination, whereas a simultaneous increase in colonization with non-vaccine serotypes was observed.[32]

In this study, we investigated the molecular epidemiological dynamics and resistance profiles of the pneumococcal isolates collected from both children in the pneumococcal vaccine (PV) and the hepatitis control vaccine group (CV) in order to obtain insight in the effect of conjugate vaccination on the genetic pneumococcal population structure.

Material and methods

Bacterial isolates. In total, 383 children, aged 1-7 years, suffering from recurrent acute otitis media, were enrolled in this double-blind randomised vaccination trial in the period April 1998 to December 2001.[32] Hundred-ninety children received once a 7-valent pneumococcal conjugate vaccine when 24 months of age and older (Prevnar®, Wyeth Lederle), or twice in children 12-24 months of age, followed by a 23-valent pneumococcal polysaccharide vaccine after 6 months for all children (Pneumune®, Wyeth Lederle). The 193 control children received, depending on the age, three times Hepatitis B (Engerix-B=AE Junior®, GlaxoSmithkline) or twice Hepatitis A vaccine (Havrix=AE Junior®, GlaxoSmithkline). Nasopharyngeal cultures were performed at study entry, just before booster vaccination at 7 months and at 14, 20 and 26 months. An additional naso-

pharyngeal sample was obtained at the first acute otitis media event after full vaccination. Pneumococcal carriage was observed in around 50% of all children. This carriage rate was maintained in both study groups in the 26 months follow-up period. Thus, no influence on overall colonization was observed during the study. Instead a decline in vaccine serotype carriage was observed in the PV group whereas the non-vaccine serotype carriage increased.[32] Nine hundred twenty-one isolates (95%) from 353 out of 383 patients participating in this study were available for further analysis by genotyping and susceptibility testing.

Bacteriological procedures. Isolation and identification of the *S. pneumoniae* isolates were performed by standard methods as described previously.[32] Susceptibility testing was performed by the agar-dilution method.[25] Resistance was defined by the breakpoint concentrations for the respective antibiotics as defined by the NCCLS.[26] Multidrug resistance was defined as resistance to ≥3 classes of antimicrobial agents.

Serotyping. Pneumococci were serotyped by the capsular swelling method (Quellung reaction) observed microscopically using commercially available antisera (Statens Serum institute, Copenhagen, Denmark).

Restriction fragment end labeling (RFEL) typing. Pneumococcal strain typing by RFEL was done as described by van Steenbergen et al.[31] and adapted by Hermans et al.[19] Briefly, purified pneumococcal DNA was digested by the restriction enzyme EcoRI. The DNA restriction fragments were end labeled at 72°C with [α-32P]dATP using DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). After the radiolabeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 *M* urea, the gel was transferred onto filter paper, vacuum dried (HBI, Saddlebrook, NY), and exposed for variable times at room temperature to ECL hyperfilm (Amersham Laboratories, Amersham, UK).

Computer-assisted analysis of DNA band patterns. RFEL autoradiographs were converted to images (Image Master DTS; Pharmacia Biotech, Uppsala Sweden) and analysed by computer (Windows version Gelcompar software version 4; Applied Math. Kortrijk, Belgium). DNA fragments were analysed as described previously.[27] For evaluation of the genetic relatedness of the isolates we used the following definitions: (1) isolates of a particular RFEL type are 100% identical by RFEL analysis; (2) an RFEL cluster represents a group of RFEL types that differ in only one band (approximately >95% genetic relatedness).

Multi locus sequence type (MLST) analysis. The genotypes of 38 isolates representing different serotypes were investigated by MLST analysis. Within the 23 largest clusters representing 29 RFEL genotypes, the most prevalent serotypes were analysed. For this purpose, a fully automated method for MLST was used as described previously.[20] The MLST types were compared with the global PMEN database (http://www.pneumo.com/physician/pmen/pmen_history.asp).

Data-analysis. P-values for differences were calculated with the Chi-square test using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, USA).

Table 1. Pneumococcal isolates with regard to study group and study phase.

	Total	Vaccine group	% vaccine-type strains	Control group	% vaccine-type strains
All phases	921	451		470	
0 months	180	96	46%	84	58%
7 months	176	81	41%	95	46%
14 months	186	84	26%	102	40%
20 months	163	81	25%	82	49%
26 months	119	64	26%	55	54%
First AOM	97	45	24%	52	44%

Results

Nine hundred twenty-one pneumococcal isolates from nasopharyngeal samples of 351 children participating in the study were available for molecular analysis by RFEL. In total, 451 of these 921 pneumococcal isolates were isolated from children in the pneumococcal vaccine group, whereas 470 isolates were isolated in the control group. Of the 921 isolates, 180 isolates were isolated at the start of the study (T=0 months; day of (first) conjugate vaccination), 176 isolates were retrieved at 7 months (day of booster vaccination) and 186, 163 and 119 isolates were isolated after completion of the vaccination scheme at 14, 20 and 26 months of study duration, respectively (table 1). The remaining 97 isolates were retrieved at the first AOM events after full vaccination (>7 months). The serotype distribution of all pneumococcal isolates collected during this study was discussed previously.[32] In summary, the contribution of vaccine serotype pneumococci to colonization gradually declined from 46% at study entry to 26% at the end of the study compared to the control group in which the contribution of the conjugate-vaccine serotypes remained approximately 50%, whereas the total pneumococcal carriage rate remained unaffected.

All 921 isolates were characterized by RFEL. We identified 275 different genotypes representing one to 49 isolates per genotype with an average of 3.35 isolates per genotype. Analysis of the per-patient follow-up revealed a high turnover rate of pneumococcal genotypes; only in 54 out of the 351 children persistent carriage was found for at most 3 consecutive samples (recurrence twice after a 6-7 months interval) (table 2).

No statistical difference in the rate of persistent carriage was found between the pneumococcal vaccine group and the control group children (15% versus 16%; $p = 0.67$). In both

Table 2. Pneumococcal colonization dynamics of the 351 children according to study and age group. Persistent carriage is shown in grey. Vaccine type pneumococci are depicted with the number code "0". Non-vaccine type pneumococci are depicted with the number code "1".

Vaccine group		Months 0	7	14	20	26	1st AOM
12-24 months	1	1	1	1		0	
	2	1		1	1	1	1
	3			1	1		
	4		1	1			
	5	1	1		1	1	
	6		1	1		1	
	7	1	0				
	8	0	0	0	0	1	
	9	0		1			
	10					1	
	11	0	1		0		
	12		0		0		
	13		1	0		1	
	14		1			1	0
	15	1		1		0	
	16	1					
	17	0	0	1			
	18	0					
	19	1	0	1	1		
	20			0	1		
	21	0			1		
	22	0	0		1		0
	23	0			1		1
	24	0	0				
	25	1	1	0			
	26	0		1			
	27		1	1			
	28	1		1	1		1
	29		1	0	1	1	
	30		0			0	1
	31					0	1
	32				1		
	33		1	1	1	1	
	34	0	0	1	0	1	
	35	0			1		
	36	0	0		0		
	37	0	1	1	1		1
	38	0	0	1	1	1	1
	39	1				1	1
	40		1				
	41	1			1	1	
	42			1			
	43	1					
	44			0			
	45		0	0	1	1	
	46	1	1				1
	47				1		
	48		1			1	
	49				1	1	
	50			1		1	
	51	1	0	1	0		
	52	0	0				
	53	1		0	1	0	
	54						0
	55		1	1	1	1	
	56		0		1		
	57	0		1			
	58		1		1	1	
	59						0
	60	0	1		1		
	61	1		0			
	62		1	1			
	63		1	1			
	64	0					
	65				1		
	66	1	1				1
	67	0	1	1			
	68		0				0
	69			0			
	70			1			
	71	0					1
	72	0	1	1			1
	73	1	0				
	74	1	0				

Vaccine group		Months 0	7	14	20	26	1st AOM
2-7 years	75	0		1	0	0	1
	76	1	1				
	77	0					
	78	0		1			
	79			1			
	80	0	0	0	0	1	
	81	1	0		1		
	82			1		0	1
	83	1	0				
	84		1	1	1		
	85				1	0	
	86	1		1	1		1
	87			1	0	1	
	88		1	1	1	1	1
	89			1	1		1
	90						1
	91	1					1
	92	0					
	93	0	1		1	1	
	94				1		
	95	1		1		1	
	96						0/1
	97				1	1	
	98				0		
	99	1	1	1			1
	100	0		1	1	0	
	101	0	0	0			
	102	1	0		1	1	
	103	1	0				
	104				0		
	105	0	0				0
	106		1	1			
	107	1	0				
	108	0		0			
	109	1			1		
	110	1			1	1	
	111			0			1
	112		1		1		
	113	0	1	1		1	0
	114					1	
	115		1		0		
	116	1		1	1		
	117		1	0	0	1	
	118	1	1	1	1		
	119	1			1	1	
	120		1	1	0		
	121		1		1	1	
	122	0				0	
	123		1	1	1	1	1
	124	1	0	1	1	1	
	125					0	1
	126	0	0	0			1
	127				1	1	
	128	0		1	1	1	1
	129		1	1			
	130	0		1	0	1	1
	131	1					
	132	1		1	0	0	
	133		1	1			
	134	1	1	1	0	1	
	135	0			1		
	136	1			1	1	
	137	1	1		0	0	
	138	0			1		
	139	0				1	
	140	1		1	1		
	141		1		1	0	
	142		1	1	1	1	1
	143	1	1		1		0
	144	1		0			
	145		1	1		1	
	146			1		1	
	147	0			1	1	1
	148	0					0
	149		0		1		
	150		1		1	1	
	151						1
	152			0	1		
	153			0	1		
	154			1	0		
	155		1				
	156				1		0
	157	1		1			
	158				1		
	159	1		1		1	
	160	1	0	0	1		
	161	1	1		1		
	162	0	0	1	0		1
	163	1			0		0
	164	0	1				
	165			0			
	166	1	0	1			
	167	0		0			
	168		1				1
	169		1	0			
	170	1					
	171		1	0			

Control group		Months 0	7	14	20	26	1st AOM
12-24 months	1	0	0	0	0		
	2	0	0		0	1	
	3	0					
	4			1	1		1
	5		0	0	0	1	
	6		0			0	
	7	0			1	0	
	8	0	1		1	0	
	9				1	0	
	10				1	0	
	11		1			1	
	12	1	0		0		0
	13		1	1	0	0	
	14					0	
	15		0		0		
	16	0		1	0	1	1
	17	0		1		0	
	18		1	1		0	
	19	1	1	1	1	0	
	20			1		0	
	21	0		1		1	
	22			0			
	23			0		0	
	24	1		0	1		
	25			1			
	26	1	1				
	27				1	0	
	28	1	1	1	0	1	1
	29		1	0		1	0/1
	30			0	1		1
	31		1	0		0	
	32		1	0	1	0	1
	33					0	
	34			1			1
	35			0			
	36		0			0	
	37	0	1		0		
	38	0	0	1	0	0	1
	39			1			
	40		0		0		0/1
	41	0	1	0	1	0	
	42		1	0	0		1
	43	1	1		0	1	
	44				0	0	0
	45				0	1	0
	46	0	0	1	0	0	0
	47		0	0	0	0	0
	48	1	1	0		0	
	49			1			
	50	0			0		
	51	0					1
	52			1			
	53			0			
	54	0	1		0		
	55			1	0		0/1
	56	0	0	0	0		0
	57	0					
	58	0					
	59			1			
	60	0		1			
	61	0	0		1		
	62			0			
	63	1		0	0		0
	64	1					
	65	0			1		
	66		1	0			1
	67		0				
	68	0		1			
	69	1					1
	70	1	1	1			
	71	0	0				0
	72	1	1				
	73	0	0	0			0
	74	1					
	75		1	0			
	76	1	0				

Control group		Months 0	7	14	20	26	1st AOM
2-7 years	77	0			1	0	1
	78			1			
	79		0				
	80		1				
	81				1		
	82	1	0	0			
	83	1		0			
	84	0	0		0	1	1
	85		0		0	1	
	86		1				0
	87		1	0	1		1
	88			0		1	
	89	1	0	0			
	90		1		0	1	
	91	1		1	1		
	92	1	0				
	93			1	1	0	
	94					1	
	95	1		1	0	1	
	96	0	1		0		1
	97	0	1		1		
	98	1	1	1			
	99	0	1	1	1		
	100				1	0	
	101				0		
	102		0	0	0		0
	103		1				
	104			1	0	1	0
	105			1			
	106	1	0	1			
	107			0			1/0
	108	1	1	0/1	0/0		
	109			1	1		
	110	0	0				
	111	0					
	112	1					
	113		0				
	114			0	1		
	115		0				
	116	0	0	0	0		
	117		1	0	1		
	118				1	0	
	119	0	1	1		0	
	120		1				
	121						0
	122	1		1			
	123		0		0		
	124	0	0	1	1		1
	125	0	1		0	1	
	126		0	1	0		
	127	1		0			
	128	1	0	0	0		
	129			1	1	1	
	130			1	1		
	131	0			1		1
	132		1	1	1		1
	133		0	1	1	1	
	134	0	0	0			
	135		0	0	0		
	136	0		1			
	137	1	0		0		
	138				0		
	139		1		1	1	1
	140	0	1	1	1	1	0
	141			0	1	1	
	142	1	1			0	0
	143		0				
	144	1	1		1		1
	145	0					
	146	0	0		1	1	
	147		1	1			
	148			1		0	1
	149			0			
	150	1		1		1	
	151					0	
	152			0			
	153	0		1		0	
	154	1				1	
	155		0			1	
	156			1	1		
	157			1			
	158	0	1	1			40
	159	0	1	0			1
	160	0					
	161			1	0		
	162		1	1			0
	163		0				
	164		0	1			
	165	0	1	1			
	166	0	0	0	1		
	167	1		1	1		
	168		0				1
	169		1				1
	170			1			
	171		1	1	1		
	172			1			
	173	0	0	1			
	174	1					0
	175		1				
	176		1				0
	177	0					
	178	1	1	1			

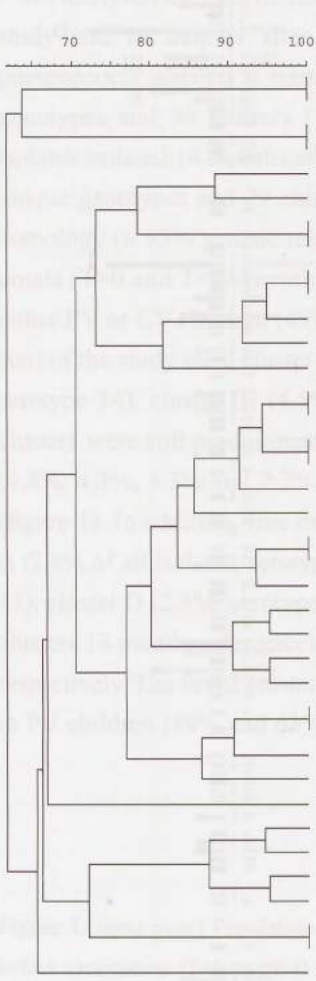
groups, the majority of the persistent isolates (60%) were non-vaccine serotypes. On three occasions, persistence of a specific genotype was accompanied by a switch in serotype. In one case, colonization with one particular genotype which is closely related to MLST type displaying serotype 15 was 6 months later followed by colonization with a second genotype of serotype 19A. Another 6 months later the initial genotype with a capsular switch to serotype 19A was identified. In the second case a switch was observed from serotype 14 to serotype 8 within the same genotype after a 6 months interval. The third case represented a capsular switch from serotype 6A to serotype 19F observed after a 6 months interval.

We analysed the genetic relatedness of the pneumococcal isolates retrieved at start of the study and 14 months after the initial vaccination from both study groups. The 180 pneumococci isolated at start of the study displayed 93 genotypes, representing 52 unique genotypes and 30 clusters (128 isolates) with an average cluster size of 4.3. The 186 isolates isolated 14 months after start of the study displayed 105 genotypes, representing 54 unique genotypes and 29 clusters (132 isolates) with an average cluster size of 4.6. Close homology (≥ 95% genetic relatedness) was found between 70% of the isolates at both time points (T=0 and T=14 months). The remaining genotypes equally represented strains from either PV or CV children (49% and 51%, respectively). The 4 most predominant clusters at start of the study were cluster I (7.8% of all isolates; serotypes 6A and 6B), cluster II (7.3%; serotype 14), cluster III (4.5%; serotype 23F) and cluster IV (4.5%; serotype 9V). These clusters were still predominant 14 months after vaccination, though slightly reduced in size (4.8%, 4.3%, 4.3% and 2.2%, respectively) and mainly observed in control group children (figure 1). In addition, five minor clusters observed in the initial phase of the study, cluster A (2.8% of all isolates; serotype 11), cluster B (1.1%; serotype 11), cluster C (2.8%; serotype 15), cluster D (2.8%; serotype 16) and cluster E (1.7%; serotype 23B), became predominant clusters 14 months after vaccination with a prevalence of 4.8%, 4.3%, 4.3%, 5.4% and 3.8%, respectively. The first 2 clusters, which resembled 85% homology, were predominantly present in PV children (89% and 63%, respectively) (figure 1).

Figure 1. (next page) Population genetic structure of the 180 and 186 pneumococcal isolates retrieved before vaccination (T=0 months) and after pneumococcal conjugate vaccination (T=14 months), respectively. Genetic relatedness is depicted in percentages. The serotypes and number of isolates per genotype are shown in bars for the two periods separately. The contribution of vaccinated children and control children is shown in grey and black, respectively. The numbers I-IV represent predominant clusters at the initial phase of the study. The letters A-E represent emerging clusters after conjugate vaccination. Clusters consisting of two or more genotypes are shown in brackets. Clusters of one genotype are depicted with x.


0 months
14 months
Number of isolates
Serotype
50 60 70 80 90 100%
160b
202b
243b
316b
348b
400b
A
B
C
D
E
I
II
III
IV

We investigated 38 isolates representing different serotypes by MLST analysis. Within the 23 largest clusters representing 29 RFEL genotypes, the most prevalent serotypes were analysed (figure 2). We found no PMEN clones in our collection of pneumococci. In addition, we observed 4 new MLST genotypes. In general, the observed homology by RFEL genotyping was confirmed by MLST, except for RFEL genotype 028 for which RFEL genotyping showed to be less discriminatory than MLST analysis (figure 2).



RFEL	sero type	MLST	*aroE*	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*
241	9V	941	8	10	15	27	2	28	71
241	NT	941	8	10	15	27	2	28	71
23	15	162	7	11	10	1	6	8	14
23	9V	162	7	11	10	1	6	8	14
599	10	097	5	7	4	2	10	1	27
535	35	446	5	7	4	19	10	40	27
12	23F	1040	1	8	9	9	6	26	6
12	23A	NEW	1	8	9	9	6	28	6
50	19F	176	7	13	8	6	10	6	14
50	3	176	7	13	8	6	10	6	14
430	14	138	7	5	8	5	10	6	14
467	6A	207	10	8	30	5	6	1	9
457	19F	NEW	7	14	4	12	1	14	14
304	15	1200	8	13	14	4	17	4	5
304	15	199	8	13	14	4	17	4	14
28	15	199	8	13	14	4	17	4	14
28	14	124	7	5	1	8	14	11	14
443	19A	416	1	13	14	4	17	51	14
101	18C	113	7	2	1	1	10	1	21
30	16	414	1	5	27	5	1	28	1
30	6A	030	1	5	27	20	1	1	1
27	4	205	10	5	4	5	13	10	18
438	23F	176	7	13	8	6	10	6	14
330	23B	439	1	8	9	2	6	4	6
329	23A	036	1	8	4	1	1	4	6
326	23F	036	1	8	4	1	1	4	6
347	19A	440	7	5	1	1	13	31	14
347	23F	440	7	5	1	1	13	31	14
56	19F	309	8	10	2	5	9	48	6
119	33	100	5	12	29	12	9	39	18
426	NT	NEW	2	5	2	NEW	2	19	14
422	11	062	2	5	29	12	16	3	14
442	11	062	2	5	29	12	16	3	14
581	6B	176	7	13	8	6	10	6	14
490	11	NEW	2	5	29	12	9	NEW	14
420	19F	395	1	5	7	12	17	1	35
420	6A	395	1	5	7	12	17	1	14
167	3	180	7	15	2	10	6	1	22

Figure 2. Genetic relatedness of the 38 MLST sequence types observed within the 23 largest RFEL clusters.

We determined the MICs for penicillin, cotrimoxazol, tetracycline, erythromycin, rifampicin, vancomycin and ciprofloxacin of 919 isolates. Resistance to at least one antibiotic was found in 128 pneumococcal isolates (14%). High-level resistance to penicillin, cotrimoxazole, tetracycline, erythromycin and ciprofloxacin was found in 2 (0.2%), 37 (4.0%), 28 (3.0%), 29 (3.2%), and 2 (0.2%) isolates, respectively. Furthermore, intermediate resistance to penicillin and cotrimoxazole was found in 8 (0.9%) and 61 (6.6%) isolates, respectively. Intermediate resistance to tetracyclin, erythromycin and cefotaxim was seen only occasionally (table 3). No cases of resistance to rifampicin and vancomycin were identified.

Table 3. Antibiotic resistance rates for the *S. pneumoniae* collection

	Nr. of resistant strains	Percentage of total	Nr. of intermediate resistant strains	Percentage of total
Penicilline	2	0.2%	8	0.9%
Cotrimoxazole	37	4.0%	61	6.6%
Tetracyclin	28	3.0%	5	0.5%
Erythromycin	29	3.2%	1	0.1%
Ciprofloxacin	2	0.2%	0	-
Cefotaxim	0	-	2	0.2%

In table 4, the resistance profiles and their rates, serotypes and number of genotypes are depicted. In total, 21 different resistance profiles were observed. We observed (intermediate) resistance to a single drug in 99 isolates (10.7% of all isolates), dual resistance in 17 isolates (1.8%) and multidrug resistance (resistance to 3 or more antibiotics) in 12 isolates (1.3%).

To evaluate the effect of vaccination on pneumococcal resistance, we compared resistance rates before full vaccination (samples at study entry plus at 7 months study duration) and after full vaccination (samples at 14, 20 and 26 months). In the PV children resistance declined from 17.5% before full vaccination to 11.8% after full vaccination, whereas in the CV children resistance was stable (14.5% and 14.3% before and after full vaccination, respectively). This difference did not reach statistical significance. We also evaluated the serotype distribution among the resistant isolates. 57% of all resistant isolates displayed vaccine serotypes. In addition, 10 out of the 12 multidrug-resistant isolates (83%) were vaccine serotype isolates, whereas the remaining two isolates displayed the cross-reactive serotype 6A.

Table 4. *S. pneumoniae* antibiotic resistance profiles, profile rates, number of genotypes and their serotype distribution.

Resistance Profile*	Nr. of strains	(%)	Nr. of genotypes	Serotypes
Co(I)	53	(5.7)	29	3/ 7/ 8/ 10/ 11/ 12/ 14/ 18C/ 18F/ 6A/ 6B/ 9A/ 9V/ 19A/ 19F/ 23F/ 31/ 33D /NT
Co	25	(2.7)	16	6A/ 6B/ 9V/ 18C/ 21/ 23F/ NT/ 34
T	7	(0.8)	6	3/ 19C/ 19F
T(I)	4	(0.4)	4	23F/18C/ 11/ 38
T(I)E	1	(0.1)	1	33
Tci	1	(0.1)	1	19F
TE	7	(0.8)	6	9N/ 19F
E	8	(0.9)	6	11/ 14/ 15/ 33
E(I)	1	(0.1)	1	23B
Co(I)E	2	(0.2)	2	6A/ 8
Co(I)T	1	(0.1)	1	6B
Co(I)TE	1	(0.1)	1	6B
CoT	2	(0.2)	2	23A/ 19F
CoTE	5	(0.5)	4	6A/6B
P(I)	1	(0.1)	1	11
P(I)CoCf(I)	1	(0.1)	1	14
P(I)Co(I)ECf(I)	1	(0.1)	1	6B
P(I)CoTE	3	(0.3)	3	19F/ 6B/ 14
Pco(I)	1	(0.1)	1	15
P(I)Co(I)	2	(0.2)	2	14/ 23F
PcoTE	1	(0.1)	1	6B
PE	1	(0.1)	1	6A
Total	128	(13.8)		

*Co: cotrimoxazole; P: penicilline; T: tetracyclin; E: erythromycin; Cf: cefotaxim; Ci: ciprofloxacin. (I): intermediate resistance

Discussion

Between 1998 and 2002 a large randomised, double-blind vaccination trial with a 7-valent pneumococcal conjugate vaccine followed by a 23-valent polysaccharide vaccine was performed among 383 children, aged 1-7 years, with a history of recurrent acute otitis media. Surprisingly, no beneficial effect was observed on the frequency of acute otitis media after pneumococcal vaccination nor on the overall colonization rate of *S. pneumoniae*. However, a shift of vaccine type pneumococci to non-vaccine type pneumococci was observed among nasopharyngeal colonization isolates. Emerging non-conjugate vaccine serotypes were serotypes 11, 15 and 16.[32] We questioned whether this shift occurred within specific genotypes or whether replacement took place with genetically different strains. If the latter was true, we wondered if these different genotypes were equally capable of horizontal dissemination and whether they represented comparable antibiotic resistance profiles.

Therefore, we analysed the 921 pneumococcal isolates retrieved from 351 of the 383 participating children. We observed 275 different genotypes, representing 106 genetic clusters and 75 unique genotypes. Analysing the per-patient follow-up revealed few episodes of persistent carriage. This implicates that pneumococcal colonization is a dynamical process with a high turnover rate of colonizing strains. No effect of vaccination was found on the limited rate of persistent strains. This was to be expected, because the majority of the persistent strains in both PV and CV group were non-vaccine serotypes.

Remarkably, in three cases of persistent carriage, a serotype switch was observed. However, one could argue whether the consecutive colonization with the serotype 15 and serotype 19A variant of a strain closely related to MLST 199 suggests the recruitment of a second isolate with identical genotype rather than a capsular switch. Importantly, in contrast to many countries including the US, this genotype is not very common in The Netherlands (3%) and a 19A serotype variant has not been previously observed. Therefore, our findings strongly suggest a capsular switch. So far, this phenomenon was only reported in vivo twice by Barnes *et al.*[3] and Sluijter *et al.*[27] Indirect prove for capsular switch was previously shown by other investigators who demonstrated the true recombinational exchanges at the capsular locus.[7-9,24] One might argue that our observations are events enhanced by conjugate vaccination due to the induction of a selective immunological pressure. Indeed, the serotype 15/19A switch was observed in a PV child but no conjugate vaccine type pneumococci were involved. The two additional cases (serotype 6A/19F and 14/8 switch) were observed in CV children. Although our data support the theory that serotype switch is a natural process which can be observed occasionally within an individual, a large number of data will be required to study the impact of conjugate vaccination on this process.

Comparison of the genetic structure of the pneumococci isolated at study entry and at 14 months after pneumococcal conjugate vaccination showed 70% homology between the pneumococcal isolates at the two time points. The non-overlapping isolates were equally

distributed among PV and CV children. Furthermore, initially predominant clusters displaying vaccine serotypes had been partially replaced by non-vaccine serotype clusters after vaccination, which displayed a similar capability to spread horizontally. Though replacement by non-vaccine serotypes has proven to occur as a result of growing age,[12] we observed this shift significantly more often in children who received the pneumococcal vaccines, indicating this process is enhanced by vaccination.

Our most predominant clusters represented multiple vaccine and non-vaccine serotypes. Since the observed genetic homology was confirmed by MLST analysis, our data suggest that a large number of recombinational events at the capsular loci have occurred within these clusters. This is in line with previous data from the USA and Latin America where the major (resistant) clones also show multiple serotypes as a result of capsular serotype switch.[9,10,16,22,29] Wolf *et al.* have shown that these events occur even more often in susceptible pneumococcal clones,[33] which is in line with our findings.

Our data support the hypothesis that serotype replacement observed after conjugate vaccination does not directly indicate a shift in the genetic structure of the pneumococcal population. Shifts towards and predominance of non-vaccine serotype variants are likely to occur within genetic clusters displaying both vaccine and non-vaccine serotypes. However, MLST analysis of the most predominant clusters of our collection of pneumococcal strains showed the presence of new genotypes and the absence of PMEN homologous clones. Therefore, this collection of strains might not be representative for countries where multidrug resistant clones are predominantly present.

To evaluate whether vaccination will have an effect on the presence of antibiotic resistance, we determined the antibiotic resistance profiles of all 921 isolates. Susceptibility testing of 919 of the 921 pneumococcal isolates was performed for penicillin, cotrimoxazole, tetracyclin, erythromycin, rifampicin, vancomycin, cefotaxim and ciprofloxacin. In agreement with previous studies performed in The Netherlands, the overall resistance is low (14% of the isolates) compared to other European countries.[5,13,14,17,18] Penicillin resistance was found rarely in our study. In contrast, we most frequently observed resistance to cotrimoxazole, tetracyclin and erythromycin. We compared our data with a previous study performed in The Netherlands, where 10,489 clinical pneumococcal isolates have been tested for drug susceptibility.[14] Compared to this study (reference year 1999), we noted a higher incidence in cotrimoxazole resistance (4.4 versus 10.6) and a lower incidence in tetracyclin resistance (3.5% versus 6.6%). Both observations can be explained by the age difference between the study groups; our study was performed in children under 7 years of age, whereas the surveillance study represented all age groups including adults. In contrast to adults, tetracyclines are contraindicated in children whereas cotrimoxazole is often first choice treatment.

We found an equal percentage of (intermediate) resistance to a single drug in our study population compared to the surveillance study (77% of the resistant isolates), comparable dual resistance (13% an 19%, respectively) and significantly higher multidrug resistance (9% and 4%, respectively; $p < 0.01$). We hypothesize that our children might select for multidrug resistant strains because of higher antibiotic consumption, which is in accordance with previous findings.[23,28]

In addition, we analysed changes in the incidence of pneumococcal resistance. To this respect, we compared resistance among the pneumococcal isolates between the initial phases of the study (before full vaccination) and the post-vaccination phases. Although a trend was seen with a decline in resistance from 17.5% to 11.8%, this was not statistically significant. Because of the low resistance rates, no subsidiary analysis could be performed for the separate sample dates. Therefore, we analysed the serotype distribution among the resistant isolates, which showed that 57% of the resistant isolates depict a vaccine serotype, which is comparable to the overall serotype distribution. However, all multidrug-resistant isolates were vaccine types or cross-reactive serotypes. Although resistance is low among *S. pneumoniae* in The Netherlands, these data implicate that vaccination with the 7-valent conjugate vaccine may reduce pneumococcal resistance in the population, particularly multidrug resistance.

In conclusion, pneumococcal conjugate vaccination did not induce a shift in the population-based structure of the pneumococci, nor decreased their tendency to spread horizontally. Our observations combined with the vaccine efficacy data of Veenhoven *et al.*[32] suggest that pneumococcal conjugate vaccination is not very useful for prevention of pneumococcal colonization in children above 1 year of age. Moreover, we strongly advice continuous and close monitoring of the pneumococcal genetic structure in areas with a conjugate vaccination policy.

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Chapter 5

Colony blot assay: a useful method to detect multiple pneumococcal serotypes within clinical specimens

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Abstract

The efficacy of pneumococal conjugate vaccines in young children may be complicated by serotype replacement. We developed a colony blot assay, which enables the identification of re-colonization with novel serotypes (replacement), overgrowth by minor co-colonizing serotypes or suppression of previously predominant vaccine serotype strains as a result of vaccination. This method allows the identification of multiple serotypes in a single specimen in a ratio of 1:1000. In order to demonstrate the potential of our method, we investigated the consecutive nasopharyngeal samples of 26 children who had shown a shift in pneumococcal colonization after conjugate vaccination. Mixed colonization was found once in 15 pre-vaccination samples and 4 times in 26 post-vaccination samples. In the remaining children 'true replacement' had presumably occurred. Hence, we conclude that the colony blot assay is an easy to apply method, which allows the identification of different pneumococcal serotypes within single clinical specimens.

Introduction

Current pneumococcal conjugate vaccines are protective against invasive diseases in children.[1] However, the impact of vaccination on otitis media and nasopharyngeal colonization is limited.[2,3] Several studies demonstrated a shift in carriage from vaccine type pneumococci towards non-vaccine types after conjugate vaccination.[4] Whether this is caused by unmasking of pneumococcal serotypes which are already present at the nasopharynx or by replacement, i.e. the acquisition of other serotypes, remains unknown.[5] We therefore developed a colony blot assay, which allows the detection of multiple serotypes, i.e. mixed colonization, within clinical specimens. Such a method is a useful tool to investigate the pneumococcal serotype dynamics, e.g. changes in pneumococcal population structure during colonization. Furthermore, this method has important applications with respect to the monitoring of pneumococcal vaccination.

Material and methods

Clinical specimens. We studied the nasopharyngeal cultures from 26 children participating in a pneumococcal conjugate vaccine trial in The Netherlands.[2] Inclusion criteria for this study were (1) two or more AOM episodes prior to study entry, and (2) age of 1 to 7 years. The number of previous AOM episodes was based on parental report. Children aged 12-24 months were immunized with PCV7 (Prevnar®, Wyeth) twice at a one-month interval followed 6 months later by PPSV23 (Pneumune®, Wyeth). Children aged 25-84 months received one dose of PCV7 followed 7 months later by PPSV23.

Nasopharyngeal swabs. At study entry, at 7 months (just before booster PPSV23 vaccination) and at 7, 14, 20 and 26 months after the last vaccination a nasopharyngeal sample was obtained for culture of *S. pneumoniae, H. influenzae*, and *M. catarrhalis*. The nasopharyngeal samples were taken transnasally by the study physicians using a flexible sterile dry cotton-wool swab (Copan Italia, Transwab, Medical Wire & Equipment Co. Ltd., Corsham, England). After sampling, swabs were placed directly in Stuart's transport medium. Samples of nasopharyngeal swabs were plated within 6 hours. Isolation and identification of the isolates was performed by standard methods.[2] In addition, in case of positive cultures for *S. pneumoniae* the gentamycin plates were rinsed with 1.0 ml THY broth containing 18.5% glycerol and stored at -80°C (primary culture). Pneumococci were microscopically serotyped by the capsular swelling method (Quellung reaction) using commercially available antisera (Statens Seruminstitut, Copenhagen, Denmark).

Colony blot assay. The colony blot assay is based on the dot blot method of Fenoll *et al.*[6] This method, used for serotyping of pneumococcal isolates, was adjusted to determine single colonies of a second serotype among predominant serotypes. In summary, serial dilutions of primary cultures in 0.9% phosphate buffered saline (PBS) were plated onto blood-agar plates and grown overnight in 5-10% CO2 at 37°C to obtain colony densities of

approximately 1000 individual colonies per plate. The colonies were blotted onto a nitrocellulose membrane (Optitran, Schleicher & Schuell, 's-Hertogenbosch, Netherlands) by applying the membrane on top of the plate for 20 minutes. After careful colony lifting, the membranes were dried for 30 minutes at room temperature (RT). The membrane was incubated in PBS for 30 minutes at 56°C to inhibit pneumococcal phosphatase activity. After incubation in blocking buffer (5% skim milk in PBS) for 1 hour at RT, the membranes were washed 3 times for 10 minutes in PBS containing 0.05% Tween 20 (PBST). To detect serotype-specific colonies the membranes were incubated with anti-capsule-specific rabbit sera (Statens Seruminstitut, Copenhagen, Denmark) diluted 1: 10,000 in PBS for 1 hour at RT. For the detection of serotype 6B pneumococci, we used a 1: 4,000 dilution of monoclonal mouse antibodies (14A2), which were kindly provided by PG van der Dobbelsteen, Netherlands Vaccine Institute, Bilthoven, The Netherlands. Prior to this incubation, these sera were preadsorbed with R6 pneumococci to remove aspecific non-capsular antibodies. The plates were washed 3 times with PBST after which antibody binding was detected by alkaline-phospatase-conjugated anti-rabbit IgG (Sigma, USA) or anti-mouse IgG (Serotec, UK) for 1 hour at RT. Conjugate binding was identified by the substrate *p*-nitrophenyl phosphate (Sigma). A colony blot using a pneumococcal strain with an identical serotype as searched for served as a positive control. After the membranes were dried at room temperature, positive colonies were identified and compared to the colonies on the original plate. The corresponding colonies were picked from the plate and re-grown overnight. Subsequently, the strains were serotyped and characterized by RFEL genotype analysis.

Cross-reactivity of the capsule-specific antisera of the Statens Seruminstitut was tested by means of colony blot analysis of at least two pneumococcal strains of each of the 7 conjugate vaccine serotypes 4, 6B, 9V, 14, 18C, 19F, 23F, the cross-reactive serotypes 6A and 23B, and the serotypes 3, 10, 11, 15, and 16. Pre-incubation of the sera with cell wall polysaccharides of strain R6 eliminated cross-reactivity for most sera. Only the serotype 6B anti-serum showed cross-reactivity with all remaining serotypes. Therefore, this antiserum was replaced by monoclonal mouse antibodies raised against serotype 6B capsule polysaccharides (14A2). Cross-reactivity remained for 19F antiserum and the pneumococcal serotypes 6A and 16 (data not shown).

In order to test the sensitivity of the colony blot method, we mixed cultures of different pneumococcal serotypes in various ratios. We were able to identify minor pneumoccocal serotypes representing 0.1% of the bacterial population.

The colony blot analysis was performed in a group of children who had shown a shift in colonization from a vaccine serotype A to a (non-) vaccine serotype B strain during 2 consecutive nasopharyngeal samples. Mixed colonization was defined as the presence of serotype A colonies among primarily serotype B colonies, or as the presence of serotype B colonies among primarily serotype A colonies. Replacement was defined as the absence of

serotype A in the serotype B culture or the absence of serotype B in the serotype A culture.

For the 26 children included in this analysis at least two primary cultures from consecutive specimens following pneumococcal vaccination were present. Only from 15 children a primary culture was available at study entry. Children displaying a shift in colonization but with less than two primary cultures available for analysis were excluded from analysis (n=5).

RFEL analysis. Pneumococcal strain typing by RFEL was performed as described previously.[7] Briefly, purified pneumococcal DNA was digested by the restriction enzyme *Eco*RI. The DNA restriction fragments were end labeled at 72°C with [α-32P]dATP using DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). After the radiolabeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 *M* urea, the gel was transferred onto filter paper, vacuum dried (HBI, Saddlebrook, NY), and exposed for variable times at room temperature to ECL hyperfilm (Amersham Laboratories, Amersham, UK). RFEL autoradiographs were converted to images (Image Master DTS; Pharmacia Biotech, Uppsala Sweden) and analysed by computer (Windows version Gelcompar software version 4; Applied Math. Kortrijk, Belgium). DNA fragments were analysed as described previously.[8] The genetic relatedness of the isolates was evaluated by computerized comparison of the individual banding patterns.

Results

We developed a colony blot assay, which enables the identification of re-colonization with novel serotypes (replacement), overgrowth by or suppression of minor co-colonizing serotypes as a result of vaccination. This method allows the identification of multiple serotypes in a single specimen in a ratio of 1:1000 (Figure 1).

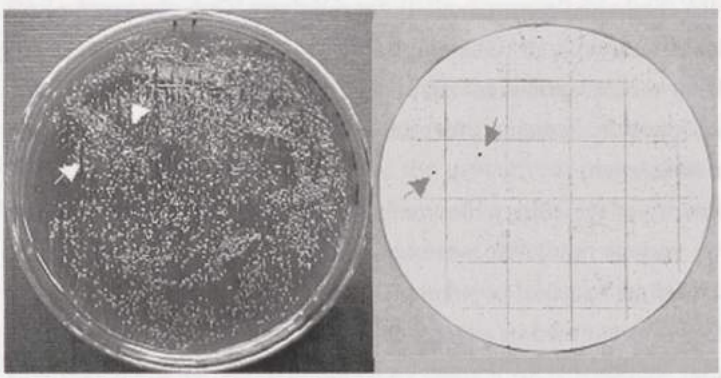

Figure 1. Culture containing multiple pneumococcal serotypes. The colonies were blotted onto a nitrocellulose membrane. The two coloured colonies marked by arrows represent a minor serotype 6A colony among predominant serotype 38 colonies.

To validate the colony blot assay, we applied the technology to clinical specimens from children vaccinated with a 7-valent pneumococcal conjugate vaccine, who were colonized with pneumococci before and after vaccination. Samples were only included when a shift in pneumococcal serotype and genotype from a vaccine-type strain before vaccination to a non-vaccine type strain after vaccination had occurred. We re-grew the primary cultures using serial dilutions to obtain densely grown bacterial plates but with individual bacterial colonies (approximately 1000 colonies per plate). Initial cultures were tested for the presence of the post-vaccination non-vaccine serotype strain, whereas post-vaccination cultures were tested for the presence of pre-vaccination vaccine serotype strains. In case the intended serotype was found, we confirmed the genetic similarity with the original strain by RFEL genotyping (figure 2).

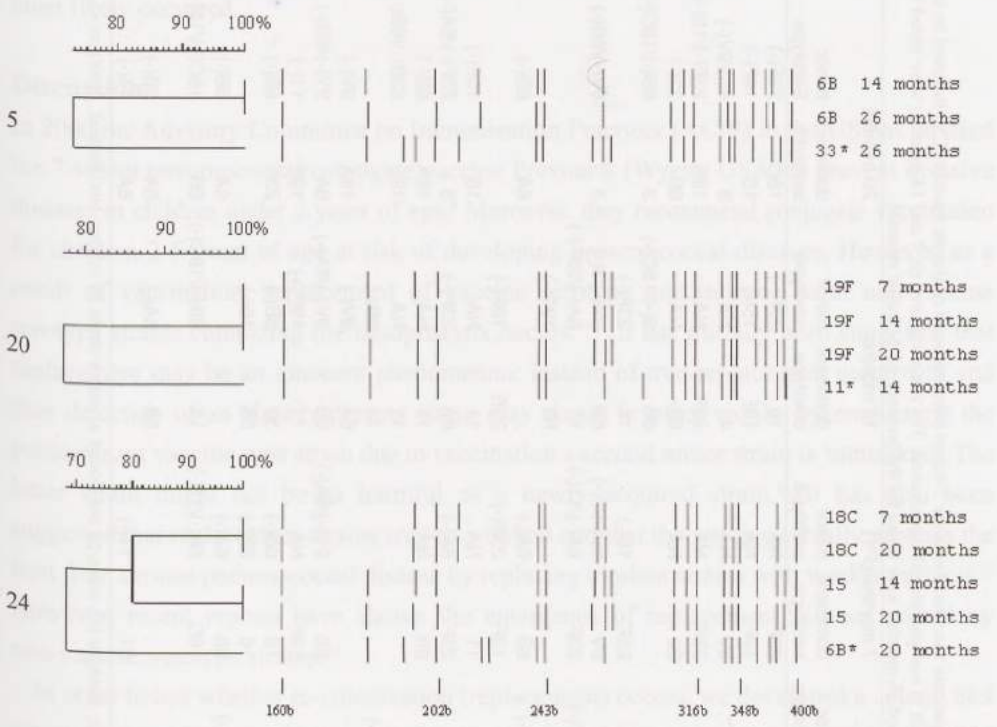

Figure 2. RFEL patterns of the consecutive isolates of the children who displayed mixed colonization by colony blot screening. Patient numbers, moment of sampling (months) and serotypes of the pneumococcal isolates are depicted. *predominant strain.

Table 1. Colony blot results of 26 patients vaccinated with the 7-valent pneumococcal conjugate vaccine (CV) followed by the 23-valent pneumococcal polysaccharide vaccine (PV) who displayed a serotype shift in nasopharyngeal colonization. The initial cultures (0, 7 and 14 months) were tested for the presence of the post-vaccination strain(s), and the post-vaccination cultures (14, 20 and 26 months) were tested for the presence of the initial strain(s).

patient	0 months (CV)		7 months (PV)		14 months		20 months		26 months		conclusion
	dominant serotype	minor serotype	dominant serotype	minor serotype	dominant serotype	minor serotype	dominant serotype	minor Serotype	dominant serotype	minor serotype	
1	23F	NA	23F	NA	10	23F(-)	18C	23F(-)	11	23F(-)	Replacement
2			19F	NA	19F	15(-)	15	19F(-)	15	19F(-)	Replacement
3	4	NA	9V	NA	4	NA	8	4(-)/9V(-)	16	4(-)/9V(-)	Replacement
4	23F	NA	23F	NA	23F	NA	19F	23F(-)/15(-)	15	23F(-)/19F(-)	Replacement
5			22	NA	6B	NA	6B	33(-)	33	6B(+)	Mixed olonization
6	7	NA			6B	3(-)/18C(-)	3	6B(-)/18C(-)	18C	6B(-)/3(-)	Replacement
7			23F	16(-)	16	23F(-)					Replacement
8	11	NA	9V	6B(-)/3(-)	6B	9V(-)/3(-)	3	9V(-)/6B(-)			Replacement
9	6A	23F(-)	23F	6A(-)/11(-)	11	6A(-)/23F(-)					Replacement
10	19F				NT	19F(-)					Replacement
11	6A	6B(-)	6B	6A(-)			6A	6B(-)	6A	6B(-)	Replacement
12	19F	NA	19F	23B(-)	23B	19F(-)					Replacement
13	19F	NA	11	NA	11	NA	16	19F(-)	16	19F(-)	Replacement
14	6A	NA	23F	6A(-)	6A	23F(-)	3	23F(-)/6A(-)			Replacement
15	6B	NA	6B	NA	15	NA	19A	6B(-)	19A	6B(-)	Replacement
16	6B	NA			11	NA	19F	23B(-)/6B(-)	23B	19F(-)/6B(-)	Replacement
17	6B	14(-)			14	6B(-)					Replacement
18	9V	NA			6A	9V(-)	19A	9V(-)	11	9V(-)	Replacement
19	9V	NA	6B	9V(-)/6A(-)	6B	9V(-)/6A(-)	6A	9V(-)/6B(-)			Replacement
20	3	NA	19F	NA	11	19F(+)	19F	11(-)	11	19F(-)	Mixed colonization
21	6B	NA	16	6B(-)	38	6B(-)	23F	6B(-)			Replacement
22	6B	NA	16	6B(-)	6B	16(-)					Replacement
23	6A	6B(-)	6B	6A(-)			6A	6B(-)			Replacement
24	6B	NA	18C	6B(-)/15(-)	15	6B(-)/18C(-)	6B	18C(+)/15(+)	23B	6B(-)/18C(-)/15(-)	Mixed colonization (2x)
25					6B	6A(-)	6A	6B(-)			Replacement
26	14	NA	21	14(-)			6A	14(-)			Replacement

NA, Initial culture is not available; serotype (-), serotype not fiound as minor strain by colony blot method; Serotype(+), serotype found as minor serotype using the colony blot method

Post-vaccination cultures were available for all 26 children and were tested for the presence of the pre-vaccination serotype. In 4 instances representing 3 children the pre-vaccination serotype strain was observed as a minor strain in the post-vaccination culture. For only 15 children pre-vaccination cultures were available and could be tested for the presence of the post-vaccination serotype as a minor strain. This phenomenon was observed in 1 child only (patient 20). The latter child showed an initial carriage of a serotype 19F strain followed 7 months later by colonization with a serotype 11 strain. Another 6 months later a serotype 19F strain with identical genotype was cultured as predominant strain again. With the colony blot assay we were able to detect the presence of the serotype 19F strain as minor strain among the serotype 11 pneumococci. The suppression of serotype 19F in specimen 14 with the subsequent predominance one culture later in specimen 20 is depicted in Table 1. In the remaining 14 children re-colonization (replacement) of the vaccine serotype strains by the newly acquired non-vaccine type pneumococci had most likely occurred.

Discussion

In 2000 the Advisory Committee on Immunization Practices (ACIP) in the US has advised the 7-valent pneumococcal conjugate vaccine Prevnar® (Wyeth, USA) to prevent invasive diseases in children under 2 years of age.[9] Moreover, they recommend conjugate vaccination for children 2-5 years of age at risk of developing pneumococcal diseases. However, as a result of vaccination, replacement of vaccine serotype pneumococci with non-vaccine serotype strains colonizing the nasopharynx occurs.[10-12] It has initially been suggested that replacement may be an innocent phenomenon: instead of true replacement outgrowth and thus detection of an already present strain may occur. In other words, by eradicating the predominant vaccine type strain due to vaccination a second minor strain is 'unmasked'. The latter strain might not be as harmful as a newly acquired strain.[13] It has also been suggested that replacement strains are less virulent and that the vaccine actually protects the host from serious pneumococcal disease by replacing virulent strains with weaker variants.[13] However, recent reports have shown the emergence of replacement disease caused by non-vaccine serotype strains.[2,3]

In order to test whether re-colonization (replacement) occurs, we developed a colony blot assay that can detect multiple and minor serotypes within a single specimen in a 1:1000 ratio. Alternative screening of individual colonies using the quellung method, is unsuitable because it is time and labour consuming. Moreover, it is inappropriate for high throughput screening of thousands of colonies.

To demonstrate the potential of the colony blot technique to answer this question, we selected a test cohort of 26 children who had shown a shift in pneumococcal carriage from

vaccine to non-vaccine serotypes after pneumococcal conjugate vaccination. Among the 26 children who were investigated by this method, four events of persistence of vaccine type pneumococci as minor strains after vaccination were observed in the presence of a predominant non-vaccine serotype strain. In one child, predominance of an initially suppressed vaccine serotype strain was observed after vaccination. These data may implicate that in the majority of these cases replacement of serotypes occurs. However, the persistence of vaccine type strains accompanied by immunological pressure due to vaccination and the availability of non-vaccine serotype strains for replacement might create ideal circumstances for recombinational exchange of capsular genes.[4] Assuming that this phenomenon occurs as a result of vaccination, future vaccine failures with respect to prevention of disease and elimination of multidrug-resistant clones may take place. To study the true effect of conjugate vaccination on replacement, obviously a larger study should be performed. For such study, the colony blot assay will be a useful additional tool.

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Chapter 6

Immunoglobulins in otitis-prone children

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Abstract

Defective or immature antibody responses to pathogens in children may explain the increased susceptibility to acute otitis media (AOM) in otitis-prone children. In literature, data on immunology have been based on studies of small groups of severely otitis-prone children and have not been consistent.

Humoral immune status was assessed in 365 children, 1- 7 years old, with two or more documented episodes of AOM in the previous year. Children with 4 or more episodes in the preceding year were defined as otitis-prone. Serum immunoglobulin levels were determined by radial immunodiffusion. Immunoglobulin levels of otitis-prone children were compared with those of children who had experienced 2-3 AOM episodes per year.

Children with recurrent episodes of AOM were found to have normal or increased serum IgA, IgM, IgG, and IgG1 levels compared with normal values for age, whereas the serum IgG2 levels were mostly in the lower normal range. Twenty-two percent of all children showed IgG2 levels lower than 2 SD below the age-specific mean. Interestingly, the otitis-prone group of children showed significantly lower median and mean levels for all immunoglobulins compared with those children with only 2-3 previous AOM episodes.

Lower immunoglobulin levels in otitis-prone children suggest a generalized decreased antibody response in otitis-prone children.

Introduction

Based on the clinical observation that some children experiences recurrent episodes of acute otitis media (AOM), the term "otitis-prone" was introduced by Howie.[1] In general, the otitis-prone condition is defined as three or more episodes of AOM in 6 months or four or more episodes in 12 months; up to 5% of all children comply with this definition.[2,3]

With respect to immunoglobulin serum levels, both normal as well as stimulated serum IgA, IgM, IgG, and IgG1 levels have been reported in otitis-prone children aged 2 months or older.[4,5] Freijd *et al.* and Sørensen *et al.* reported lower levels of IgG2 in children with recurrent AOM as compared to age-matched controls,[5,6] but others did not confirm these observations.[4,7]

With respect to antibody activity against the two main bacterial pathogens in AOM *Streptococcus pneumoniae* and nontypable *Haemophilus influenzae* (NTHI), subnormal or absent antibody responses have been reported in otitis-prone children, [8-11] as well as decreased antibody responses upon immunization with Hib conjugate and rubella vaccine.[12,13]

These findings may suggest decreased antibody responses upon both T-cell-dependent as well as T-cell independent antigens in otitis-prone children.[14] Hitherto, immunologic evaluations have been performed only in small groups of otitis-prone children. The need to obtain more substantial data led us to analyse immunoglobulins in a large group of 365 children with varying susceptibility to acute otitis media.

Methods

This study was conducted in a general hospital (Spaarne Hospital Haarlem) and a tertiary care hospital (University Medical Center Utrecht), the Netherlands. The Medical Ethics Committees of both participating hospitals approved the design of the study. A signed informed consent was obtained from the parents or legal guardians of all children before evaluation.

From April 1998 to February 2001, 365 children aged 1-7 years with 2 or more episodes of AOM in the previous year were included in the study. The number of previous AOM episodes was based both on parental report, with AOM defined as having one or more of the following symptoms: acute earache, new-onset otorrhea, irritability and fever, and on clinical confirmation of the diagnosis AOM by a physician. Patients with previously recognized congenital or acquired immunodeficiencies were excluded from the study.

Total serum immunoglobulin concentrations of IgA, IgM, and IgG as well as IgG1 and IgG2 subclass concentrations were determined by radial immunodiffusion (Behring Werke, Mannheim, Germany and Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). Serum immunoglobulin levels within the range of 2 SD below or above the age-specific mean were considered normal.[15,16] Total deficiency of IgA was defined as a serum level of less than ≤ 0.05 g/L. Total deficiency of IgG2 was defined

as a serum level less than ≤ 0.02 g/L. To address the question whether otitis-prone children are immunologically different from children with fewer episodes of AOM, the children were divided in two groups: 231 otitis-prone children with 4 or more AOM episodes in the preceding year and 134 children with 2-3 AOM episodes in the preceding year.

Statistics. Differences in the number of children with low serum immunoglobulin levels according to age (12-24 mo *versus* 25-84 mo) were analyzed using χ^2 tests or Fisher exact tests when appropriate. Differences between mean immunoglobulin levels between children with 2-3 AOM and children with 4 or more episodes were analysed with *t* test for independent samples or the Mann-Whitney U test when appropriate. Group differences were considered statistically significant at $p < 0.05$. Linear regression modeling was used to analyse the potential effect of age differences on immunoglobulin levels.

Results

Table 1 provides general characteristics for all children. The median age of the total group of children was just above 2 years.

Table 1. General characteristics of total patient group (n = 365).

Male sex (%)	231	(63.3)
Median age in years (minimum-maximum)	2.15	(1-6.99)
Age (%)		
12-24 months	153	(41.9)
25-84 months	212	(58.1)
median number of AOM in preceding year (minimum-maximum)	4.0	(2-12)
No. of AOM episodes in preceding year (%)		
2-3 AOM	134	(36.7)
4 or more AOM	231	(63.3)

Serum concentrations of IgA, IgM, IgG, IgG1, and IgG2 according to age for the whole group of children are illustrated in figure 1. The IgA, IgM, IgG, and IgG1 levels were generally in the high-normal range or higher than 2 SD above the age-specific mean. In contrast, the IgG2 levels were mostly in the low-normal range and 22.5% of all children showed IgG2 levels lower than 2 SD below the age-specific mean. Table 2 shows the number of children with low immunoglobulin levels (<2 SD below the age-specific mean) according to different age groups. In children aged 12 to 24 months significantly higher percentages of children with low serum levels of IgA (18.3% *vs* 7.1%, p=0.001) and IgG2 (32.7% *vs* 15.1%, p=0.001) were found as compared to older children.

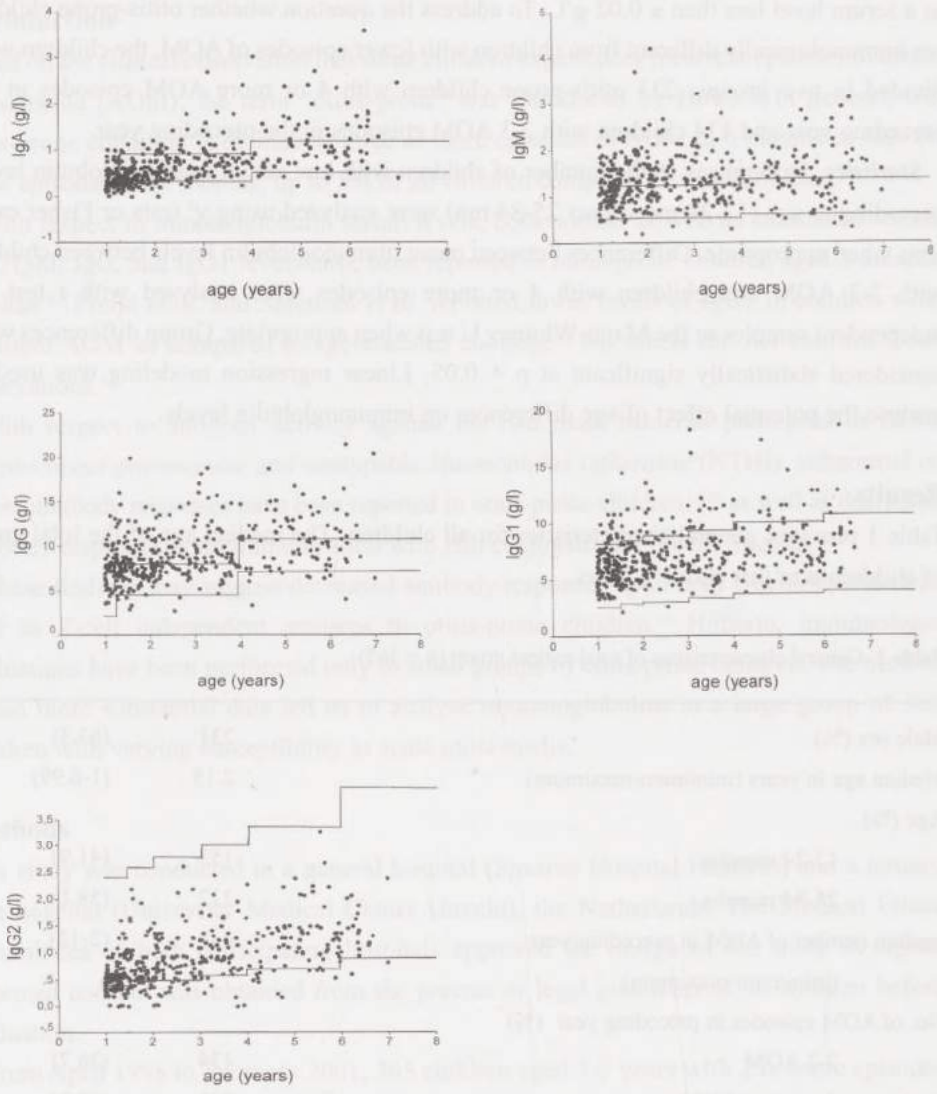

Figure 1. Distribution of serum concentrations of IgA, IgM, IgG, IgG1, and IgG2 according to age for all children. Solid lines represent ± 2 SD of the age-specific mean.

Total IgA deficiency was found in 3 children with 2-3 previous AOM episodes and 2 children with 4 or more AOM episodes. In contrast, the nine children with absent IgG2 serum levels all belonged to the group with 4 or more AOM episodes. These children with total IgG2 deficiency suffered from significantly more recurrent AOM episodes per year as compared to the whole group of children with subnormal or normal IgG2 serum levels (8.00 and 4.97 episodes respectively; p=0.003).

Table 2. Number (%) of children with serum concentrations of IgA, IgM, IgG, IgG1, and IgG2 less than 2 SD of the age-specific mean according to age at the time of evaluation.

	12-24 months (n=153)	25-84 months (n=212)	p-value
low IgA	28 (18.3%)	15 (7.1%)	0.001*
low IgM	9 (5.9%)	7 (3.3%)	0.24
low IgG	5 (3.3%)	7 (3.3%)	0.99
low IgG1	1 (0.7%)	1 (0.5%)	1.00
low IgG2	50 (32.7%)	32 (15.1%)	0.001*

Differences in the number of children with low serum immunoglobulin levels between both age groups were analysed using Chi-square tests or Fisher exact tests when appropriate.

* Group differences were considered statistically significant at $p < 0.05$.

Table 3 shows the mean total serum levels of IgA, IgM, IgG, IgG1, and IgG2 now according to number of AOM episodes in the previous year. Levels of serum immunoglobulins in otitis-prone children with 4 or more AOM episodes were significantly lower than in children with 2-3 previous AOM episodes. These differences in the serum immunoglobulin levels were not influenced by differences in age according to linear regression analyses.

Table 3. Serum immunoglobulin concentrations according to the number of AOM episodes per year.

	2-3 AOM episodes/year (n=134)	≥ 4 AOM episodes/year (n=231)	p-value
IgA g/l	0.71 (0.01-3.58)	0.59 (0.01-3.15)	0.03
IgM g/l	1.44 (0.05)	1.30 (0.04)	0.02
IgG g/l	9.67 (0.28)	8.80 (0.18)	0.02
IgG1 g/l	7.80 (0.26)	6.95 (0.14)	0.005
IgG2 g/l	0.99 (0.05)	0.83 (0.03)	0.005

Values are means (SEM), except for IgA for which the medians (range) are provided due to skewed distributions. P-values obtained from Student's T test or Mann Whitney U-test when appropriate. Group differences were considered statistically significant at $p<0.05$.

Discussion

In this large group of 365 children aged 1-7 years with recurrent AOM episodes, in general normal or stimulated levels of IgG, IgM, IgA, and IgG1 were found, whereas IgG2 levels proved to be in the lower normal range or depressed as compared to normal control values. Markedly, 32.7% of the children aged 12-24 months showed IgG2 levels lower than 2 SD below the age-specific mean. Most probably, due to spontaneous recovery of IgG2 levels this percentage was statistically significantly lower in older children but still impressive with 15.1%, suggesting a specific role for IgG2 in susceptibility to AOM. A higher percentage of low IgA levels was only found in the youngest group of children aged 12 to 24 months (18.3%), at older age this percentage nearly normalized (7.1%).

IgG2 may be important in the defense against otitis pathogens like *S. pneumoniae*. Effective host defense against *S. pneumoniae* depends primarily on opsonizing antibodies against the capsular polysaccharides. In adults, pneumococcal anticapsular antibodies reside primarily in the IgG2 subclass. Our finding of low IgG2 antibody levels in children with recurrent AOM is in agreement with a previous report of otitis-prone children at 30 months of age, who showed lower IgG2 anti-pneumococcal antibody levels as compared to healthy age-matched children and adults. In contrast to IgG2, in the same report the IgG1 anti-pneumococcal antibody levels in the otitis-prone group of children were even higher than those in adults.[17] Also after vaccination with pneumococcal polysaccharide vaccine low to absent IgG2 anti-pneumococcal antibody responses were observed in otitis-prone children.[10] Some in vitro data showed effective phagocytosis of pneumococci to be primarily related to IgG2 anti-pneumococcal antibodies.[18,19,20] This dependency on IgG2 antibodies may also be reflected in the low expression of FcγRIIaH131, the Fc receptor for IgG2 on effector cells, in patients with recurrent respiratory tract infections, or bacteremic pneumonia.[21,22] Our findings support the hypothesis that clinical protection against mucosal infections like AOM depends more on IgG2 levels, and not on IgG1.

In addition to the relative IgG2 immunodeficiency found in our population, overall serum immunoglobulin levels were lower in children with 4 or more AOM episodes as compared to children with 2-3 AOM episodes per year. It should be noted that suffering from 2-3 AOM episodes in the first years of life is not comparable to suffering from 2-3 episodes at the age of 4 to 7 years. These older children with 2-3 AOM episodes per year might also be regarded as otitis-prone. This is reflected in the fact that at the age of 2 to 4 years children with 4 or more AOM episodes show decreased IgA, IgM, and IgG2 levels as compared to children with 2-3 AOM episodes. In children aged 4 to 7 years no significant differences in any of the immunoglobulin isotypes existed anymore between children with 2-3 AOM episodes per year and 4 or more (data not shown). Low serum immunoglobulin levels may indicate decreased antibody responses despite recurrent infections. Normally, recurrent

infections induce high antibody levels due to repeated stimulation as for example in patients with cystic fibrosis or defective granulocyte functions. In otitis-prone children the observed humoral hyporesponsiveness may be one of the causes of the ongoing susceptibility to AOM pathogens. This hypothesis is supported by the fact that, apart from absent or low responses towards T-cell independent pneumococcal polysaccharides antigens, diminished responses towards protein (T-cell dependent) antigens are also observed in otitis-prone children. Hotomi *et al.* demonstrated that 11 of 20 otitis-prone children older than 18 months exhibited reduced anti-P6-IgG antibody levels to NTHI as compared to healthy age matched controls despite repeated infections with NTHI.[11] P6 is one of the six outer membrane proteins of NTHI. Furthermore, decreased responses to T-cell independent polysaccharide Hib vaccine, as well as polysaccharide protein Hib conjugate vaccine were observed in children aged 22 to 158 months with a history of recurrent respiratory tract infections and with normal IgG subclasses.[12] Finally, 13 children with recurrent AOM showed a significantly lower antibody response to the viral rubella vaccine than did 29 children without AOM.[13]

Immune responses to infectious agents are regulated by immune effector and cytokine producing cells. The cytokines IL-1, IL-6 and tumor necrosis factor-alpha (TNF-alpha) are glycoproteins produced by different cell types when exposed to bacteria and viruses.[23] These cytokines trigger acute phase responses and induce proliferation and differentiation of T and B cells. One study showed children with recurrent AOM episodes to produce significantly lower nasopharyngeal IL-1β, IL-6 and TNF-alpha in nasopharyngeal secretions upon colonization with *Haemophilus influenzae* than healthy children.[24] Such a local defect in cytokine production could contribute to the defective immune reactivity in otitis prone children.[25]

In conclusion, a relative high percentage of low IgG2 levels is found in children with recurrent otitis media. In the youngest children, many also show low IgA levels, but this disappears at older age. Otitis-prone children in general show lower total IgM, IgA, IgG, IgG1, and IgG2 levels as compared to those with fewer episodes of AOM.

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Chapter 7

Immunogenicity of a 7-valent pneumococcal conjugate vaccine followed by a 23-valent pneumococcal polysaccharide vaccine in children with recurrent acute otitis media.

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Abstract

Anti-polysaccharide antibody responses after combined vaccination with a 7-valent pneumococcal conjugate vaccine followed by a 23-valent pneumococcal polysaccharide vaccine were evaluated in a well-defined group of children aged 1-7 years with a history of recurrent AOM. Children aged 1-2 years received the conjugate vaccine twice and older children once before booster vaccination with the polysaccharide vaccine. The vaccinations induced adequate (> 1.0 mg/L) IgG serum antibody responses to all pneumococcal serotypes included in the conjugate vaccine, except for serotype 6B in the youngest age group 1-2 years. In otitis-prone children above the age of 2 years, anti-pneumococcal antibody responses were diminished compared with age-matched children with 2-3 AOM episodes in the year before study entry. For optimal systemic IgG antibody induction including for serotype 6B in children with recurrent upper respiratory tract infections, we suggest to prime children aged 12-24 months with at least 3 doses of PCV7.

Introduction

Streptococcus pneumoniae is one of the leading causes of bacterial infections worldwide, ranging from acute otitis media (AOM) and sinusitis to pneumonia and invasive diseases like bacteremia and meningitis. Both persisting high morbidity and mortality associated with pneumococcal infections despite use of antibiotics, and the rapid emergence of multidrug-resistant *S. pneumoniae* have prompted the search for preventive strategies like vaccination to control pneumococcal disease.[1,2] Especially high-risk groups like young children, elderly and those with primary or secondary immunodeficiency could benefit from such pneumococcal vaccines.

The traditional pneumococcal polysaccharide vaccines are known for their limited immunogenicity in children aged < 2 years and in patients with immunodeficiencies like those suffering from HIV infection or following stem cell transplantation.[3,4] Patients with recurrent bacterial respiratory infections are also known to respond poorly to polysaccharide antigens, such as the capsular polysaccharides of *Haemophilus influenzae* type b (Hib) and *S. pneumoniae*, despite normal serum IgG levels and adequate responses against protein antigens.[5-7] In a consequence, the protective value of these pneumococcal polysaccharide vaccines is poorest in those at highest risk for pneumococcal disease.

After the successful introduction of the Hib conjugate vaccine in infants resulting in a sharp decline in Hib invasive disease, similar conjugate vaccines were developed for *S. pneumoniae*. The currently available protein-polysaccharide combinations contain 7-11 selected pneumococcal capsular polysaccharides bound to a protein carrier. They induce a T-cell dependent immune response and, in contrast to polysaccharide vaccines, they proved to be immunogenic in healthy infants[8,9] as well as in certain patients with recurrent respiratory tract infections[10,11] and in HIV infected children.[12] By inducing immunologic memory because of the T-cell dependent character, pneumococcal conjugate vaccines also prime for an effective booster IgG response, not only to repeated vaccination with conjugate vaccine but also to that with the pneumococcal polysaccharide vaccine.[13,14,15] Booster vaccination with a polysaccharide vaccine may result in even higher quantitative serum IgG antibody concentrations than following the conjugate vaccine.[15] Clinical efficacy of combined vaccination with 7-valent pneumococcal conjugate vaccine (PCV7) followed by 23-valent pneumococcal polysaccharide vaccine (PPSV23) was studied in a well-defined group of 383 children with recurrent AOM episodes.[16] In this article we will present the serum antibody responses to conjugate pneumococcal serotypes in children aged 12-24 months who received PCV7 twice and older children who received PCV7 once before booster vaccination with PPSV23.

Subjects, materials, and methods

Immunogenicity of combined pneumococcal conjugate and polysaccharide vaccinations

was studied as part of a double-blind, randomised efficacy trial in Dutch children with recurrent AOM.[16] From April 1998 to February 2001, children aged 1-7 years, with 2 or more episodes of AOM in the previous year were included in the study. The number of previous AOM episodes before study entry was based on both parental report and clinical confirmation of the diagnosis by a physician. Patients with previously recognized congenital or acquired immunodeficiencies were excluded from the study. Children who were found at baseline to have IgA deficiency defined as a serum level less than ≤ 0.05 g/L or IgG2 deficiency defined as a serum level less than ≤ 0.02 g/L, were also excluded from analyses. Of the 383 children participating in the trial, anti-pneumococcal antibody responses were measured in 120 randomly selected children; 92 from the pneumococcal vaccine group and 28 control vaccinees.

Vaccination schemes. Children aged 12-24 months were immunised twice with PCV7 (with a 1-month interval), and 6 months later with PPSV23. Children aged 25-84 months received PCV7 once, followed 7 months later by PPSV23. Children in the control group aged 12-24 months received three hepatitis B vaccinations and those aged 25-84 months received hepatitis A vaccine twice.

Vaccines. PCV7 (Prevnar®, Wyeth, Rochester, NY, USA) consisted of 2 μg each of capsular polysaccharides of pneumococcal serotypes 4, 9V, 14, 19F, and 23F, 4 μg of serotype 6B polysaccharide, and 2 μg of serotype 18C oligosaccharide, each conjugated individually to the CRM197 protein. PPSV23 (Pneumune®, Wyeth) consisted of 25 μg of capsular polysaccharides of each of the pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. Control vaccines were hepatitis B vaccine (Engerix-B=AE Junior(®), GlaxoSmithkline, Rixensart, Belgium) and hepatitis A vaccine (Havrix=AE Junior®, GlaxoSmithkline).

Antibody measurements. Before and 1 month after a complete course of PCV7 (once or twice) or control vaccination(s) and 1 month after the last vaccination with PPSV23 or control vaccine, a blood sample was taken for immunological assessment. Pre- and post-vaccination IgG levels to the seven pneumococcal serotypes in the conjugate vaccine were measured in serum by ELISA as described previously.[6,17] All serum samples were preincubated overnight with excess free common cell wall polysaccharide (CPS) to remove anti-CPS antibodies. The pneumococcal antibody reference serum (lot 89-SF) was used for assay standardization.[18]

Adequate IgG antibody responses to an individual serotype after PCV7 or after PCV followed by booster PPSV23 were defined as a post-immunization antibody concentration > 1.0 mg/L.[8,11] The overall response to pneumococcal vaccinations was considered successful if postvaccination IgG titers were > 1.0 mg/L for at least five out of seven measured conjugate vaccine serotypes (serotypes 4, 6B, 9V, 14, 18C, 19F, 23F).[11,19]

Statistical analysis. Geometric mean (GM) serum antibody concentrations and percentages

of children reaching IgG antibody titers of > 1.0 mg/L for the individual conjugate vaccine serotypes are presented separately for children aged 12-24 months and 25-84 months. The relationship between age and number of AOM episodes in the year before study entry and antibody concentrations was evaluated by Mann-Whitney U test using non-transformed data. Differences in percentages of children who reached protective antibody concentrations and percentages of children who reached successful vaccination were analysed by Chi-square test or Fisher's exact when appropriate.

This study was performed in a general hospital (Spaarne Hospital, Haarlem) and a tertiary care hospitals (Wilhelmina Children's Hospital of the University Medical Center Utrecht in the Netherlands). The medical ethics committees of the participating hospitals approved the study protocol. A signed informed consent was obtained from the caregivers of all children participating in this study.

Table 1. Patient characteristics and immunological data at baseline.

Characteristics	**Pneumococcal vaccine group (n=92)**		**Control vaccine group (n=28)**	
Male sex	58	(63%)	21	(73%)
Median age, years (range)	2.1	(1.02 - 6.58)	2.3	(1.02-5.95)
Age 12-24 months	42	(46%)	13	(46%)
24-84 months	50	(54%)	15	(54%)
Number of AOM episodes in preceding year (%)				
2-3	42	(46%)	12	(43%)
4-5	28	(30%)	10	(36%)
6 or more	22	(24%)	6	(21%)
Median level of immuno-globulins, g/L (range)				
IgA	0.65	(0.08-5.50)	0.64	(0.16-2.37)
IgM	1.29	(0.62-2.50)	1.17	(0.22-2.42)
IgG	8.96	(3.40-18.70)	7.88	(3.00-18.80)

Results

General characteristics and baseline immunological data of the children are presented in table 1. Median age of the children of the pneumococcal and control vaccine group was approximately 2 years. In both groups more than 50% of all children were otitis-prone with 4 or more AOM episodes per year. In the pneumococcal vaccine group, the median number of AOM episodes in the year before study entry was the same (4 episodes) in children aged 12-24 months and those aged 25-84 months. With respect to age, 20 of 42 children with 2-3 AOM and 22 of 50 children with 4 or more AOM were younger than 2 years of age, and therefore received PCV7 twice followed by booster PPSV23.

Antibody concentrations at baseline. Despite previous recurrent AOM episodes, all children had low pre-vaccination GM IgG anti-pneumococcal antibody concentrations (< 1.0 mg/L) irrespective of age, with the exception of serum IgG levels for serotype 14 (table 2). Children aged 25-84 months had higher pre-vaccination IgG concentrations than those aged 12-24 months for serotypes 14 (p=0.02), 18C (p=0.005), 19F (p=0.02), and 23F (p=0.001); similarly, more children aged 25-84 months had serum IgG levels above 1.0 mg/L than those aged 12-24 months for serotypes 14 (55% *vs.* 35%, p=0.02), serotype 19F (22% vs. 6%, p=0.01), and serotype 23F (22% *vs.* 7%, p=0.03). For the remaining serotypes, less than 10% of children in both age groups had an IgG antibody titer of > 1.0 mg/L. With respect to the history of the number of previous AOM episodes, all GM IgG concentrations were similar in children with 2-3 AOM and 4 or more AOM episodes per year (data not shown).

Antibody responses with respect to different vaccination schemes related to age. After conjugate vaccination(s) alone, GM IgG antibody concentrations were already higher than 1.0 mg/L for 6 of 7 serotypes in every age category (table 2). The highest GM concentrations were achieved for serotype 14. However, antibody concentrations against serotype 6B remained low (<1.0 mg/L) despite a 7-10 fold rise from baseline; 0.43 mg/L in children aged 12-24 months and 0.59 mg/L in older children. Overall, GM IgG antibody responses after two doses of PCV7 as administered in the younger age group did not significantly differ from those after one PCV7 as applied in the older children, with the exception of the IgG response for serotype 18C, which was higher in the older age group (p=0.02). Percentages of children who reached antibody levels over 1.0 mg/L after vaccination were roughly similar in both age groups and varied per serotype from 22% for serotype 6B in children aged 12-24 months to more than 90% for serotypes 14 and 18C in both age groups (figure 1). Furthermore, the percentage of children that by our definition fell into the category "successful vaccination" after receiving only PCV7 vaccinations was similar in both age groups: 71% and 74%, respectively.

After booster vaccination with PPSV23, GM IgG antibody responses increased markedly in both age groups (table 2). In the youngest children aged 12-24 months, however, no

Table 2. Serum GM IgG anti-pneumococcal antibody concentrations (mg/L) to the seven conjugate vaccine serotypes at baseline, after vaccination with PCV7 (children aged 12-24 months twice and older children once), and after PPSV23 compared with control vaccinations.

Pneumococcal Serotype	Age	GM concentrations (mg/L)				
		Baseline	After PCV7 or control vaccine		After PPSV23 or control vaccine	
		all patients	PV	CV	PV	CV
4	12-24 months	0.05	1.60	0.14	5.85*	0.12
	25-84 months	0.05	1.35	0.12	3.38	0.13
6B	12-24 months	0.04	0.43	0.05	0.56	0.06
	25-84 months	0.05	0.59	0.04	1.52*	0.05
9V	12-24 months	0.17	2.10	0.81	28.39	0.59
	25-84 months	0.21	2.42	0.26	25.42	0.30
14	12-24 months	1.05	14.62	6.39	78.06	15.59
	25-84 months	2.26*	19.93	3.81	77.36	3.95
18C	12-24 months	0.15	4.38	0.33	9.63	0.48
	25-84 months	0.27*	6.58*	0.49	9.13	0.32
19F	12-24 months	0.21	2.96	0.41	12.34	0.64
	25-84 months	0.39*	2.46	0.48	13.01	0.35
23F	12-24 months	0.41	2.18	0.68	4.23	0.99
	25-84 months	0.61*	1.85	0.53	3.65	0.71

PV = pneumococcal vaccine group

CV = control vaccine group

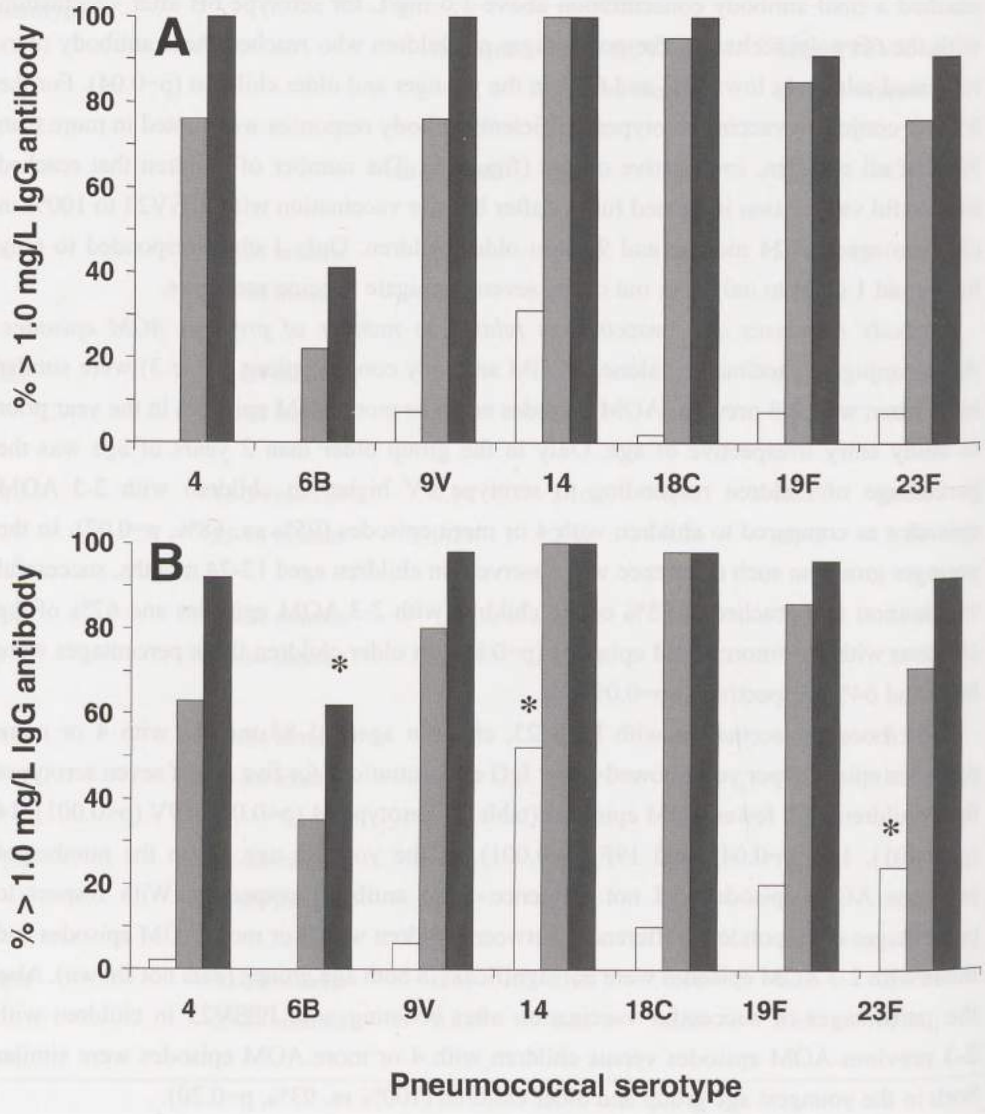
* Value significantly higher ($p<0.05$) with Mann-Whitney U test as compared with other age group

further increase in antibody concentration of serotype 6B (0.56 mg/L) was found. In contrast, a 3-fold rise to 1.52 mg/L for serotype 6B was noted in older children. This difference in GM antibody concentrations between both age groups was significant (p=0.02). With respect to all other serotypes, only the IgG antibody response for serotype 4 (p=0.02) was significantly higher in the younger children, who had received PCV7 twice, than in the older children, who had received PCV7 once. Although overall more children reached a final antibody concentration above 1.0 mg/L for serotype 6B after vaccination with the 6B polysaccharide, the percentages of children who reached these antibody titers remained relatively low: 41% and 62% in the younger and older children (p=0.04). For the 6 other conjugate vaccine serotypes, sufficient antibody responses were noted in more than 90% of all children, irrespective of age (figure 1). The number of children that reached successful vaccination increased further after booster vaccination with PPSV23 to 100% in children aged 12-24 months and 96% in older children. Only 1 child responded to only three, and 1 child to only four out of the seven conjugate vaccine serotypes.

Antibody responses after vaccination related to number of previous AOM episodes. After conjugate vaccinations alone, all GM antibody concentrations (table 3) were similar in children with 2-3 previous AOM episodes and 4 or more AOM episodes in the year prior to study entry irrespective of age. Only in the group older than 2 years of age was the percentage of children responding to serotype 9V higher in children with 2-3 AOM episodes as compared to children with 4 or more episodes (95% *vs.* 68%, p=0.02). In the younger group no such difference was observed. In children aged 12-24 months, successful vaccination was reached in 75% of the children with 2-3 AOM episodes and 67% of the children with 4 or more AOM episodes (p=0.56). In older children these percentages were 86% and 64%, respectively (p=0.09).

After booster vaccination with PPSV23, children aged 25-84 months with 4 or more previous episodes per year showed lower IgG concentrations for five out of seven serotypes than children with fewer AOM episodes (table 3); serotypes 4 (p<0.001), 9V (p<0.001), 14 (p=0.001), 18C p=0.04), and 19F (p=0.001). In the younger age group the number of previous AOM episodes did not influence these antibody responses. With respect to percentages of responders, differences between children with 4 or more AOM episodes and those with 2-3 AOM episodes were not significant in both age groups (data not shown). Also the percentages of successful vaccination after boosting with PPSV23 in children with 2-3 previous AOM episodes versus children with 4 or more AOM episodes were similar both in the youngest age group and older children (100% *vs.* 93%, p=0.20).

Figure 1. Children aged 12-24 months (panel A) or 25-84 months old (panel B) were vaccinated with 7-valent pneumococcal conjugate vaccine (PCV7), followed 6 months later by 23-valent pneumococcal polysaccharide vaccine (PPSV23). Serum IgG anti-pneumococcal polysaccharide antibodies were determined before vaccination (open bars), 4 weeks after PCV7 (gray bars) and 4 weeks after PPSV23 (black bars)



*Value significantly higher ($p<0.05$) with Chi-square tests as compared with corresponding data in panel A

Table 3. Serum GM IgG anti-pneumococcal antibody concentrations to the seven conjugate vaccine serotypes at baseline, after vaccination with PCV7 alone (children aged 12-24 months twice and older children once) and after PPSV23 for both age groups according to the history of previous number of AOM.

Pneum. Serotype	Age	Geometric mean concentrations (mg/L)					
		Baseline		After PCV7		After PPSV23	
		2-3 AOM	≥ 4 AOM	2-3 AOM	≥ 4 AOM	2-3 AOM	≥ 4 AOM
4	12-24 mo	0.06	0.05	1.63	1.57	6.28	5.48
	25-84 mo	0.04	0.04	1.47	1.26	5.32*	2.36
6B	12-24 mo	0.04	0.03	0.51	0.37	0.63	0.51
	25-84 mo	0.04	0.05	0.58	0.59	2.14	1.15
9V	12-24 mo	0.19	0.17	1.82	2.41	28.51	28.29
	25-84 mo	0.21	0.20	3.57	1.80	52.44*	14.39
14	12-24 mo	1.20	0.90	17.52	12.31	89.21	69.13
	25-84 mo	3.47*	1.21	28.44	15.27	135.28*	49.87
18C	12-24 mo	0.17	0.13	4.23	4.52	10.80	8.67
	25-84 mo	0.34	0.26	7.23	6.13	12.13*	7.30
19F	12-24 mo	0.28	0.17	3.63	2.44	19.33	8.20
	25-84 mo	0.52	0.27	3.19	2.03	25.41*	7.69
23F	12-24 mo	0.45	0.40	2.36	2.02	4.81	3.76
	25-84 mo	0.56	0.64	1.68	1.99	4.42	3.14

* value significantly higher ($p<0.05$) with Mann-Whitney U test for children with 2-3 AOM compared with children with 4 or more AOM episodes.

Discussion

In this group of children aged 1-7 years with a history of recurrent AOM, immunisation with 7-valent pneumococcal conjugate vaccine followed by 23-valent pneumococcal polysaccharide vaccine resulted in serum GM IgG antibody concentrations well above 1.0 mg/L for 6 out of 7 conjugate vaccine pneumococcal serotypes in more than 90% of the children.

However, the response to serotype 6B remained low in 59% of children aged 1-2 years and 38% of those aged 2-7 years.

The final serum IgG antibody concentrations for all serotypes found in our population except serotype 6B were similar or higher than those reported previously in healthy infants and toddlers immunised with conjugate vaccines only.[8,9,12,20,21] Limited responses to polysaccharide 6 in otitis-prone children have been described earlier.[22] Poor responses to serotype 6B after vaccination with one or two doses of PCV7 have also been found in infection-prone patients aged 2-18 years who also failed to mount adequate responses to the polysaccharide vaccine.[10,11] Similar to our results, in these studies the percentages of children who reached an adequate response (>1.0-1.3 mg/L) for serotype 6B was also low and maximally 35%. However, Barnett *et al.* found more or less similar GM IgG antibody concentrations for serotype 6B after a single dose of PCV7 in otitis-prone children (1.19 mg/L) and otitis-free control children (1.43 mg/L).[23] Our study design did not allow us to compare vaccine responses with those of an otitis-free group of children. The variance in results between Barnett's and our study could be explained by differences in age, for we did find higher responses in children aged 2-7 years compared with younger children. Our children also may have been more prone to recurrent AOM and more impaired in the response against polysaccharide antigens than the children in the study of Barnett *et al.* and therefore better matched with children in the studies of Sorensen *et al.*[10] and Zielen *et al.*[11] The fact that booster polysaccharide vaccination after a single priming with the conjugate vaccine did increase anti-6B antibody titers in older children but not in those aged 1-2 years despite priming twice with the conjugate vaccine, points to the role of age in the maturation of the response to serotype 6B. To improve the antibody response against serotype 6B in children with recurrent AOM, in particular in the youngest age group, an additional dose of PCV7 may be required.

With respect to the final antibody concentrations after immunisation it is difficult to compare our results with PCV7 followed by PPSV23 with those of vaccination with multiple doses of PCV7 alone, as was done in other studies in infants and otitis-prone children. Further, in interpreting various study results one should take into account that different laboratories were involved in measuring antibody concentrations and also that population-based differences in antibody responses to same vaccine antigens have been described.[24,25] However, it has already been shown that after immunisation with conjugate vaccines at ages 2, 4 and 6 months, children who received a booster PPSV23 at 14 months had higher IgG serum antibodies to the pneumococcal serotypes included in the conjugate vaccine than those receiving a booster PCV7 at the same age.[15] This could be the result of the higher doses of each of the polysaccharides (25 µg) in the polysaccharide vaccine than in the conjugate vaccine (2-4 µg). Boosting with PPSV23 instead of PCV7 is worth considering because the polysaccharide vaccine is less costly than the conjugate vaccine and has the potential

advantage of broader serotype coverage for the remaining 16 serotypes. The vaccination scheme we used, however, failed to prevent nasopharyngeal replacement by non-conjugate serotypes 11 and 15, both included in PPSV23, despite high serum IgG antibody responses against these serotypes (45 mg/L and 12 mg/L, respectively).[16] Apparently, without priming by conjugate vaccines, these antibodies may be only marginally functionally active and therefore unable to influence acquisition and carriage of pneumococcal serotypes included in the polysaccharide vaccine.[26,27] Hence, no broader serotype coverage with respect to mucosal carriage and possibly also mucosal infections was observed. Furthermore, despite potentially higher serum IgG antibodies after the PPSV23 booster, a PCV7 booster may nevertheless be more effective because the conjugate vaccine is thought to recruit new memory B-cells whereas the polysaccharide vaccine might primarily induce terminal differentiation of already present polysaccharide specific B-cells.[28] In this context we found that only those children who had received the conjugate vaccine twice showed a significant decrease of nasopharyngeal carriage of pneumococcal conjugate vaccine serotypes, whereas children who had received the vaccine only once did not, despite similar final serum IgG levels after boosting with the polysaccharide vaccine.[29] Other possible disadvantages of a polysaccharide booster, such as ultimately a potential depletion of the memory pool and lack of affinity maturation should also be considered.[30] For this reason, although at present IgG antibody concentrations are the principle immunologic parameters used to compare study results, the use of functional antibody levels like opsonophagocytic activity, and avidity might be preferable as the basis for long-term protection.[31]

With respect to the frequency of previous AOM episodes, this was found to influence the antibody response only in children older than 2 years of age: otitis-prone children with 4 or more previous AOM episodes per year showed lower antibody responses for five serotypes than children of the same age with fewer AOM episodes. Their ultimate GM antibody concentrations, however, were still well above 1.0 mg/L. Whether such reduced antibody concentrations as were found in the otitis-prone children do influence clinical efficacy of the vaccine is not exactly clear, for we observed that neither children with 2-3 previous AOM episodes nor otitis-prone children benefit clinically from pneumococcal vaccinations.[16] Moreover, no difference in the impact of pneumococcal vaccination on pneumococcal carriage in more or less otitis-prone children was observed.[29]

In conclusion, in children aged 1-7 years with a history of recurrent AOM, vaccination with PCV7 followed by a booster with PPSV23 induces good IgG antibody responses to 6 out of 7 pneumococcal serotypes included in the conjugate vaccine. PCV7 vaccination(s) apparently failed to induce systemic anti-6B antibodies and the polysaccharide booster did not further increase those antibodies, in particular in the youngest children. Therefore priming with an additional dose of PCV7 may be required to overcome these low anti-6B antibody responses.

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Chapter 8

Summarising discussion

The trial results

In this thesis we have reported the results of our study on vaccination with a 7-valent pneumococcal conjugate vaccine (PCV7, Prevnar®, Wyeth) followed by a 23-valent pneumococcal polysaccharide vaccine (PPSV23, Pneumune®, Wyeth) in 383 Dutch children, aged 1-7 years, with a history of recurrent AOM. In contrast to earlier studies in healthy infants vaccinated at a younger age,[1,2] pneumococcal vaccination did not reduce AOM episodes in our population, neither in children immunised at ages 12-24 months, nor at ages 24-84 months. Exclusion of severely otitis-prone children with 6 or more AOM episodes in the year before study entry did not chance the outcome of our study (chapter 2). The main reason for this lack of efficacy of pneumococcal vaccinations in our study population is a shift from conjugate vaccine to non-conjugate vaccine pneumococcal serotypes at the nasopharyngeal level. As a result, overall pneumococcal carriage remained the same throughout the study. Pneumococcal replacement was most pronounced in children aged 12-24 months, who were primed with PCV7 twice, and far less so, and not significant, in older children, who were primed with PCV7 once before booster vaccination with PPSV23 (chapter 3). The question whether this serotype replacement occurred within specific genotypes or took place with genetically different strains we answered in chapter 4: only 3 of 54 children who carried a genotype identical pneumococcus for at least 12 months exhibited a capsular serotype switch. Because this switch was observed once in pneumococcal vaccinees and twice in control vaccinees, this phenomenon is most probably due to a natural process observed occasionally within an individual. The same chapter also reports a decline in overall antibiotic resistance among *S. pneumoniae* from 17.5 to 11.8% as a result of pneumococcal vaccination. Although penicillin resistance is low among *S. pneumoniae* in the Netherlands, these data support earlier observations that vaccination with PCV7 may reduce pneumococcal resistance in the population, particularly multidrug resistance.[3] In chapter 5 a newly developed colony blot assay is described, that allows the detection of multiple serotypes within a single specimen. This assay was applied in a subgroup of 26 children of the original study who had demonstrated a shift in both pneumococcal serotype and genotype from a conjugate vaccine strain before vaccination to a non-conjugate vaccine strain after pneumococcal vaccination. In the majority of cases this shift was due to true replacement of serotypes; persistence of conjugate vaccine type pneumococci as minor strains was rarely found. Finally, our study population was assessed for baseline immunological status (chapter 6) and anti-pneumococcal antibody responses after pneumococcal vaccination (chapter 7). Low serum IgG2 levels were remarkable for our group of children with recurrent AOM. Similarly, otitis-prone children with 4 or more previous AOM episodes per year had lower overall levels for IgM, IgA, IgG and lower levels for subclasses IgG1 and IgG2 than children with fewer previous episodes. This may suggest a generalised

decreased antibody response in otitis-prone children. Antibody responses towards the conjugate *Haemophilus influenzae* type b vaccine as well as to vaccines of diptheria, tetanus and measles were, however, normal in our study population.[4] After vaccination with the conjugate and polysaccharide vaccine, we found high overall serum IgG anti-pneumococcal antibody titres for 6 out of 7 conjugate vaccine serotypes. The antibody response against serotype 6B, however, remained markedly low, particularly in the youngest children (<1.0 mg/L). The relatively low anti-6B response may be related to an impaired response in otitis-prone children to this weak immunogenic serotype, as was described previously for the cross-reactive serotype 6A.[5] Despite high overall IgG antibody responses, otitis-prone children aged 2 years and older had lower anti-pneumococcal antibody responses after pneumococcal vaccination than those with fewer AOM episodes. These data of decreased antibody responses against pneumococcal polysaccharides in children with recurrent AOM are in accordance with earlier observations in patients with recurrent respiratory tract infections.[6-9]

Our trial results raise a number of issues:

1. *Why do pneumococcal conjugate vaccinations prevent recurrent AOM episodes in healthy infants but not in older children with a history of recurrent AOM?*
Apart from a different vaccination regime with a final vaccination with PPSV23 after priming with PCV7 in our study, the different outcomes of the studies in healthy infants[1,2] and ours in older children with a history of recurrent AOM may be explained for the greater part by differences in age at which vaccination was started. Nasopharyngeal pneumococcal carriage develops during the first year of life, and the onset of AOM episodes due to *S. pneumoniae* is directly related to acquirement of such carriage.[10-12] When vaccination is started as early as at 2 months of age, pneumococcal carriage of the 7 most prevalent serotypes, and thereby also the onset of pneumococcal AOM episodes brought on by these serotypes, may be delayed until a later age, when the child is immunologically and anatomically more mature and therefore more capable of handling an AOM infection. *S. pneumoniae* is a frequent pathogen in early and initial AOM episodes, and by changing middle ear conditions pneumococcal AOM may predispose to further AOM episodes with less pathogenic micro-organisms like untypable *Haemophilus influenzae*.[11] It is plausible that prevention of early pneumococcal AOM by early vaccination may reduce development of the otitis-prone condition in toddlers and older children, as was found in the Kaiser Permanente study.[1] In our population, vaccinations were started in children after the age of 12 months, when pneumococcal carriage was already established. Because of naso-pharyngeal replacement of conjugate vaccine to non-conjugate vaccine pneumococcal

serotypes in the children aged 12-24 months, pneumococcal vaccination was not effective in preventing AOM. Most likely the same replacement took place at the middle ear level. Such replacement at the middle ear level after PCV7 vaccinations has already been established in the two Finnish infant studies;[2,13] in these studies, the occurrence of AOM caused by the replacing serotypes in all likelihood limited the clinical efficacy of conjugate vaccines against AOM. In children in our study who were vaccinated after 2 years of age, vaccinations did not influence pneumococcal carriage at all and therefore did not prevent AOM recurrences. The fact that we immunised these children only once with the conjugate vaccine appears to have been insufficient to influence their mucosal conditions despite good serum IgG anti-pneumococcal antibody concentrations. This lends further credence to the theory that a polysaccharide booster increases serum IgG levels but does not influence mucosal immunity.[14]

2. *Why did we find a higher rate of AOM recurrences in pneumococcal vaccinees than in control vaccinees?*

Unexpectedly, all analyses showed a trend towards a higher recurrence rate of AOM following pneumococcal vaccination than following control vaccination. Only the per-protocol analysis after complete vaccination showed a significant increase in AOM episodes (rate ratio 1.29, 95% CI 1.02-1.62). There are several possible explanations for this observation. Pneumococcal nasopharyngeal replacement by itself might induce occurrences of AOM episodes, for newly acquired carriage of *S. pneumoniae* is associated with a higher risk for development of AOM than established carriage.[11] Besides, other micro-organisms, which potentially interfere with growth of pneumococci, may be involved.[13] Remarkably, following pneumococcal vaccinations we isolated *S. aureus* significantly more often from spontaneously draining ears than after control vaccines. Although the otopathogenicity of *S. aureus* is still debated, our study results point to an effect of pneumococcal vaccination on the growth conditions of this bacterium. Bogaert *et al.* made a similar observation in their nasopharyngeal carriage study in 3,200 healthy Dutch children aged 1-19 years; in children aged 4-9 years carriage of *S. pneumoniae* and *S. aureus* were found to be inversely related.[15] This phenomenon merits further investigation in future studies of pneumococcal vaccination.

3. *How to explain the different impact of pneumococcal vaccination on the occurrence of pneumococcal AOM and invasive disease?*

With respect to AOM, the othopathogenic capacities of the replacing non-conjugate vaccine type pneumococci have been established in the two trials of PCV7 conducted in infants in Finland; both studies have shown a reduction of AOM episodes caused by vaccine type pneumococci, but this reduction was accompanied by an increase of AOM due to non-vaccine type pneumococci by 27-33%.[2,13] Irrespective of serotype, it was found that pneumococci may cause AOM at approximately the same frequency as that in which they

are present in the nasopharynges of the children.[2,16] This is in contrast to the effects of pneumococcal conjugate vaccination on invasive pneumococcal disease; up until 2002, 2 years after distribution of Prevnar® in the USA, only a small increase in invasive disease caused by non-vaccine serotypes has been observed and this increase is minimal as compared to the decrease of invasive disease caused by conjugate vaccine serotypes.[1,3] Brueggeman *et al.* [2003] have recently confirmed that invasive isolates appear to be less diverse than those colonising the nasopharynx; non-conjugate serotypes, in particular serotypes 3, 11, and 15, rarely seem to cause invasive disease in infants. Although these serotypes may lack virulence factors required for invasive disease, their capsular polysaccharides seemed to be the most important factor for their being less invasive.

In general, systemic IgG antibodies of the IgG1 isotype are effective against invasive disease with encapsulated bacteria such as *S. pneumoniae* and *Haemophilus influenzae* type B. Prevention of mucosal disease, however, may be more complex. This may be the context in which to interpret the results of the infant studies, that showed that the efficacy of PCV7 against conjugate vaccine type pneumococci to be much lower for AOM (around 57%) than for invasive disease (97%).[1,2,13] Protection at the mucosal level where secretory and direct physical defences are also operative, probably requires higher antibody concentrations and different immunoglobulin isotypes such as IgA and IgG2 than protection against invasive disease.[17]

Finally, simply the fact that the nasopharynx is directly connected to the middle ear by the eustachian tube could contribute to the higher impact of nasopharyngeal replacement on mucosal disease than on invasive disease and to a less important role for systemic IgG responses.

4. *Which pneumococcal vaccination scheme is most optimal for induction of antibodies?*

We will focus on the optimal vaccination scheme for mucosal disease, since serum IgG anti-pneumococcal antibodies above 1 mg/L obviously protect against invasive disease.[1,18] The question is how many doses of PCV7 are required to achieve optimal protection at the mucosal level. In our study population, pneumococcal vaccination was most effective against conjugate vaccine type pneumococci at the nasopharyngeal level in children aged 12-24 months, who were primed with PCV7 twice before booster vaccination with PPSV23. In older children, who were primed with PCV7 once, this efficacy was far less outspoken and no longer significantly different from controls. This age-related difference in impact of pneumococcal vaccination on carriage of conjugate vaccine type pneumococci could not be explained by differences in final serum IgG antibody concentrations, because these were roughly comparable in both age groups. Most likely, the difference is explained by the fact that the younger children had received an extra conjugate vaccination. This could have enabled them to recruit more memory B-cells at the nasopharyngeal mucosal level, resulting

in higher mucosal antibody responses and better mucosal protection upon natural challenge with conjugate vaccine type pneumococci.[14,19] Apparently, at least two immunisations with the conjugate vaccine are required to influence nasopharyngeal carriage.

Despite high IgG antibody concentrations for 6 out of 7 pneumococcal serotypes, the antibody response to pneumococcal serotype 6B remained relatively low in our study group, in particular in the youngest age group. This poor anti-6B response most likely explains why vaccination did not reduce serotype 6A carriage, since for protection against cross-reactive serotype 6A a higher anti-6B response is warranted than for protection against serotype 6B.[20] Final anti-6B concentrations below 1 mg/L in children aged 12-24 months, however, were sufficient for a reduction in carriage of serotype 6B by 61%. For adequate antibody induction in children at risk for pneumococcal infections, in particular for those aged 12-24 months, a vaccination scheme with at least 3 doses of PCV7 could be considered in order to obtain higher antibody concentrations for frequent pathogens like serotypes 6B and 6A.

This study does not establish the role of the polysaccharide vaccine in mucosal disease. Booster vaccination with PPSV23 failed to prevent the emergence of the non-conjugate serotypes 11 and 15 at the nasopharyngeal level, even though these serotypes were included in PPSV23 and sufficiently high IgG antibody responses against these serotypes were obtained by vaccination. Without priming by conjugate vaccines these antibodies may be only marginally functionally active at the mucosal level.[21,22] Therefore, boosting with PPSV23 will most probably not broaden the coverage of pneumococcal serotypes at the mucosal site. In the most negative scenario polysaccharide boosting after priming with a conjugate vaccine might even frustrate future antibody responses on subsequent natural challenge with polysaccharides.[23,24] Therefore, with respect to prevention not only of mucosal infections but also of pneumonia, booster vaccination with polysaccharide vaccine should be reconsidered. Furthermore, several aspects of a polysaccharide booster, in particular its effects on immunological memory, should be further clarified through experimental research.

Recommendations for pneumococcal vaccination against AOM

Based on results of previous studies of PCV7 in healthy infants and our study in children with a history of recurrent AOM, we conclude that to prevent pneumococcal AOM in general and to protect children from developing recurrent AOM it is crucial to start pneumococcal conjugate vaccinations early in life, at least before 12 months of age, and preferably before the first AOM episode has occurred. Contrary to the recommendations made by the American Academy of Pediatrics and Advisory Committee on Immunization

Practices,[25,26] there is no evidence for efficacy of PCV7 in the management of AOM episodes in children older than 12 months of age with a history of recurrent AOM. For optimal antibody induction and impact on the nasopharyngeal carriage of conjugate vaccine type *S. pneumoniae* we recommend that children aged 12-24 months be immunised with at least 3 doses of PCV7, and older children with at least two doses. The role of a PPSV23 booster is still debatable and seems not indicated for prevention of mucosal infections.

What to expect from experimental strategies for prevention of AOM in future?

Currently, 9-valent (heptavalent plus types 1 and 5), and 11-valent (9-valent plus types 3 and 7V) conjugate vaccines have been studied or are underway in phase 3 trials in children.[18,27-30] These vaccines have primarily been developed to increase coverage against invasive disease in particular in developing countries. However, because they do not cover the majority of potentially replacing non-conjugate vaccine serotypes at the mucosal level it is doubtful whether these vaccines will increase efficacy against AOM in infants.

In the future, pneumococcal protein vaccines could offer an alternative approach in the prevention of mucosal disease. These proteins are common in most of the pneumococcal serotypes and have been identified as virulence factors. Since these proteins belong to the T-cell dependent antigens, they are also potentially effectively immunogenic at infant age. In animal studies immunisation with pneumococcal surface protein A has been shown to be effective against AOM, while pneumococcal surface adhesion A protein protects against pneumococcal nasopharyngeal carriage.[31,32] To this point, human studies with protein vaccines have not been initiated. Inclusion of these proteins in conjugate vaccines might enhance their efficacy against AOM by providing coverage against serotypes not included in the conjugate vaccine.

Strategies that prevent colonising nasopharyngeal bacteria to become pathogenic are attractive. In this context, viral vaccines are promising, since viral upper respiratory tract infections are known to induce bacterial AOM.[33,34] Thus far only the influenza vaccine has been evaluated in children; in three trials the incidence of AOM episodes during the influenza season was reduced by 30-36% after vaccination with inactivated or live attenuated influenza virus.[35-37] In a recent large study in infants and toddlers with a longer follow-up of one year, however, the influenza vaccine did not reduce overall AOM episodes and medical consumption related to AOM.[38] New vaccines for respiratory syncytial virus and rhinovirus, as they might also substantially reduce AOM incidence, must be evaluated. Another candidate preventive measure for AOM is the administration of xylitol, a widely used sugar substitute that inhibits growth and adherence of *S. pneumoniae*.[39,40] Although oral xylitol (chewing gum or mixture) did reduce AOM incidence by 40%, the dosing schedule (five times daily) is

unacceptable to most families.[41] Xylitol administered only during upper respiratory tract infection was ineffective in preventing AOM.[42]

Last but not least, studies have shown that both aerobic and anaerobic bacteria of the normal flora in the upper respiratory tract can hinder the growth of common AOM pathogens and the establishment of a renewed infection. With respect to the normal flora, alpha-hemolytic streptococci predominates in the healthy nasopharynx. Lower numbers of these streptococci have been found in the nasopharynx of otitis-prone children compared with non-otitis-prone children.[43] The first two attempts to recolonise the nasopharynx of these children with alpha streptococci by spray showed a reduction of AOM episodes of maximally 20% during a short follow-up of 3 to 6 months.[44,45] Lactobacilli are also used to restore the ecological equilibrium of different mucosal areas in humans. In animals, intranasally inoculated *Lactobacillus fermentum* decreased the number of *S. pneumoniae* in the respiratory tract. Most interestingly, these animals produced higher anti-pneumococcal antibodies upon challenge with *S. pneumoniae* as compared to controls, suggesting that the mucosal immune system could be involved in the protective effect.[46] In the first large trial in children aged 1-6 years attending day care centres,[47] daily consumption of milk with *Lactobacillus rhamnosus* decreased the occurrence of AOM by 21% (p=0.08). It seems worthwhile to study the effects of probiotic administration in children with recurrent AOM, not only for its efficacy against AOM, but also for its impact on nasopharyngeal carriage and its immune modulating properties.

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Samenvatting

Streptococcus pneumoniae is op dit moment de belangrijkste bacteriële verwekker van meningitis, pneumonie en otitis media acuta (OMA) bij kinderen. De laatste decennia is veel onderzoek gedaan naar de preventie van pneumokokkeninfecties door vaccinatie. De sinds de jaren tachtig beschikbare polyvalente pneumokokkenpolysaccharidevaccins blijken bij zuigelingen en peuters niet effectief vanwege een onvoldoende antistofrespons op kapselpolysacchariden op deze leeftijd. Analoog aan het *Haemophilus influenzae*-type-b (Hib)-conjugaatvaccin, waarin het kapselpolysaccharide van Hib gekoppeld is aan een dragereiwit, zijn recent ook multivalente pneumokokkenconjugaatvaccins ontwikkeld. Een conjugaatvaccin kan in tegenstelling tot een polysaccharidevaccin al vanaf de leeftijd van 2 maanden een goede antistofrespons induceren na herhaalde vaccinaties. Het 7-valente pneumokokkenconjugaatvaccin (pneumokokkenserotypen 4, 6B, 9V, 14, 18C, 19F, 23F, Prevnar®, Wyeth) heeft bij gezonde zuigelingen een effectiviteit van 95% ten aanzien van het voorkómen van invasieve infecties (meningitis, bacteriemie) veroorzaakt door deze 7 in het vaccin opgenomen pneumokokkenserotypen, mits toegediend op de zeer jonge leeftijd van 2, 4 en 6 maanden met een herhalingsvaccinatie op 12-15 maanden. Ten aanzien van het voorkómen van OMA is de effectiviteit echter slechts 6-7%. Zuigelingen met een verhoogd risico op het krijgen van oorontstekingen lijken het meest profijt te hebben van het pneumokokkenconjugaatvaccin; het aantal kinderen met 3-6 OMA-episoden per jaar verminderde met 9-23% en het aantal kinderen dat met trommelvliesbuisjes behandeld werd, nam met 20% af. Op grond van deze gegevens verkregen bij gezonde zuigelingen hebben de American Academy of Pediatrics (AAP) en de Advisory Committee on Immunization Practices (ACIP) in 2000 een advies uitgebracht om alle kinderen met recidiverende of ernstige episoden van OMA, en kinderen met trommelvliesbuisjes vanwege recidiverende otitiden, in te enten met het 7-valente pneumokokkenconjugaatvaccin. Binnen onze Nederlandse OMAVAX-studie gaan we in op de vraag of gegevens die verkregen zijn bij een populatie van gezonde zuigelingen geëxtrapoleerd mogen worden naar oudere kinderen die al een aantal OMA-episoden hebben doorgemaakt. In dit proefschrift beschrijven wij de resultaten van vaccinatie met een 7-valent pneumokokken-conjugaatvaccin, gecombineerd met een 23-valent pneumokokkenpolysaccharidevaccin in een populatie van 383 kinderen van 1-7 jaar oud die tevoren tenminste twee OMA-episoden hadden doorgemaakt. Deze combinatie van conjugaat- en polysaccharidevaccin werd gekozen op basis van eerder onderzoek dat aantoonde dat na een eerste vaccinatie met het conjugaatvaccin, de zogenaamde boostervaccinatie met het polysaccharidevaccin vervolgens zowel bij gezonde zuigelingen als bij oudere kinderen met een verhoogd risico op OMA goede antistoftiters induceert. Tevens beschermt het 23-valente polysaccharide-vaccin kinderen in potentie tegen meer pneumokokkenserotypen dan de 7 die opgenomen zijn in het conjugaatvaccin.

In hoofdstuk 2 tonen wij aan dat een dergelijke pneumokokkenvaccinatie in onze

populatie kinderen die tevoren al meerdere OMA-episoden hadden doorgemaakt, nieuwe OMA-episoden niet voorkomt. Dit geldt zowel voor kinderen van 12-24 maanden als voor oudere kinderen. Ook wanneer kinderen met meer dan zes OMA-episoden in het jaar voorafgaande aan de studie van de analyses worden uitgesloten, verandert de uitkomst van onze studie niet. De belangrijkste verklaring voor deze klinische ineffectiviteit van pneumokokkenvaccinatie in onze groep kinderen is dat er in de nasofarynx na vaccinatie een verschuiving optreedt van pneumokokkenserotypen die in het conjugaatvaccin zijn opgenomen naar serotypen (type 11, 15 en 16) die niet in het vaccin zijn opgenomen. Het percentage kinderen dat een pneumokok draagt in de nasofarynx blijft hierdoor na vaccinatie, zowel in de pneumokokkenvaccingroep als in de controlegroep, gelijk met zo'n 50%. Deze verschuiving van typen pneumokokken na pneumokokkenvaccinatie blijkt het meest uitgesproken bij kinderen van 12-24 maanden, die tweemaal waren ingeënt met het conjugaatvaccin (hoofdstuk 3). Bij oudere kinderen, die eenmaal waren ingeënt met het conjugaatvaccin beïnvloedde de pneumokokkenvaccinaties slechts zeer beperkt het dragerschap van conjugaatvaccintypepneumokokken en niet-vaccintypen.

In hoofdstuk 4 beantwoorden wij de vraag of genetisch identieke pneumokokken spontaan of onder druk van vaccinatie kunnen veranderen van kapselserotype (de pneumokok zou dan als het ware een ander jasje aantrekken). Bij slechts drie van de 54 kinderen in onze studiepopulatie die met tussenpozen van ten minste 6 maanden drager waren van een genetisch identieke pneumokok vond een dergelijke verandering van serotype plaats. Aangezien het hier twee kinderen betrof uit de controlegroep en slechts één uit de pneumokokkenvaccingroep, konden wij geen relatie leggen tussen de pneumokokkenvaccinatie en het optreden van deze veranderingen. In hetzelfde hoofdstuk bevestigen wij de resultaten van eerdere studies die aantoonden dat pneumokokkenvaccinatie leidt tot een afname van antibioticaresistentie van pneumokokken; in onze studie daalde de 'overall' antibioticaresistentie van 17.5% naar 11.8%.

In hoofdstuk 5 beschrijven wij een nieuwe 'colony blot' methode, die het mogelijk maakt meerdere pneumokokkenserotypen te isoleren van een kweekplaat. Deze methode werd toegepast bij kinderen die voor de pneumokokkenconjugaatvaccinatie drager waren van een vaccintypepneumokok en daarna van een niet-vaccintype, om te beoordelen of een echte vervanging van pneumokokken plaatsvond of dat er sprake was van een verschuiving in dominantie van pneumokokkenstammen. Er werd bij 26 kinderen gekeken of de niet-vaccintypepneumokok die na de vaccinatie geïsoleerd werd, ook al voor de vaccinatie aanwezig was. Tevens werd gekeken of de vaccintypepneumokok die voor de vaccinatie aanwezig was ook nog gevonden kon worden na de vaccinatie. Bij het merendeel van deze kinderen bleek er sprake te zijn van een echte verschuiving, slecht zelden persisteerde er na vaccinatie een conjugaatvaccintypepneumokok.

De immunologische resultaten van ons onderzoek worden beschreven in hoofdstuk 6 en 7.

Bij aanvang van de studie, voorafgaande aan de eerste vaccinatie, vinden wij in onze groep kinderen opvallend lage serum IgG2 spiegels. Kinderen die 4 of meer OMA-episoden per jaar hebben doorgemaakt blijken verder gemiddeld lagere serum spiegels te hebben van IgA, IgM, IgG en IgG-subklassen dan kinderen met minder frequente OMA-episoden per jaar. Dit terwijl we gezien de recidiverende infecties juist meer stimulatie van het immuunsysteem zouden verwachten. Deze bevindingen zouden kunnen passen bij verlaagde antistofresponsen bij kinderen met frequent recidiverende oorontstekingen. In onze studiegroep bleken de antistofresponsen tegen Hib-conjugaat, difterie, tetanus en het mazelenvaccin echter normaal te zijn (Wiertsema 2004). Bovendien induceerde vaccinatie met het 7-valente pneumokokkenconjugaatvaccin gevolgd door het 23-valente pneumokokkenpolysaccharidevaccin adequate IgG antipneumokokkenantistoftiters tegen 6 van de 7 pneumokokkenserotypen van het conjugaatvaccin. De antistofrespons tegen pneumokokkenserotype 6B bleef echter opvallend laag, vooral bij kinderen van 12-24 maanden (< 1.0 mg/l). Dit kan wijzen op een subtiele immuunstoornis betreffende het zwak immunogene serotype 6B bij kinderen met recidiverende otitiden. Kinderen van 25-84 maanden oud met een voorgeschiedenis van 4 of meer otitiden per jaar hadden ondanks leeftijdsadequate serumtiters (met uitzondering van type 6B) gemiddeld toch lagere antipneumokokkenantistoffen dan kinderen met 2-3 otitiden per jaar, hetgeen opnieuw zou kunnen wijzen op een verminderde antistofrespons bij deze groep kinderen.

In hoofdstuk 8 beschrijven wij hoe de bevindingen van de OMAVAX-studie geïnterpreteerd kunnen worden en wat de implicaties zijn voor de klinische praktijk en toekomstig wetenschappelijk onderzoek. Het moment van vaccineren lijkt de verschillende resultaten van onze studie met het pneumokokkenconjugaatvaccin bij oudere kinderen met een verhoogd risico op OMA en die van de studies bij gezonde zuigelingen te kunnen verklaren. Wanneer kinderen vanaf de leeftijd van 2 maanden worden ingeënt dragen nog maar weinig kinderen een pneumokok in de nasofarynx en hebben zij meestal nog geen otitis doorgemaakt. Waarschijnlijk kan het conjugaatvaccin op dit moment het dragerschap van de 7 meest prevalente pneumokokkenserotypen vertragen of zelfs voorkomen. Hiermee kan ook een eerste OMA-episode door pneumokokken worden voorkómen of worden uitgesteld tot een oudere leeftijd waarop zij immunologisch en anatomisch beter in staat zijn deze pneumokokkeninfectie te klaren. Omdat het doormaken van een OMA door pneumokokken op zuigelingenleeftijd predisponeert tot het recidiveren van OMA op latere leeftijd, zou pneumokokkenvaccinatie ook op deze wijze de kans op oorontstekingen kunnen verkleinen. In onze studie induceerde pneumokokkenvaccinatie bij de jonge kinderen van 12-24 maanden op nasofaryngeaal niveau verschuiving van conjugaatvaccintypepneumokokken naar niet-vaccintypen. Omdat deze niet-vaccintypen ook OMA kunnen veroorzaken was pneumokokkenvaccinatie niet effectief ten aanzien van het voorkómen van OMA bij deze kinderen. Bij de oudere kinderen in onze studie werd het pneumokokkendragerschap veel

minder beïnvloed door pneumokokkenvaccinatie, hetgeen waarschijnlijk verklaart waarom het ook bij hun ineffectief was ten aanzien van OMA.

Tegen verwachting toonden de per-protocol analyse zelfs een hoger aantal recidieven van OMA na pneumokokkenvaccinatie dan na de controlevaccinatie. Dit zou verklaard kunnen worden door de waargenomen verschuiving van pneumokokken in de nasofarynx, aangezien een nieuw verworven pneumokok in de nasofarynx een hoger risico geeft op het ontwikkelen van OMA dan een al langer in de nasofarynx aanwezige pneumokok. Verder werd *S. aureus* significant vaker gekweekt uit oorvocht van kinderen die ingeënt waren met pneumokokkenvaccins dan van kinderen uit de controlegroep. Of *S. aureus* een echte OMA-pathogeen is staat nog ter discussie, maar deze bevinding wijst erop dat pneumokokkenvaccinatie de groei van andere bacteriën kan beïnvloeden.

Bij gezonde zuigelingen is pneumokokkenvaccinatie veel effectiever ten aanzien van het voorkómen van invasieve infecties dan van OMA. Hiervoor is een aantal verklaringen mogelijk. Ten eerste wordt ook in studies bij zuigelingen na inenting met het conjugaatvaccin een verschuiving van pneumokokkenserotypen waargenomen. De daardoor opkomende niet-vaccintypen blijken goed in staat te zijn oorontstekingen te veroorzaken, maar lijken minder geneigd te zijn tot invasiviteit. Ten tweede blijkt bescherming tegen infecties op mucosaal niveau toch complexer dan bescherming tegen invasieve ziektes. Bij zuigelingen is pneumokokkenconjugaatvaccinatie vrijwel 100% effectief ten aanzien van invasieve ziekten door vaccintypepneumokokken, terwijl deze effectiviteit ten aanzien van OMA gemiddeld 57% bedraagt. Tot slot is het waarschijnlijk dat, doordat het middenoor in directe verbinding staat met de nasofarynx via de buis van Eustachius, pneumokokkenverschuiving veel meer gevolgen heeft voor het optreden van OMA dan voor invasieve ziekten.

Als laatste overwegen wij wat het beste vaccin schema zou kunnen zijn om mucosale infecties te voorkómen. Opvallend was dat in onze studiepopulatie alleen bij kinderen van 12-24 maanden die tweemaal met het conjugaatvaccin waren ingeënt een duidelijke afname in dragerschap van conjugaatvaccintypepneumokokken werd gevonden; bij oudere kinderen die slechts eenmaal het conjugaatvaccin hadden gekregen was dit veel minder het geval. Aangezien in beide groepen kinderen de antipneumokokkenantistofresponsen vrijwel gelijk waren zou het zeer wel mogelijk kunnen zijn dat twee giften van het conjugaatvaccin meer B-geheugen cellen rekruteren dan één gift, met als gevolg een betere antistofrespons op mucosaal niveau en daarmee ook een betere bescherming tegen dragerschap van vaccintypepneumokokken. Daarom zou overwogen kunnen worden om ook oudere kinderen tweemaal het conjugaatvaccin te geven. Daarnaast vertoonden de kinderen van 12-24 maanden een opvallend lage serum respons tegen pneumokokkenserotype 6B met echter goede vermindering van nasopharyngeaal dragerschap. Om de antistofrespons tegen type 6B te verbeteren in de hoop op betere preventie van infecties zou overwogen kunnen

worden om jonge kinderen een derde dosis van het conjugaatvaccin te geven. Onderzoek moet echter aantonen of dit ook nut heeft ten aanzien van de uiteindelijke klinische effectiviteit van de vaccinaties. De discussie betreffende het geven van een polysaccharidebooster na priming met conjugaatvaccin is evenmin gesloten. Hoewel er goede serum antistoftiters worden geïnduceerd met een polysaccharidevaccinbooster lijkt er geen enkele effectiviteit ten aanzien van het pneumokokkendragerschap te bestaan. Daarmee is het nut van de polysaccharidebooster voor de preventie van mucosale infecties waarschijnlijk gering.

Op dit moment echter, zijn de optimistische verwachtingen uit de jaren negentig dat vaccinatie de oplossing zou bieden voor OMA nog niet bewaarheid. Pneumokokkenvaccins die niet zijn samengesteld uit kapselpolysacchariden maar uit eiwitten kunnen in de toekomst een oplossing bieden, ofwel als aanvulling op de polysaccharidevaccins, ofwel als vervangers. Veelbelovend zijn eiwitten als pneumococcal surface protein A, pneumococcal surface adhesin A en pneumolysine. Dierexperimentele studies hebben fraaie resultaten opgeleverd ten aanzien van invasieve en mucosale pneumokokkeninfecties en het pneumokokkendragerschap, maar op dit moment zijn nog geen humane studies gestart. Van belang is ook strategieën te ontwikkelen die voorkómen dat bacteriën in de nasofarynx zich als pathogeen gaan gedragen. Preventie van OMA blijkt mogelijk door de ertoe predisponerende virale infecties te voorkómen. In recente trials met influenzavaccins werd bij gevaccineerde kinderen gedurende het griepseizoen een relatieve afname van otitiden gevonden van 30-36%. Het suikersubstraat xylitol blijkt de groei van pneumokokken te kunnen remmen. Het profylactisch toedienen van xylitol als drank of kauwgom bij kinderen leidde tot een reductie van OMA van maximaal 40%. Aan de frequente toediening van xylitol (5x daags) kleven echter nogal wat practische bezwaren. Het toedienen van xylitol alleen tijdens bovenste luchtweginfecties, voorkomt OMA niet. Tenslotte blijkt dat het toedienen van alpha-hemolytische streptokokken en lactobacillen, die tot de normale flora van de bovenste luchtwegen behoren, middels een drank of spray, de groei van pneumokokken te kunnen remmen. De eerste studies met deze bacteriën tonen inderdaad een reductie van OMA-episoden van maximaal 20%. Het lijkt de moeite waard deze behandelingsstrategieën verder te onderzoeken.

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Een belangrijk onderdeel van het OMAVAX-onderzoek betrof het kweken en analyseren van pneumokokken. De basis van dit alles lag in het Streeklaboratorium Haarlem. Jacob Bruin en Ed IJzerman waren hierin de onmisbare schakels. Indrukwekkend was jullie inzet en belangstelling voor het OMAVAX-onderzoek. Zelfs buiten kantooruren en tijdens de weekenden konden wij bij jullie medewerkers terecht. Dank voor jullie ondersteuning. Wanneer er een pneumokok werd gekweekt was het Debby Bogaert, arts-assistent kindergeneeskunde in het Sophia Kinderziekenhuis te Rotterdam die zich vervolgens ontfermde over de collectie pneumokokken. Met veel inzet en geduld werd door haar iedere pneumokok geserotypeerd en ook moleculair geanalyseerd. Na het verbreken van de code bleek hoe belangrijk deze resultaten waren voor mijn en ook haar proefschrift.

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Curriculum Vitae

Reinier Veenhoven werd geboren op 28 april 1959 te Heemstede. In 1977 behaalde hij het gymnasiumdiploma aan het Willem de Zwijger College te Bussum. In hetzelfde jaar begon hij met de studie geneeskunde aan de Rijks Universiteit Groningen. Het doctoraal examen geneeskunde werd in 1983 afgelegd en het artsexamen in 1985. Vervolgens was hij werkzaam als arts-assistent kindergeneeskunde (AGNIO) in het Ziekenhuis de Weezenlanden te Zwolle. Van 1986 tot 1991 volgde hij de specialisatie Kindergeneeskunde in het St. Elisabeth Ziekenhuis te Tilburg (Opleider: Drs. J.A. Rammeloo) en het Wilhelmina Kinderziekenhuis te Utrecht (Opleiders: Prof.dr. J.W. Stoop en Prof.dr. J.L. Van den Brande). Na zijn registratie als kinderarts in 1991 was hij gedurende 1 jaar fellow op de afdeling Neonatologie in het Wilhelmina Kinderziekenhuis (hoofd: Dr. L.J. Gerards). Vervolgens besloot hij zijn werkzaamheden voort te zetten in een algemeen ziekenhuis. Gedurende 2 jaar heeft hij gewerkt in het Ziekenhuis Gooi-Noord te Blaricum en sedertdien vanaf 1994 in het Spaarne Ziekenhuis te Haarlem. In 1998 is in samenwerking met de afdeling Immunologie van het Wilhelmina Kinderziekenhuis (Prof.dr. E.A.M. Sanders, hoofd afdeling: Prof.dr. W. Kuis), de afdeling KNO (Dr. A.G.M. Schilder) en het laboratorium Immunologie (Dr. Ir. G.T. Rijkers) het OMAVAX-onderzoek gestart wat heeft geleid tot deze dissertatie.

List of publications

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