Clinical and experimental studies with pneumococcal and *Haemophilus influenzae* type b conjugate vaccines

Cover:

Front: Splenic localization of pneumococcal polysaccharide 19F after vaccination with pneumococcal polysaccharide (left) and pneumococcal conjugate (right). (chapter 2, figures 3a and 3b).

Back: Localization of pneumococcal polysaccharide 19F after vaccination with pneumococcal polysaccharide (left) and pneumococcal conjugate (right), with simultaneous immunohistochemical characterization of splenic cells. (chapter 2, figure 4).

ISBN: 90-393-2663-0

This study was supported by grant no. 920-02-091 from the Netherlands Organization for Scientific Research (NWO)

Clinical and experimental studies with pneumococcal and *Haemophilus influenzae* type b conjugate vaccines

Klinische en experimentele studies met pneumococcen-
en *Haemophilus influenzae* type b conjugaat vaccins

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. dr. W.H. Gispen
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen
op maandag 9 april 2001, des namiddags te 2.30 uur

door

Maria Albertina Breukels

Geboren op 11 september 1968 te Eindhoven

Promotores: Prof. dr. B.J.M. Zegers
Prof. dr. W. Timens

Co-promotores: Dr. Ir. G.T. Rijkers
Dr. E.A.M. Sanders

Afdeling Pediatrische Immunologie
Wilhelmina Kinderziekenhuis, Universitair Medisch Centrum Utrecht

Afdeling Pathologie en Laboratoriumgeneeskunde
Academisch Ziekenhuis Groningen / Rijksuniversiteit Groningen

Financial support by Wyeth Vaccines for the publication of this thesis is gratefully acknowledged.

"…you can't help respecting anybody who can spell TUESDAY, even if he doesn't spell it right; but spelling isn't everything. There are days when spelling Tuesday simply doesn't count."

The Tao of Pooh, Benjamin Hoff

Aan papa

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Chapter 1

Introduction

Partly based on:

"Responsiveness of infants to capsular polysaccharides: impact for vaccine development."
Rijkers,G.T., Sanders,E.A.M., Breukels,M.A., and Zegers,B.J.M.
Reviews in medical microbiology (1996). *7*, 3-12.

Encapsulated bacteria

Polysaccharide-encapsulated bacteria comprise a major group of human pathogens. In this thesis, encapsulated bacteria are represented by *Haemophilus influenzae* type b and *Streptococcus pneumoniae*. Another notable example is *Neisseria meningitides*. Infections with these organisms result in substantial morbidity and mortality, particularly in young children, elderly and immunocompromised patients.
Host immunity against encapsulated bacteria depends primarily on the presence of antibodies against their capsular polysaccharides. These specific antibodies confer protection against infections with the corresponding bacteria by activating phagocytosis.
In infants and young children the antibody response to capsular polysaccharides is immature, resulting in increased incidence of diseases such as pneumonia, meningitis, otitis media and other forms of bacterial disease. Polysaccharide vaccines are ineffective in this age group. Polysaccharide vaccines are also ineffective in many other patient groups with increased susceptibility to encapsulated bacteria due to insufficient antibody responses to these antigens. The antibody response to polysaccharides can be improved by coupling the polysaccharide to protein carriers. The resulting conjugate vaccines are effective in inducing antibody responses to the polysaccharide antigens and reducing the occurrence of invasive disease.

***Haemophilus influenzae* type b**

Haemophilus influenzae is among the bacteria normally found in the pharynx. Up to 80% of persons are carriers. Most people are colonized with unencapsulated strains, but in 3-5% of people the isolates have capsules [1]. *Haemophilus influenzae* is the causative agent of a spectrum of human diseases, the most serious of which is meningitis. Other invasive diseases caused by this organism include pneumonia, epiglottitis, cellulitis and septic arthritis. Nearly all invasive disease caused by *Haemophilus influenzae* is due to serotype b, one of the six (a-f) antigenically distinct capsular types [2]. Non-encapsulated variants and mutants unable to synthesize a capsule are usually not able to cause invasive disease [3;4]. These nontypable *Haemophilus influenzae* isolates are common etiologic agents in mucosal infections such as otitis media and sinusitis.

A striking epidemiologic feature of invasive *Haemophilus influenzae* type b (Hib) disease has been the age distribution. More than 90% of all infections occur in children less than 5 years of age. The peak attack rate in developed countries occurs at 6-12 months of age. Before introduction of conjugated Hib vaccines in the childhood vaccination schedules, Hib appeared to be the most common cause of bacterial meningitis among children in the United States, accounting for approximately 70% of bacterial meningitis cases among children <5 years of age [5]. The incidence of invasive disease varies between different countries and in certain populations. Populations identified to have an increased incidence of invasive disease include Alaskan Eskimos, Apaches, Navajos and African Americans [6-10]. Before introduction of vaccination against Hib, yearly an estimated number of 700 cases of invasive Hib diseases occurred in the Netherlands, 50% of which were cases of meningitis and 15-30% epiglottitis [11].

Type b *Haemophilus influenzae* strains produce the type b capsular polysaccharide, composed of repeating units of polyribosylribitol phosphate (PRP). This capsule protects Hib against engulfment and killing by phagocytic cells and complement mediated killing [12;13]. The protective action of the capsule can be overcome by antibodies that bind to it and activate complement. Both antibodies and complement deposited on the bacterial surface bind to receptors on phagocytic cells, and initiate uptake and killing of the bacteria.

Streptococcus pneumoniae

Streptococcus pneumoniae regularly colonizes the upper respiratory tract, and can be an invasive pathogen. It is the most common cause of community-acquired bacterial pneumonia and otitis media and the third common cause of meningitis [14]. Almost every clinical isolate of *S. pneumoniae* contains an external capsule; only rarely have unencapsulated strains been implicated as the cause of infection, nearly always in cases of conjunctivitis [15;16]. In recent years, the emergence of antibiotic-resistant strains of *S. pneumoniae* has made pneumococcal disease management more difficult, thereby increasing morbidity and mortality and urging development of preventive vaccination-strategies.

Many healthy individuals carry *S. pneumoniae* in their upper respiratory tract. Carriage rates are highest in infants and young children attending out-of-home care, with carriage rates

ranging from 21% in point-prevalence studies up to 65% in longitudinal studies [17-20]. Carriage rates tend to decrease with increasing age. Colonization and pneumococcal disease are season-related, and peak during winter months [21].
Transmission of antibiotic-resistant strains of pneumococci is an increasing problem. Factors associated with carriage of antibiotic resistant pneumococci include age younger than two years, frequent upper respiratory tract infections, use of prophylactic antibiotics, frequent use of antibiotics for febrile illnesses, and a history of recurrent acute otitis media or acute otitis media unresponsive to antibiotic treatment [17-20;22;23].
Infections due to pneumococci are most frequently observed in infants and elderly persons, and in patients with a variety of underlying conditions such as a poor splenic function, asplenia, congenital malformations, malignancies and various other chronic diseases [24-27]. Among children < 5 years of age, it is estimated that pneumococcal infections account for at least one million deaths annually [28]. Almost all of these childhood deaths occur in developing countries. Population-based data on invasive pneumococcal disease suggest an annual incidence of over 50 cases per 100,000 elderly adults (above the age of 65 years) [29]. *S. pneumoniae* is cultured from middle ear fluid in 18-52% of the cases of otitis media [30]. When antigen detection methods are employed, *S. pneumoniae* is found in even >60% of the cases [31;32].

The importance of the pneumococcal capsule as a virulence factor has long be known. A clear relationship was observed between capsular serotype and pneumococcal disease in humans [21;33]. Pathogenic pneumococci produce polysaccharide capsules that show considerable molecular variety: more than 90 pneumococcal serotypes have been described to date. The serotypes that are responsible for disease differ between adults and children. Serotypes 14, 6, 19, 18, 9, 23, 7, 4, 1 and 15 are, in descending order, responsible for >85% of invasive pneumococcal infections in children from developed countries. In developing countries the order is 6, 14, 8, 5, 1, 19, 9, 23, 18, 15 and 7 [34]. In adults serotypes 1, 3, 4, 7, 8, 9, 12 and 14 cause most invasive pneumococcal infections; the relative frequency of isolated pneumococcal serotypes varies per region [35;36].

Host defence against encapsulated bacteria

The key component of the host defence against encapsulated bacteria is opsonophagocytosis of the micro-organism. For this process, specific antibodies to the capsular polysaccharides, complement, and phagocytes are required. The spleen is important in the initiation of the anti-polysaccharide antibody response. Fc receptors on phagocytic cells form an essential bridge between the humoral branch and the effector cells of the immune system. These elements in host defence against encapsulated bacteria will be discussed.

The antibody response to bacterial capsular polysaccharides

Anti-polysaccharide antibodies

Specific antibodies to the capsular polysaccharides play a crucial role in the defence against encapsulated bacteria. The protective role of antibodies to the Hib polysaccharide has been shown convincingly. The inverse correlation of disease occurrence and the presence of bactericidal antibodies in the serum of subjects at different ages was shown as early as 1933 [37]. The same relationship was demonstrated later for specific anticapsular antibodies [38]. Natural antibodies specific for Hib begin to occur at approximately 2 years of age and reach adult levels by about 5 years. The increased susceptibility for invasive Hib diseases in early childhood is thus correlated with the absence of serum bactericidal anti-Hib polysaccharide antibodies. In the pre-antibiotic era it was also recognized that immune sera could be used for the prevention and treatment of pneumococcal infections [39]. Protective antibodies are directed against the capsular polysaccharide, and are therefore serotype specific.

Bacterial capsular polysaccharides

Bacterial capsular polysaccharides are linear or branched polymers composed of repeating units of two to five different saccharide moieties. The virulence of a given encapsulated bacterium depends primarily, though not exclusively, on the chemical composition and structure of the capsular polysaccharide, and not on other capsular or cell wall components. The various capsular polysaccharides are different in immunogenicity, and antibody responses are age-related. Infants respond infrequently, and with low antibody levels to the PRP polysaccharide of Hib [40]. The responses mature at the age of 18 months, but children

aged 18-24 months do not respond as well as those older than 2 years [40]. The different pneumococcal capsular polysaccharides also vary in immunogenicity. In young children, capsular polysaccharides of pneumococcal serotypes 6 and 23 are poorly immunogenic, serotypes 14 and 19F are intermediately immunogenic, and types 8, 12, 1, 7, 4, 9 as well as serotype 3 induce good antibody responses as compared to adults after immunization with pneumococcal polysaccharide vaccine [41-44].

T cell independent type 2 antigens

Capsular polysaccharides belong to the class of T cell independent type 2 (TI-2) antigens: they are able to induce antibody responses in athymic nude mice, but not in mice with an X-linked B cell maturation defect (CBA/N mice) [45]. A number of features distinguish polysaccharides from proteins, belonging to the class of T cell dependent (TD) antigens (Table 1). TI-2 antigens are high molecular weight molecules, carrying repeating epitopes. TI-2 antigens are poorly metabolized *in vivo*, and may be retained in lymphoid organs for prolonged periods. Up to months after immunization, polysaccharides can be demonstrated on marginal zone macrophages of the spleen [46]. At high doses, polysaccharides can be tolerogenic. TI-2 antigens do not induce classic immunological memory [40]. Importantly, the onset of the responsiveness to polysaccharides is relatively late. Following vaccination, affinity maturation of antibodies does not occur [47].

	TI-2	**TD**
prototype antigen	capsular polysaccharides	proteins
T cells required	no	yes
T cell regulation	yes	yes
onset of responsiveness in ontogeny	late	early
induction of memory	no	yes
isotype/idiotype restriction	yes	no

Table 1. Characteristics of T cell independent type 2 and T cell dependent antibody responses.

Compared to protein antigens, the antibody response to TI-2 polysaccharide antigens is more restricted in use of immunoglobulin variable (V) regions: following vaccination, infants and adults produce serum antibody populations derived from one to three clones (reviewed by [48]). The immunoglobulin isotypes of anti-polysaccharide antibodies are limited as well. The human antibody response upon immunization with polysaccharide involves IgM, IgG1, IgG2 and IgA isotypes. In infants and children, the IgG anti-polysaccharide antibodies

predominantly reside in the IgG1 subclass. With increasing age, a shift towards IgG2 occurs [49-52]. The seemingly superiority of IgG2 anti-pneumococcal polysaccharide antibodies compared to IgG1 antibodies remains to be explained. IgG1 is more effective in activating the classical complement pathway [53], but IgG2 seems to be superior to other IgG isotypes in activating the alternative pathway in the presence of high epitope density [54]. In phagocytic assays, both IgG1 and IgG2 antibodies are effective [55-58], but phagocytosis of *S. pneumoniae* [57;59]and group B streptococci [55] may be more dependent on IgG2 than on IgG1 type antibodies, compared to *H. influenzae* type b [56;58].
Altogether, these data indicate that IgG2 represents the main IgG isotype in conferring clinical protection against pneumococcal infection.

For better understanding it would be convenient if all bacterial capsular polysaccharides could be considered TI-2 antigens. However, only the capsular polysaccharides of *S. pneumoniae* serotype 4 [60] and 2 [61], the capsular polysaccharide PRP of *H. influenzae* type b [62] and that of type III Group B *Streptococcus* [63] formally have been proven to behave as human TI-2 antigens. For other capsular polysaccharides the relative late onset of responsiveness in ontogeny and the restriction of antibodies to the IgG2 subclass are considered sufficient for qualification as TI-2 antigen. For a number of polysaccharides, e.g. pneumococcal polysaccharide type 3, observations such as early onset of reponsiveness make it unlikely that they represent true TI-2 antigens.

Regulatory T cells

TI-2 antigens can induce an antibody response in the absence of T cells, but regulatory T cells may influence the magnitude of the response. T cells that augment the anti-polysaccharide antibody response are not antigen specific in their action and are not MHC-restricted. The *in vitro* anti-PRP B cell response can be augmented either by autologous or allogeneic T cells [62]. The T cells that augment the anti-polysaccharide B cell response are termed amplifier cells in order to distinguish them from classic MHC-restricted $CD4^+$ helper cells that participate in B cell activation by (TD) protein antigens. The amplifier activity resides in the $CD4^+$ TCR T cell population, although some $\gamma\delta$ TCR T cell lines also can augment the *in vitro* anti-polysaccharide B cell response [61]. How amplifier T cells become activated is unclear. Up till now, neither processing nor antigen presentation in the context of class II MHC molecules has been demonstrated for polysaccharides [64]. Potentially, CD1 molecules

present on dendritic cells, which recently have been shown to be able to present lipid and glycolipids [65], could present polysaccharides to the TCR.

The ontogeny of polysaccharide responsiveness and the role of complement receptor 2 (CD21) in B cell activation by polysaccharides

As mentioned above, the onset of responsiveness to polysaccharides is relatively late. This defective anti-polysaccharide response at early age probably is due to immaturity of B cells of neonates and infants: neonatal T cells are equally well capable as adult T cells in supporting an *in vitro* anti-polysaccharide antibody response of adult B cells. However, the unresponsiveness of neonatal B cells to polysaccharide could not be overcome by adult T cells. Polysaccharide-specific B cells are present at birth, and responsiveness to polysaccharides can be induced *in vitro* by treatment with 8-mercaptoguanosine [66], or *in vivo* by protein conjugated polysaccharide vaccines (see below). These data indicate that if physiological B cell activation routes are bypassed, neonatal B cells can respond to polysaccharides.

T cell dependent protein antigens are bound and internalized by the B cell antigen receptor (mIg), and re-expressed as processed peptides on class II MHC molecules. The peptide-MHC complex on B cells can activate peptide-specific T cells provided that a costimulatory signal is given to the B cell via CD40-CD40L interaction [67]. This model can not explain B cell activation by polysaccharides since neither processing nor antigen presentation in the context of class II MHC molecules has been demonstrated for polysaccharides [64].

The first step in B cell activation by polysaccharides is ligation and cross-linking of antigen receptors (mIg). Indirect evidence points towards a role for complement receptor 2 (CR2; CD21) in providing a second activation signal. CD21 is a 145 kD glycoprotein of the complement receptor family which is expressed on mature B cells and also on follicular dendritic cells (FDC) and at a low level on a subpopulation of T cells [68;69]. The highest expression of CD21 is found on B cells which reside in the splenic marginal zone (MZ) [70]. CD21 is the receptor of the complement component C3 split products iC3b, C3dg and C3d. CD21 also serves as the cellular receptor for the Epstein-Barr virus, and as an alpha-interferon receptor [71;72]. Bacterial capsular polysaccharides can activate complement via the alternative pathway of complement activation, without the need for anti-polysaccharide specific antibodies. C3 split products are generated, which become deposited on the polysaccharide. Thus complexed C3d can be bound by CD21 [73]. Complexes of polysaccharides and C3d are better *in vitro* immunogens than native polysaccharide [74].

Immunohistochemical studies have shown that 15 to 30 minutes after intravenously administration of 23-valent pneumococcal polysaccharide vaccine to rats, polysaccharides are localized on splenic marginal zone B lymphocytes. Double staining for polysaccharides and follicular dendritic cells (FDC) indicates that at 3-5 days after immunization, polysaccharides are localized on germinal center FDC [75]. *In vitro* incubation of polysaccharide on rat [75] or adult human [76] spleen cryostat sections only results in binding of polysaccharides to marginal zone B cells and germinal center FDC when the polysaccharides are preincubated in C3 containing serum. Marginal zone B cells and FDC are the cells with the highest expression of CD21. These data strongly suggest a sequence of events that start with activation of C3 and subsequent deposition of C3d on pneumococcal polysaccharides. Through binding of C3d to CD21, polysaccharides can localize on B cells in the marginal zone, where the anti-polysaccharide antibody response can be initiated.The gradual disappearance of polysaccharide from the marginal zone and the appearance of polysaccharide on germinal center FDC that is seen 3-5 days after immunization may indicate a migration of marginal zone B cells to the germinal center.

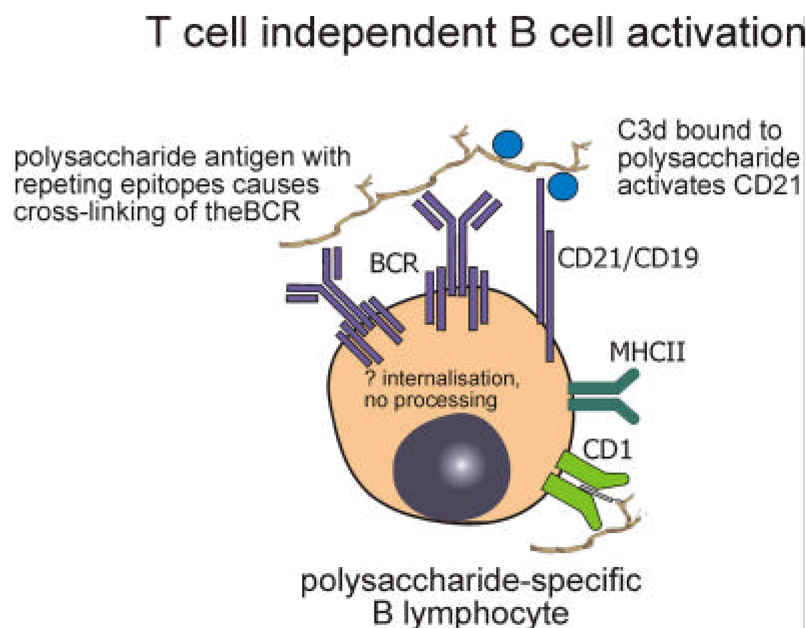

Figure 1. T cell independent B cell activation

The emerging mechanism is that C3d enables crosslinking of mIg and CD21 on polysaccharide specific B cells, and thus bypassing the need for engagement of CD40/CD40L in B cell activation (Figure 1). This mechanism also would explain why children up to the age

of 2 years do not respond to polysaccharide antigens: newborn blood B cells as well as splenic marginal zone B cells of children up to 2 years of age have a reduced expression of CD21 [70;74]. Acquisition of adult levels of CD21 expression coincides with onset of anti-polysaccharide responsiveness [70]. The importance of marginal zone B cells, complement receptors, and complement in the antibody response to a synthetic TI-2 antigen is shown directly in recent studies in $C3^{-/-}$, $CRI/II^{-/-}$, and $Pyk2^{-/-}$ mice [77].

The role of the spleen in the defence against encapsulated bacteria

For many years, the spleen has been considered as a rudimentary organ without known function. Consequently, surgical removal of the spleen because of trauma or disease was thought to have little consequences for the health of the patient (historical review by [78]). Although incidental reports mentioned a relationship between splenectomy and infection, it was not until 1952 that a causative association was reported between splenectomy and the occurrence of meningitis with sepsis. Since then the increased risk of septicaemia directly related to splenectomy has been well defined. Such infections are generally termed "overwhelming post splenectomy infections" (OPSI), and are most often caused by *S. pneumoniae* (50%), followed by *N. meningitides* (12%), *Escherichia coli* (11%), *H. influenzae* (8%) and *Staphylococcus aureus* (8%) [79-81]. The highest incidence of OPSI is generally found in infancy and childhood. Patients who have undergone splenectomy for haematologic diseases, reticuloendothelial diseases or portal hypertension have a higher incidence than those undergoing splenectomy for trauma [81-85].

The spleen consists of two general components: the white pulp (5-10%) and the red pulp (±85%).

White pulp

The white pulp is composed of three major compartments: the periarteriolar lymphocyte sheet (PALS), the lymphoid follicle (LF) and the marginal zone (MZ) [86;87]. The PALS is the T cell compartment of the white pulp, surrounding a central artery. The LF of the spleen are globular structures attached to the PALS with similar structure as LF in other lymphoid organs [88]. The primary LF consists of a homogeneous aggregate of small B cells in an inactivated state. Upon activation, the primary LF will become a secundary LF with a

germinal centre (GC) surrounded by a small rim of remaining small B cells, the lymphocyte corona (LC). The GC of the secondary LF consists of differentiated B cells, a few T cells and follicular dendritic reticulum cells. In the LF a special type of dendritic cells (follicular dendritic cells (FDC)) is found, which are able to bind immune complexes. They can maintain immune complexes on their surface for a long period without phagocytosis. GC provide a site for rapid proliferation of B cells, with immunoglobulin isotype switch and affinity maturation. The GC is surrounded by the LC, which in its turn is surrounded by the MZ. The MZ consists of medium-sized lymphocytes, mostly B cells; the area is an anatomical demarcation between the white and the red pulp. In the MZ the blood flow is low, and thus the MZ provides an environment that allows prolonged and intimate contact between antigens in the bloodstream and the lymphocytic system [86]. Because of the low flow in combination with the presence of a special type of B cells, the MZ is supposed to have an unique and important role in the primary immune response to TI-2 antigens, as is discussed above.

Red pulp

The red pulp consists of a loose reticular tissue rich in capillaries and venous sinusoids. The main function of the red pulp is blood filtration. Macrophages in the red pulp have secretory capacities. They synthesize components of the complement system, interferon, haematopoetic colony-stimulating factors and fibroblast stimulating factors. They are part of the so-called mononuclear phagocytic system (MPS).

The red pulp has a special role in phagocytosis of poorly opsonized particles. The greatly retarded blood flow in the red pulp cords allows a very intimate and prolonged contact between antigens and phagocytes. Thus, particles can be ingested without specific and/or high affinity ligand-receptor interactions. An implication of this specialized phagocytosing capacity is that the spleen is the most important site of clearance during the early phase of bacterial invasion, before sufficient amounts of specific antibodies have been produced.

The role of FcgReceptors

Effective host defence against encapsulated bacteria depends on the presence of opsonic anti-capsular polysaccharide antibodies that trigger phagocytic uptake and killing by polymorphonuclear neutrophils (PMN). Receptors for the constant region of these antibodies (FcR) play a crucial role in phagocytosis. Specific receptors have been described for most

immunoglobulin (Ig) isotypes, i.e. IgA, IgD, IgE, IgG and IgM. IgG represents the dominant antibody class in plasma, and receptors for IgG (FcγR) have been intensively studied. Within the various types of FcγR, genetic polymorphisms have been described which have an impact for susceptibility to disease.

Leucocyte FcγR belong to the Ig superfamily and fall into three main classes, with their genes located on the long arm of chromosome 1 [89]. Each class is divided in subclasses: FcγRI (CD64) in FcγRIa1, Ib1 and 2, and Ic; FcγRII (CD32) subdivided in FcγRIIa1 and 2, IIb1, 2, and 3, and IIc; and FcγRIII (CD16) in FcγRIIIa and b. These FcγR are capable of initiating a variety of cellular effector functions such as phagocytosis, antibody dependent cytotoxicity (ADCC), superoxide generation, antigen presentation, cytokine release, cell degranulation, and regulation of antibody production.

The three classes differ in their affinity for and interaction with IgG subclasses. FcγRIa is the only high affinity receptor and binds monomeric IgG1, 3 and 4. FcγRII are low affinity receptors, as is IIIb; FcγRIIIa is a medium affinity receptor. FcγRII and III interact mainly with immune-complexed IgG. For the various leucocyte FcγR genetically determined polymorphisms have been described.

In the FcγRIIa gene, a *G* to *A* single base pair substitution results in an arginine (R) or histidine (H) residue at position 131 in the membrane proximal Ig-like domain, which affects receptor affinity and specificity. FcγRIIa-H131 exhibits higher affinity for human IgG3 and IgG1 and represents the sole leucocyte FcγR capable of interaction with IgG2. PMN from FcγRIIa-R131 homozygous individuals phagocytose IgG2-opsonized particles far less efficiently than PMN from FcγRIIa-H131 homozygous individuals [58;90;91]. FcγRIIIb represents the most abundant FcR on neutrophils, and bears the neutrophil antigen (NA) polymorphism [92] with the isoforms NA1 and NA2. FcγRIIIb-NA1 exhibits a higher affinity for immune-complexed IgG3, and IgG1- and IgG3-opsonized particles are more efficiently phagocytosed via FcγRIIIb-NA1 than via IIIb-NA2 [93;94].

It is convincingly shown that the FcγRIIa class is important in phagocytosis of encapsulated bacteria [58;91]. Inefficient phagocytosis might increase the risk of infections by encapsulated bacteria. Since IgG2 is important in host defence against encapsulated bacteria, and FcγRIIa-H131 represents the sole leucocyte FcγR capable of efficient interaction with IgG2, FcγRIIa polymorphisms should influence phagocytosis mediated by antibodies of the IgG2 isotype.

Increased frequencies of meningococcal disease were indeed reported in late complement component deficient patients with the combined FcγRIIa-R/R131 -FcγRIIIb-NA2/2 genotype [95]; and the FcγRIIa-R131 allotype is associated with more serious disease in these patients [96]. Skewed distribution of FcγRIIa genotypes are also found in survivors from fulminant meningococcal septic shock [97], although another study found comparable distributions of FcγRIIa, as well as FcγRIIIa and FcγRIIIb genotypes among meningococcal disease patients and controls (Van de Pol, thesis, University Utrecht). In children, the combination of the FcγRIIa-R131 allele with low IgG2 anti-pneumococcal antibody titers seems to constribute to a higher risk for recurrence of bacterial respiratory tract infections [98].

Risk groups for infections with encapsulated bacteria

Effective removal of encapsulated bacteria by phagocytosis requires the presence of anti-capsular polysaccharide antibodies, complement, a functional spleen and phagocytes. Absence or impaired function of one or more of these elements leads to increased susceptibility to disease. Groups at increased risk for infections with encapsulated bacteria because of defective anti-polysaccharide antibodies are the following:

Infants and young children

As already discussed above, young children up to the age of 18-24 months have a deficient anti-polysaccharide antibody response possibly related to reduced expression of CD21 on marginal zone B cells. This unresponsiveness to polysaccharides results in a high susceptibility for infections with encapsulated bacteria as soon as maternally derived antibodies diminish.

Elderly

Adults above 65 years of age generally are more susceptible to infections than younger adults. It is unclear whether the immune changes associated with aging are sufficient in themselves to explain the higher susceptibility to infections, or whether age-related disease and its impact on host resistance are more important in this process [99]. Elderly are, next to infants and young children, an important group at increased risk for pneumococcal infections. Retrospective studies have shown some clinical effectiveness [100] and cost-effectiveness [101] of

vaccination with pneumococcal polysaccharides in preventing invasive pneumococcal disease in otherwise healthy elderly, but efficacy in high-risk elderly is poor. For preventing pneumococcal pneumonia, prospective studies have been disappointing [102] or inconclusive [100]. It is questionable whether limited protective efficacy of vaccination is due to an inadequate antibody response [103-105].

Selective IgA deficiency

Selective IgA deficiency, defined as an isolated absence or near absence (<0.02 g/l) of serum and secretory IgA, is the most common immunodeficiency order, with an estimated frequency of 1:700 in the Caucasian polpulation [106]. The clinical expression varies widely: both healthy individuals and patients with serious recurrent infections may present with IgA defciency. A significant proportion of patients show aberrant anti-polysaccharide antibody responses. Although total serum IgG2 levels may be normal, antibodies are preferentially expressed in the IgG1 and IgG3 subclasses, whereas in healthy individuals anti-polysaccharide antibodies reside predominantly in the IgG2 subclass [107-109]. IgA deficient patients with frequent pneumococcal infections failed to produce IgG2-type anti-polysaccharide antibodies after vaccination [110;111]. The basic defect leading to IgA deficiency is unknown. IgA deficiency and common variable immunodeficiency may be based on the same underlying genetic defect. The susceptibility for these two defects may reside in the MHC class III region on chromosome 6 [112].

IgG2 deficiency and other subclass deficiencies

Some individuals have partial or total deficiencies of one or more subclasses of IgG -defined as serum levels below 2 SD of the age-adjusted mean- despite normal or even elevated total serum IgG concentrations. Since the initial description of selective IgG subclass deficiency [113], many reports have described IgG subclass deficiencies in patients with recurrent respiratory tract infections [50;114;115]. Most often these reports concern correlations between susceptibility to infections and IgG2 deficiency, but in adults also an isolated IgG1 deficiency can be found [116;117]. In literature, 35-40% of children who developed invasive Hib disease after a single dose of *conjugated* Hib vaccine at the age of >15 months were described to have subnormal concentrations of serum immunoglobulins. Specifically, a high prevalence of subnormal concentrations of IgG2 was observed [118;119]. In a recent large series of Hib *conjugate* vaccine failures, an immunological deficiency, most frequently a deficiency of IgG2, was detected in 30% of children who were vaccinated before 12 months of age. Those

children with IgG2 deficiency presented at younger age, and had lower convalescent-phase anti-Hib antibody levels than those with normal IgG2 concentrations [7]. Reports on Hib *polysaccharide* vaccine failures showed that almost all of these children had normal serum concentrations of immunoglobulins, including IgG2 [119;120]. Since human antibodies to polysaccharide antigens reside predominantly in the IgG2 subclass, it is suggested that susceptibility to infections in patients with low levels of IgG2 is caused by a specific deficiency in the antibody response to polysaccharides. However, it became evident that no strict causal relationship exists between low serum IgG2 and susceptibility to infections with encapsulated bacteria; and that other IgG subclasses, particularly IgG1, contribute significantly to the antibody response to several polysaccharides [49;121-124]. In fact, people who lack the IgG2 heavy chain gene but are completely healthy have been described [125;126]. Furthermore, healthy adults [127] as well as healthy children [128], both with subnormal IgG2 levels, but normal responses to PRP have been described. This is in contrast with symptomatic children with low IgG2 levels who showed aberrant responses to PRP polysaccharide vacccination [128]. It therefore appears that not the IgG subclass level as such, but rather specific antibody deficiencies to polysaccharide antigens seem to be the hallmark for increased infections with encapsulated bacteria.

Selective anti-polysaccharide antibody deficiency

Patients with frequently recurrent bacterial respiratory tract infections, especially otitis media, may have a selective anti-polysaccharide antibody deficiency, despite normal serum immunoglobulin and IgG subclass levels. Only few of these patients with selective anti-polysaccharide antibody deficiency have invasive infections [129]. They respond normally to protein antigens, but fail to mount adequate antibody responses to polysaccharides [130-136]. The unresponsiveness to polysaccharides, resembling the unresponsiveness to polysaccharides in infants, can sometimes be overcome by polysaccharide conjugate vaccines [115;129;132;137-139]. However, these patients may need repeated vaccinations, a phenomenon also observed in infants under the age of 6 months [115;137]. The fact that the unresponsiveness to polysaccharides can be overcome by vaccination with conjugate vaccines indicates that precursor B cells should be present, and that the defect of B cell maturation and/or activation can be bypassed by activation of T cells.

Complement component deficiencies

The occurrence of infections with encapsulated bacteria in patients with congenital deficiencies of complement components has been well documented [140]. A clinical picture of recurrent infections in patients with complement deficiencies is not unexpected, since the importance of complement in the opsonophagocytosis of encapsulated bacteria. The importance of C3 in the initiation of the anti-polysaccharide antibody response [73;77] is underlined by a study in a group of patients with inherited deficiencies of complement components: three of four patients with a homozygous C3 deficiency were almost totally deficient in anti-polysaccharide antibodies, whereas other deficiencies of complement components were not associated with reduced anti-polysaccharide antibody levels [141].

Functional or anatomical hypo/asplenia

The increased risk of overwhelming postsplenectomy infections (OPSI), most often caused by *S. pneumoniae*, may be explained by the special role of the spleen in host defence against encapsulated bacteria (see before). The combination of impaired initiation of antibody responses to polysaccharide antigens (which takes place in the marginal zone of the spleen) and decreased phagocytic function of the spleen may determine the susceptibility for pneumococcal sepsis in persons with anatomic or functional asplenia.

Indications for performing splenectomy are traumatic injury of the spleen, staging of Hodgkin's disease and conditions in which excessive MPS activity of the spleen results in depletion of thrombocytes (such as idiopathic thrombocytopenic purpura, ITP) or erythrocytes (such as spherocytosis). The frequency of OPSI is dependent on age and the cause of splenectomy [81-83;142-145].

Functional asplenia is most often related to sickle cell disease (SCD), but may also be found in SLE. Before the use of penicillin prophylaxis and pneumococcal polysaccharide vaccination, rates of invasive disease in children with SCD exceeded those in healthy children 20-100 fold, with the greatest risk in children younger than 5 years [146]. These high rates of invasive pneumococcal disease are also found in children with congenital asplenia [147].

The phagocytic function of the spleen can not easily be restored, but protective antibody levels may reduce the risk of infection and enable the liver to remove optimally opsonized bacteriae. When elective splenectomy is scheduled, pneumococcal vaccine should be administered at least two weeks before surgery. Persons with functional or anatomic asplenia should also be vaccinated with pneumococcal vaccine [148]. Although the overall efficacy for preventing infection caused by serotypes included in the vaccine was reported to be 77%

(95% CI, 14% to 95%) among persons with anatomic asplenia [149], results on immunogenicity in asplenic or hyposplenic patients are conflicting [150-153]. Pneumococcal conjugate vaccines may be more effective than pneumoccocal polysaccharide vaccines in all asplenic patients, not only in the children < 2 years of age [154], because antibody responses to conjugate vaccines may be less dependent on a functional spleen [155-157].
Apart from vaccination, other methods of prevention could be used. Oral penicillin is recommended for prevention of pneumococcal disease in children with functional or anatomic asplenia [158]. Autotransplantation of splenic tissue after splenectomy may restore defects in the immune response [159-165].

Wiskott-Aldrich Syndrome (WAS)

This X-linked recessive syndrome is characterized by severe trombocytopenia, atopic dermatitis, and, with age, progressive defects in both cellular and humoral immunity. Patients have an impaired humoral response to polysaccharide antigens, as evidenced by markedly diminished isohaemagglutinins, and poor or absent antibody responses to polysaccharides [166]. Consequently, they have increased susceptibility for infections with encapsulated bacteria. Later, gradual deterioration of T cell function affects the antibody response to TD antigens as well.The abnormal gene, located on the proximal arm of the X-chromosome, encodes for the WAS-protein (WASP), an important regulator of lymphocyte and platelet function [167].

Ataxia-telangiectasia

Ataxia-telangiectasia (A-T) is a complex syndrome with neurologic, imunologic, endrocrinologic, hepatic and cutaneous abnormalities. Recurrent, usually bacterial sino-pulmonary infections occur in roughly 80% of the patients. Cells from patients as well as those of heterozygous carriers have increased sensitivity to ionizing radiation, defective DNA-repair and frequent chromosomal abnormalities. The sites of chromosomal breakages may involve the genes that code for the T cell receptor and/or immunoglobulin heavy chains, probably accounting for the combined T- and B cell abnormalities. The most frequent humoral abnormality is selective IgA deficiency; IgG2 or total IgG may also be decreased. Anti-polysaccharide antibody production has been shown to be impaired [168]. The gene responsible for the clinical and cellular phenotype is the ATM gene [169;170].

Other risk groups

The risk for infections with encapsulated bacteria will also be high for persons with a decreasing function of the immune system, including deterioration of the responsiveness to polysaccharide antigens, or for patients with an increased rate of decline in serum antibody concentrations as a result of: a) acquired immunodeficiency as e.g. infection with immunodeficiency virus (HIV) [171], and conditions as leukemia, lymphoma, multiple myeloma, or Hodgkins disease [172;173]; b) organ or bone marrow transplantation; c) therapy with alkylating agents, antimetabolites, or systemic corticosteroids [174]; or d) chronic renal failure or nephrotic syndrome [175;176].

Prevention of disease caused by encapsulated bacteria by vaccination

The aim of vaccination against encapsulated bacteria is to prevent disease caused by these bacteria. Since antibodies to their capsular polysaccharides play a crucial role in the defence, induction of protective levels of these antibodies should be achieved.

Polysaccharide vaccines

Pneumococcal, meningococcal and *H. influenzae* type b polysaccharide vaccines can effectively induce anti-polysaccharide antibodies, and confer protection to disease in those individuals able to respond to polysaccharide antigens [38;177-183].

Prevention of pneumococcal pneumonia by vaccination with heat-killed bacteria has been undertaken initially by Wright in goldminers in South Africa [184], resulting in a 50% reduction in mortality. In the period following, capsular polysaccharide vaccines have been developed [39], resulting in the 23-valent PS vaccine that is licensed for use in persons above 2 years of age since 1983. This pneumococcal polysaccharide vaccine contains 25 μg of purified 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. Based on US surveillance data on respiratory tract and invasive pneumococcal isolates, these 23 capsular polysaccharide antigens provide potential serotype-specific protection against at least 75% of pneumococcal infections and potential serogroup cross-protection against an additional 14%

of isolates. Thus, these vaccines provide potential protection against 85% to 90% of invasive and respiratory infections caused by pneumococci [34;185].
Most pneumococcal polysaccharide-based vaccines induce protective immunity in adults and children above the age of 2 years. The antibody response to most pneumococcal serotypes is poor or inconsistent in children less than 2 years of age. Age-specific immune responses vary by serotype, and the response to some common ‘pediatric’ pneumococcal serotypes (e.g. 6A and 14) also is decreased in children aged 2-5 years [42;44;186].
Anti-pneumococcal antibody responses do occur in the elderly, in persons who have alcoholic liver cirrhosis, COPD, and insulin-dependent diabetes mellitus [100;105;175], although responses may be lower than those obtained after polysaccharide vaccination of young adults. Immunocompromised patients, and those with a variety of clinical conditions, form a group at increased risk of disease and most in need of protection. However, these patients often fail to mount an adequate antibody response upon polysaccharide vaccination.
Despite suboptimal immunogenicity, effectiveness of pneumococcal polysaccharide vaccines in protecting against invasive disease generally has ranged from 56%-81% in case-control studies [149;187] in groups for which pneumococcal polysaccharide vaccination is currently recommended in the USA [148]. Efficacy may be lower in high-risk, immunocompromised populations [188].

Consideration of active immunization against Hib has been prompted by the frequency of diseases produced by this pathogen, the high incidence of morbidity associated with Hib meningitis [189], the significant mortality despite the availability of effective antibiotics [190] and the emergence of resistence to ampicillin, the usual antibiotic of choice for therapy of Hib infections [191]. *H. influenzae* type b polysaccharide vaccine was found to be 80% effective in preventing invasive type b disease in children 24-35 months old, but it was ineffective in children < 18 months of age, the group most susceptible to *H. influenzae* type b disease. Efficacy was uncertain in children 18-23 months of age [38;182].

Polysaccharide protein conjugate vaccines

Vaccination with purified capsular polysaccharides has been only partially successfull [192], since they do not reliably induce protective antibody responses in children younger than 2 years, the age group that shows the highest incidence of invasive infections with encapsulated

bacteria. In addition, polysaccharide vaccine appears to confer only limited protection to patients with underlying illnesses such as immunodeficiencies and hematologic malignancies, and may not be effective against poorly immunogenic capsular types.
The immunogenicity of polysaccharides can be increased by covalent linking to a carrier protein [193]. This approach is thought to work through recruitment of T cell help. The exact mechanism of recruitment of T helper cells, and the specificity of these cells, remains a matter of debate. The prevailing model is depicted in Figure 2. B cells with a B receptor complex specific for the polysaccharide bind the covalent complex, internalize it, and process the protein part into peptides. These peptides (T cell epitopes) are then presented, in the context of class II major histocompatibility complex (MCH) molecules, to helper T cells bearing specific T cell receptors (TCR). The activated helper T cells in turn stimulate B cell proliferation and differentiation into antibody-secreting cells and/or into memory cells [194-196].

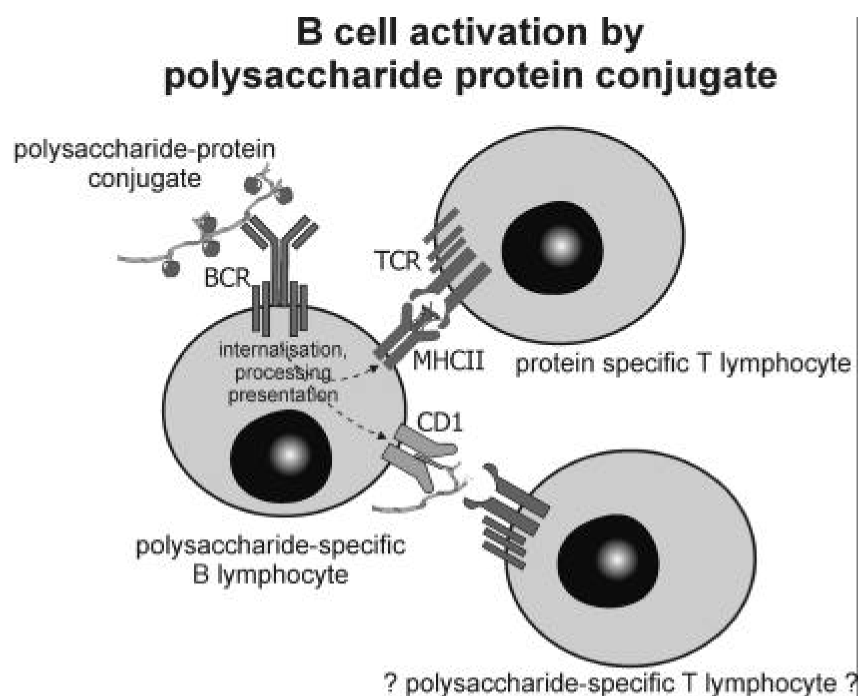

Figure 2. B cell activation by polysaccharide protein conjugate

Conjugate vaccines of capsular polysaccharides have been prepared from *H. influenzae* type b, *S. pneumoniae*, *N. meningitidis* and group B *Streptococcus* as well as from several other bacterial species [197;198]. The anti-polysaccharide antibody response to these

polysaccharide-protein conjugate vaccines bears the characteristics of a TD antibody response: onset of responsiveness early in life and induction of memory.

H. influenzae type b conjugate vaccines have been successful examples of this appoach. Conjugate vaccines, developed by coupling PRP to different carrier proteins, have proved to be immunogenic in infants, children and adults [197;199-201]. Since their introduction in childhood immunization schemes in several countries, the incidence of invasive Hib disease in these countries has dropped dramatically [202-204].
A very important finding is that immunization with Hib conjugates has effect on the oropharyngeal carriage of Hib. This is in contrast to what has been found with the PRP polysaccharide vaccine which has no effect on the carriage rate of Hib [38;205]. All available Hib conjugate vaccines have now been shown to reduce the carriage rate of Hib in a vaccinated population [205-209]. The importance of this finding lies in its implications for the transmission of the bacteria. The fewer the number of carriers, the fewer are the chances of spread of the infection. This situation could lead to herd immunity -also protecting those not vaccinated- and to eventual eradication of the disease.

Pneumococcal conjugate vaccines are developed to prevent invasive as well as mucosal infections. The initial strategy has been to design a vaccine containing about seven of the epidemiologically most important serotypes. These include types 4, 6B, 9V, 14, 18C, 19F, and 23F, which together account for 52-81 % of the pneumococci causing invasive infections in children [34], and 58% of those causing acute otitis media (AOM). In the 9-valent vaccine serotypes 1 and 5 are added, and the 11-valent vaccines includes also serotypes 3 and 7V. Addition of these 4 serotypes to the 7-valent vaccine increases the coverage to 73 to 92% of invasive infections [210]. From a manufacturing point of view, preparation of this multivalent vaccine poses formidable challenges, since at least seven separate vaccines must be produced, controlled and combined in appropriate concentrations.
Safety and immunogenicity of candidate vaccines have been demonstrated in healthy adults [211;212], and in children and infants [213-215]. A three dose series is usually administered in infants, starting at two months of age, with doses 2 months apart. Potential target groups for conjugate vaccines are, in addition to infants and young children, several risk groups where the immunogenicity of polysaccharide vaccines is less than optimal. These include various groups of immunocompromised patients as well as elderly. In these groups conjugate vaccines seem able to induce a modest or good antibody response [154;173;211;216;217].

Pneumococcal conjugate vaccine has also been demonstrated to be capable of inducing antibody responses in patients with recurrent infections who had failed to mount an adequate response to the polysaccharide vaccine [139]. In addition to immunogenicity in groups not generally responding to pneumococcal polysaccharide vaccines, another important characteristic of pneumococcal conjugate vaccine is its ability to induce immunologic memory [214;218-221].

A recent clinical trial of the heptavalent CRM197 pneumococcal conjugate vaccines shows that this vaccine is highly effective (efficacy in intention-to-treat analysis 93.9%, 95% confidence interval 82.7-99.9%) in preventing invasive disease caused by isolates of the vaccine serotypes [222]. No increase of disease caused by non-vaccine serotypes was observed. The pneumococcal conjugate vaccine had little impact on otitis media irrespective of etiology; in analysis of spontaneous draining ears, serotype-specific effectiveness was 66.7% [222]. In a Finnish trial an estimated vaccine efficacy of 57% (95% CI 44%-67%) against culture-confirmed AOM caused by vaccine serotypes was found. Efficacy against AOM irrespective of etiology was 6% (95% CI 14%-16%) [223]. In the Netherlands, a trial measuring the efficacy of the 7-valent CRM197 pneumococcal conjugate vaccine followed by booster vaccination with the 23valent polysaccharide vaccine in children with recurrent otitis media acuta (OMAVAX) is currently ongoing.
Unlike pneumococcal polysaccharide vaccines, pneumococcal conjugate vaccines affect the prevalence of nasopharyngeal carriage [224;225]: the prevalence of nasopharyngeal carriage of pneumococci of the vaccine serotypes decreases [226]. Potential positive consequences may include induction of herd immunity. A 'new' role may be to reduce carriage and spread of disease caused by antibiotic resistant pneumococci [227], since pneumococcal conjugate vaccines include most of the serotypes associated with increasing antimicrobial resistance -i.e the serotypes 6B, 9V,14, 19A, 19F and 23F [228], of which only 19A is not included in the heptavalent pneumococcal vaccine. A negative effect of widespread vaccination may be replacement of vaccine serotypes with other bacteria or non-vaccine serotype pneumococci [229].

Correlates of protection

Several approaches have been used to determine the protective concentration of anti-polysaccharide antibodies [230]. Since invasive *H. influenzae* type b disease is extremely rare in adults, and in adult sera anti-PRP concentrations are all > 0.15 μg/ml, this level is considered to be protective [231]. The anti-PRP antibody concentrations in infants offer an other possibility to estimate protective antibody concentrations. Maternally transferred antibodies decline with time. The concentration at the age when invasive *H. influenzae* disease starts to appear would indicate the level required for protection. A more direct approach is to determine the serum anti-PRP concentration of passively given antibodies which is needed for protection [131;232]. The latter type of study led to an estimated concentration of 0.05-0.15 μg/ml specific antibody needed for protection. Analysis of protection afforded by Hib polysaccharide vaccine learned that post-immunization titers of 1 μg/ml predicted protection over the following year [233].

All these estimates for protective antibody levels are based on the assumption that protection from invasive Hib disease is mediated solely by serum antibodies and the role of cell-mediated immunity is negligible. This assumption was justified since Hib polysaccharide is a T cell independent antigen. But this matter becomes quite different when the character of the polysaccharide vaccine is altered by conjugating it to a protein carrier and the immunological memory plays a role in protection. The requirement for existing antibodies for protection may be less, since the immunized infants are thought to be able to respond with a rapid and high antibody response after exposure to the bacterium, even at an age when encapsulated bacteria would normally not be able to evoke any immune response. After vaccination with Hib conjugate, high antibody concentrations have been found in sera of children colonized by *H. influenzae* type b [206]. Booster responses after Hib conjugate vaccination can even be demonstrated in infants who did not show a detectable antibody response to the first vaccination [202]. So after immunization with conjugate vaccines, protection can be seen at lower serum antibody concentrations than after polysaccharide vaccine.

The protective concentration of anti-pneumococcal polysaccharide antibodies is not known. It may be not the same for different clinical entities and/or for different pneumococcal serotypes. A protective level against invasive infections of adults, 200-300 ng ab N/ml has

been suggested [234]. Antibody concentrations > 0.15 µg/ml and >1.0 µg/ml are assumed to be required for short-term and long-term protection, respectively [235]. These antibody levels are extrapolated from studies of protection against Hib [232;233;236]. It has been shown that anti-pneumococcal polysaccharide concentrations of > 1.0 µg/ml could be protective in chinchillas [237] and mice [238]. Infant rats could be protected from bacteraemia and death by 0.1-1.15 µg/ml (depending on serotype) passively acquired anti-pneumococcal antibodies [239], and infant mice could be protected from bacteraemia by 0.05->0.4 µg/ml specific antibodies [240].

Evaluation of the immunogenicity of the newly developed pneumococcal conjugate vaccines is usually based on the measurement of serum antibody concentrations, measured by enzyme immunoassay. It needs to be emphasized that estimates for protective antibody levels are based on the assumption that protection from disease is mediated solely by serum antibodies. After immunization with pneumococcal conjugate vaccines, immunological memory plays a role in protection, and protection can be expected at lower serum antibody concentrations than after pneumococcal polysaccharide vaccination.
Furthermore, qualitive characteristics of antibodies may be at least as important as the antibody concentration [241-243]. Opsonophagocytic activity and relative avidity of anticapsular antibodies both are believed to measure their functional activity [238;240;244]. Notable increases in opsonophagocytic activity are induced by conjugate vaccines in adults and infants [245]. Different conjugates induce antibodies of different avidities [246], and avidity further increases after boosting with conjugate, but not with polysaccharide vaccine [247]. Pneumococcal conjugates thus are able to induce functionally active antibodies. An important aim of the large-scale efficacy trials is to establish laboratory surrogates of protection.

Aim and Outline of this Thesis

Capsular polysaccharides of encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae* type b are of major fundamental and applied interest. Anti-polysaccharide antibodies confer clinical protection against infections caused by encapsulated bacteria. A characteristic feature of the immune response to polysaccharides is the late onset of responsiveness in ontogeny. Infants and young children are therefore particularly at risk for infections with these bacteria, and prevention of disease by vaccination with capsular polysaccharides is ineffective in this age group. Polysaccharide vaccines are also suboptimally immunogenic in several other groups at risk for infections with encapsulated bacteria. Because of this limited use of polysaccharide vaccines, polysaccharide-protein conjugate vaccines have been developed. Clinical and epidemiological data indicate that this conjugation of polysaccharides to protein carriers results in conjugate vaccines that induce protective immunity in risk groups otherwise not covered by polysaccharide vaccines. The way the immune system handles and reacts to polysaccharide conjugate vaccines is as yet incompletely understood. This thesis applies an *in vivo* rat model to address this issue with the aim to extrapolate the results to the clinical setting.
In addition the immune response to pneumococcal and *Haemophilus influenzae* type b conjugate vaccine was studied in selected patients groups with the aim to contribute to the development of new vaccination strategies to encapsulated bacteria.

Chapter 1 reviews the importance of anti-capsular polysaccharide antibodies in host defence against encapsulated bacteria, including the immunobiology of the anti-polysaccharide antibody response. The initiation of the antibody response to polysaccharides is assumed to take place in the marginal zone of the spleen. Polysaccharides activate and bind complement component C3d, and localize on splenic marginal zone B cells, specialized cells expressing high levels of complement receptor 2 (CD21) on their surface. Activation of polysaccharide-specific B cells is achieved by signaling via CD21-C3d interaction in combination with cross-linking of B cell receptors by the polysaccharide. The mechanism of co-ligation of mIg and CD21 may account for the fact that antigen-specific T cells are not strictly required for induction of an anti-polysaccharide B cell response.
We hypothezised that the initiation of the anti-polysaccharide antibody response by conjugate vaccines could be independent of signaling via CD21-C3d interaction. We addressed this

hypothesis in **chapter 2**. In a rat model we studied the influence of complement depletion on localization of pneumococcal polysaccharide and conjugate vaccines, and on the induction of antibody responses.
Extending the hypothesis that initiation of the anti-polysaccharide antibody response might be independent of CD21-C3d interaction, we reasoned that the antibody response to conjugate vaccines might even be spleen- independent. In **chapter 3** we studied, in the same rat model, the effect of splenectomy on the antibody response to pneumococcal polysaccharide- and conjugate vaccines. In order to achieve adequate antibody responses to as many serotypes as possible –and not only to serotypes present in the conjugate vaccine-, we studied different vaccination regimes, the effect of admixture of polysaccharides with the conjugate vaccine and booster vaccination with polysaccharides after primary vaccination with the conjugate.

As mentioned earlier, the mechanism by which conjugate vaccines induce T and B lymphocyte activation, still remains a matter of debate. In **chapter 4** we investigated the cellular interactions between B and T cells which could determine the magnitude and nature of the anti-polysaccharide antibody response.

Polyvalent pneumococcal polysaccharide vaccines normally induce IgM, IgG1, IgG2 and IgA type antibodies, of which especially in adults and older children IgG2 is the predominant isotype. The inability to produce IgG2- and IgA-type anti-pneumococcal polysaccharide antibodies appears to define a clinical entity of high susceptibility for mucosal infections with bacteria like *S. pneumoniae*. Pneumococcal conjugate vaccines induce primarily an IgG1 type anti-polysaccharide antibody response. It remains to be determined whether IgG1 type antibodies only will be sufficient to prevent mucosal infections like acute otitis media. The induction of IgG2 or IgA anti-pneumococcal polysaccharide antibodies may be necessary for optimal protection against mucosal respiratory tract infections. We hypothesized that priming with a conjugate vaccine, followed by vaccination with a pneumococcal polysaccharide vaccine might induce an increased IgG antibody response and favors a shift towards an IgG2 type antibody response. In **chapter 5a** we present the results of a study in five otitis-prone children vaccinated with a pneumococcal conjugate vaccine, followed by booster vaccination with a pneumococcal polysaccharide vaccine. In **chapter 5b**, we describe antibody responses obtained with this vaccination schedule in a larger group of patients, including patients with partial IgG2 and/or IgA deficiency.

The ultimate parameter for evaluation of vaccine efficacy is the incidence of disease in the vaccinated group versus that in the nonvaccinated group. Since opsonophagocytosis is the crucial defence mechanism against encapsulated bacteria, functional antibody levels measured by phagocytosis assay employing human PMN may be used as correlates for protection. PMN constitutively express two different receptors for IgG: FcγRIIa and FcγRIIIb. These receptors show two genetically determined polymorphisms: the biallelic FcγRIIa-R131 and -H131 polymorphism, and the NA1/NA2 FcγIIIb polymorphism. In humans, only FcγRIIa-H131 can efficiently interact with IgG2. Pneumococcal conjugate vaccines induce good antibody responses, residing predominantly in the IgG1 subclass. Since only the FcγRIIa-H131 can efficiently interact with IgG2, Fcγ-receptor polymorphisms should mainly influence phagocytosis mediated by antibodies of the IgG2 isotype. Therefore, in **chapter 6** we studied whether PMN Fcγ-receptor polymorphism has an effect on in vitro phagocytosis of *S. pneumoniae* mediated by antibodies induced by a pneumococcal conjugate vaccine.

The introduction of *H. influenzae* type b conjugate vaccines in childhood vaccination programs has resulted in a dramatic reduction in invasive Hib infections. Despite this success, *H. influenzae* type b conjugate vaccine failures are occasionally reported. In **chapter 7a** we evaluated the *H. influenzae* type b conjugate vaccine failures reported in the Netherlands. In **chapter 7b** we studied whether quantitative or qualitative (avidity) defects exist in anti-*H. influenzae* type b antibodies in patients with *H. influenzae* type b conjugate vaccine failures.

Chapter 8 comprises a general discussion of the findings described in this thesis.

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Chapter 2

The immune response to pneumococcal conjugate vaccine does not depend on the presence of complement

Mijke A. Breukels[1], Ger T. Rijkers[1], Monique E. Lodewijk[2], Andre Zandvoort[2], Pieter A. Klok[2], Geert Harms[2], Wim Timens[2]

[1)]Department of Pediatric Immunology, Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands.
[2)]Department of Pathology, University Hospital Groningen, The Netherlands.

Abstract

The antibody response to polysaccharides is evoked when polysaccharides bind complement factor C3d, and these polysaccharide-C3d complexes subsequently localize on splenic marginal zone B cells strongly expressing CD21 (complement receptor 2). Infants and children under the age of two years express only low levels of CD21 on their marginal zone B cells, and consequently do not adequately respond to polysaccharides. However, polysaccharide-protein conjugate vaccines are able to induce antibodies at this young age. Conjugate vaccines apparently overcome the necessity for CD21-C3d interaction for an anti-polysaccharide antibody response.
We demonstrate in a rat model that splenic localization of pneumococcal polysaccharides indeed is complement-dependent, and that the antibody response to pneumococcal polysaccharides is impaired after complement-depletion. We show that pneumococcal conjugates are able to initiate antibody responses without depending on the presence of complement. Furthermore, polysaccharide conjugates do not specifically localize on splenic marginal zone B cells. Thus, the induction of anti-polysaccharide antibodies by conjugate vaccines apparently can occur independently of CD21-C3d interaction. These basic findings may explain their effectiveness in young children, and may open the way for application of conjugated vaccines in patient groups.

Introduction

Antibodies directed to capsular polysaccharides are essential in the defense against infections with encapsulated bacteria such as *Streptococcus pneumoniae.* In infants and young children, the antibody response to polysaccharides is inadequate, resulting in increased incidence of disease caused by encapsulated bacteria.

Polysaccharide molecules are made up of a number of repeating epitopes. As a consequence, soluble polysaccharides have the capacity to cross-link antigen receptors on the surface of B cells. Furthermore, polysaccharides can activate the complement system via the alternative pathway, without the need for anti-polysaccharide antibodies [1]. Complement fragment C3d, that is generated during this process, is bound by the polysaccharide molecules to form polysaccharide-complement complexes [2]. These polysaccharide-C3d complexes are better *in vitro* immunogens than native polysaccharides [3]. They have the ability to bind to B cells via interaction of C3d with CD21 (complement receptor 2, CR2) [4]. The co-ligation of CD21 and antigen receptors may account for the fact that the antibody response to polysaccharides (so-called T cell independent type 2 (TI-2) antigens [5]) can occur in the absence of a functional thymus, although T cells can augment antibody production [6-8].

Splenic marginal zone B cells express the CD21 surface molecule in particularly high density, and blood flow in the marginal zone is low. These two characteristics enable polysaccharide-complement complexes to localize in the marginal zone by interaction of the complement fragment C3d with CR2 on B cells [9-12], providing maximum opportunity for induction of a humoral immune response [13;14]. The marginal zone of the spleen is thus a major site of initiation of the anti-polysaccharide antibody response.

Infants and young children do not yet express high levels of CD21 on their splenic marginal zone B cells, which explains their inability to respond to polysaccharide antigens [10]. Covalent conjugation of the polysaccharides to protein carriers overcomes this anti-polysaccharide unresponsiveness in early life [15;16]. This suggests that conjugates might initiate the anti-polysaccharide antibody response in a way that is not dependent on polysaccharide-C3d complex localization on splenic marginal zone B cells by C3d-CD21 interaction.

The importance of marginal zone B cells, complement receptors, and complement in the antibody response to a synthetic TI-2 antigen is shown in recent studies in $C3^{-/-}$, $CRI/II^{-/-}$, and $Pyk2^{-/-}$ mice [17]. No such data exist for the antibody response to natural TI-2 antigens like bacterial capsular polysaccharides, nor is it known whether presence of complement and localization on marginal zone B cells is important for induction of antibody responses to polysaccharides conjugated to proteins.
We studied the effect of complement depletion on the antibody response to pneumococcal polysaccharide- and conjugate-vaccines in a previously described rat model, in which the *in vitro* complement dependency of pneumococcal polysaccharide localization on splenic marginal zone B cells was already shown [13].

Experimental Procedures

Animals, immunization, blood drawing and tissue preparation
Young adult male Wistar rats (Harlan, Horst, the Netherlands), of approximately 200 g, were housed under standard laboratory conditions on a 12 h light-dark cycle. They were fed standard laboratory rat food (Hope Farms, Inc., Woerden, the Netherlands) and tap water ad libitum. The rats were immunized intravenously (i.v.) in the tail vein with either one of the vaccines described below.

For serological studies, five rats per group were used. 1, 3, 6, 8, 14 and 21 days after intravenous immunization, blood was obtained from the tail vein, and allowed to clot at 0ºC. All rats were individually marked, so antibody titers could be analyzed separately for each rat, and thus followed over time.

For pneumococcal polysaccharide localization studies and antigen detection in serum, at least three rats per group were used. Rats were sacrificed at 15 minutes, 7 hours, and 5 days after vaccination (the timepoints were based on previous experience [13]). Blood was drawn by cardiac puncture and allowed to clot at 0ºC. Tissue blocks of spleen and liver were immediately frozen by immersion in liquid 2-methylbutane (cooled in a freezer to -80ºC). The tissue blocks were stored in a freezer at -80ºC until sectioned.

***In vivo* complement inactivation.**

Decomplementation was performed by i.v. injection of 1200 U of CVF, isolated as described [18] in 200 µl of NaCl 0.9%. CVF-treatment was given 24 and 18 hours before immunization and, when prolonged complement depletion was required -for serological studies- also 1, 3, 6 and 8 days after immunization.

Vaccines

For serological studies, the following vaccines were used:

1) A heptavalent PCV (Prevnar®; Wyeth Lederle Vaccines and Pediatrics, Rochester, NY) including polysaccharides of pneumococcal serotypes 4, 6B, 9V, 14, 19F and 23F and an oligosaccharide of serotype 18C, conjugated to CRM_{197} carrier protein. Each dose PCV contains 2 µg polysaccharides of serotypes 4, 9V, 14, 18C, 19F and 23F, and 4 µg of serotype 6B in 0.5 mL aluminum phosphate reconstitution fluid.

2) As unconjugated equivalent of this vaccine, pneumococcal polysaccharide serotypes 4, 6B, 9V, 14, 18C, 19F and 23F (PPS, American Type Culture Collection, Rockville, MD) were used. Each dose contained 20 µg of each of the pneumococcal polysaccharides type 4, 6B, 9V, 14, 18C, 19F and 23F in 0.5 mL saline.

For pneumococcal polysaccharide localization studies we used:

3) heptavalent PCV

4) an equivalent dose (2 µg per serotype) of the corresponding unconjugated polysaccharide serotypes 4, 6B, 9V, 14, 18C, 19F and 23F

5) a ten-fold higher dose of one pneumococcal polysaccharide serotype (20 µg of PS19F)

Measurement of serum complement activity

Efficiency of decomplementation was assessed by a hemolytic assay of the classical complement pathway employing sensitized sheep erythrocytes. Clotting of serum was allowed to take place at 0°C to minimize complement activation. Inactivation of complement was performed by heating rat serum at 56ºC for 30 min. 1:10 dilutions of the rat sera were incubated with sensitized sheep erythrocytes for 30 mins at 37ºC. The OD at 405 nm of the supernatant was used as a measure of complement activity. To correct for possible hemolysed serum (resulting from the blooddrawing procedure) OD values resulting from incubation of heat-inactivated serum with sensitized sheep erythrocytes were subtracted.

Immunohistochemical detection of *in vivo*-administered PPS and PCV.

Sections (4 µm) of spleen and liver tissue were prepared, air dried for 20 min, fixed for 10 min in acetone (100%), air dried, washed in PBS (pH 7.4) for 5 min, incubated with a type-specific polyclonal rabbit anti-PPS antibody (State Serum Institute, Copenhagen, Denmark) in PBS. Endogeneous peroxidase activity was blocked with 0.075% (vol/vol) H_2O_2. Sections were next incubated with peroxidase-conjugated swine anti-rabbit immunoglobulin (SAR^{PER}, Dakopatts, Glostrup, Denmark). Peroxidase activity was visualized by using 3-amino-9-ethylcarbazole plus H_2O_2 as a reagent. Finally, sections were counterstained in Mayer's hematoxylin and embedded in Kaiser's glycerin-gelatin.

Detection of *in vivo*-administered PPS and PCV simultaneously with immunohistochemical characterization of splenic cells.

For the simultaneous detection of PPS and immunohistochemical characterization of splenic cells, sections were incubated with a type-specific polyclonal rabbit anti-PPS antibody (State Serum Institute, Copenhagen, Denmark) in PBS and stained with SAR^{PER} as described above. The sections were next incubated with a given monoclonal antibody (MoAb, Table 1) and subsequently with an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Dakopatts). Alkaline phosphatase activity was visualized with naphthol AS-MX phosphate as a substrate (Sigma Chemical Co., St. Louis, MO) and fast blue BB as a chromogen; peroxidase activity was visualized by the 3-amino-9-ethylcarbazole reaction. Finally, sections were embedded in Kaiser's glycerin-gelatin.

The following MoAbs were used for immunohistochemistry: ED1 and ED3 [19](Serotec Ltd, Oxford, UK); T cell receptor (TCR) (Pharmingen, San Diego, CA, USA) and CD45R [20;21], IgM, IgD, and HIS57 [22] (Table 1).

MoAb	Clone	Reactivity	Reference
IgM	HIS40	Immunoglobulin M, B cells	[22]
IgD	MaRD3	Immunoglobulin D, B cells	[22]
CD68	ED1	Macrophages	[19]
ED3	ED3	Marginal metallophilic macrophages	[19]
TCR	R73	αβ T cell receptor	[27]
HIS57	HIS57	Marginal zone B cells	[22]
CD45R	HIS24	B cells, leukocyte common antigen	[20;21]

Table 1: Reactivity of monoclonal antibodies (MoAb) used.

Detection of pneumococcal polysaccharide serotype 19F in rat serum

10µL of rat serum samples were spotted onto nitrocellulose filters (BioRad Trans-Blot, 0.45 µm) and dried at 37°C. Pre-incubation with blocking buffer (0.1 M Tris HCl, pH 7.5; 2.5% milk powder; and Tween-20 0.05% vol/vol in 0.9% NaCl) for 30 min at room temperature was followed by incubation with rabbit anti-PPS19F (State Serum Institute, Copenhagen, Denmark) 1:10,000 in blocking buffer for 90 min at room temperature. After washing (0.1 M Tris HCl, pH 7.5; Tween-20 0.05% vol/vol in 0.9% NaCl), the nitrocellulose filters were incubated with peroxidase-labelled donkey anti-rabbit antibodies (Amersham NA934) 1:10,000 in blocking buffer for 60 min at room temperature. After treatment with ECL^{TM} (Amersham Pharmacia) for 1 min, a photofilm was developed for 1 min. 1:10 dilutions of PPS 19F (ATCC), starting concentration 10 µg/ml) in 0.9% NaCl were used as a standard. This assay has a sensitivity of detecting 0.1 µg/ml polysaccharide.

Anti-pneumococcal antibody determination by ELISA.

IgG- and IgM- antibodies against three (PS6B, PS19F and PS14) of the seven capsular polysaccharide serotypes present in both vaccines were measured by ELISA in pre- and post-immunization sera.

All serum samples were preincubated overnight with excess free common cell wall polysaccharide (CPS) to remove anti-CPS antibodies [23;24]. Microtiter plates (Greiner Labortechnik, Langerthal, Germany) were coated with pneumococcal capsular polysaccharides (ATCC, 10 µg/ml in saline solution) at 37°C, overnight. Subsequently, plates were washed with phosphate buffered saline (PBS), Tween-20 0.05% vol/vol and incubated with serial dilutions of serum samples in PBS, 0.05% Tween-20, 1% bovine serum albumin (BSA, vol/vol). After washing (PBS-0.05 % Tween-20), the plates were incubated for two hours (37°C) with peroxidase labelled goat anti-rat IgM (Santa Cruz Biotechnology, Inc.) or goat anti-rat IgG antibodies (Southern Biotechnology Associates, Birmingham, AL). After washing and incubation with enzyme substrate for 20 min at room temperature, absorbance was read at 450 nm on a Milennia ELISA reader (Flow Laboratories, Irvine, CA). The antibody concentrations in the serum samples were calculated by comparison with a rat hyperimmune serum pool generated from sera of both PCV and PPS vaccinated rats; this hyperimmune serum pool was included in every ELISA-run as a standard. The antibody concentrations of the different serotypes in this pool were assigned 100 U/ml (100%) for each serotype.

Serum samples obtained pre-vaccination and at the different time points post-vaccination from individual rats were analyzed simultaneously.

Statistical Analysis

Anti-pneumococcal antibody concentrations from rats of the different vaccine groups (having received either the non-conjugated PPS vaccine or the 7-valent PCV, with or without CVF treatment, 5 rats per group) were analyzed and compared for statistically significant differences by repeated measurement analysis using the General Linear Model procedure of the statistical package SPSS version 9.0 (SPSS Inc., Chicago, IL).

OD values as measure of serum-complement activity were compared for significant differences between treated and untreated groups of rats at different time-points after CVF-treatment by using the Mann-Whitney *U* test. P-values <0.05 were considered significant.

Results

Complement depletion by cobra venom factor

In vivo complement depletion was induced by cobra venom factor (CVF) administration. The efficiency of complement depletion was assessed by a hemolytic complement assay.

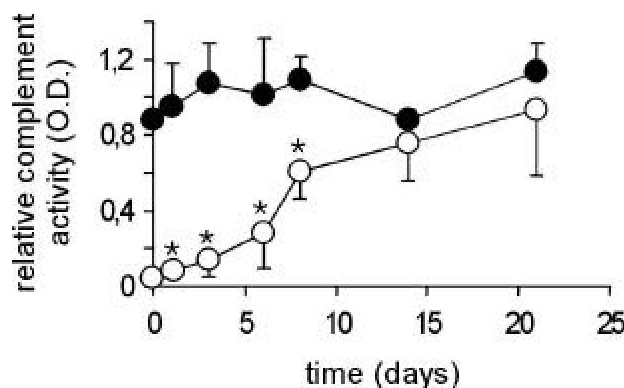


Figure 1. Complement activity in rat serum

Rats were treated with 1200 U cobra venom factor (CVF) at T= -24 h, -18 h, and T= 1, 3, 6, and 8 days. Degree of lysis of sensitized sheep erythrocytes by rat serum, expressed as OD, was used as a measure of complement-activity.

Open symbols: CVF-treated animals; closed symbols: non-treated animals. Each data-point represents the mean ± SD of five animals. Statistically significant differences are indicated (*).

The relative complement activity in serum decreased immediately after the first two injections with CVF. By a regimen of alternate day CVF injections, significantly lower complement activity as compared with control rats could be maintained for approximately 8 days. Despite ongoing treatment with CVF, complement activity in serum from CVF-treated rats was completely recovered after 14 days (Figure 1). CVF-treatment did not affect the overall histological architecture of the spleen, nor did it alter the distribution of lymphocyte and macrophage subpopulations in the spleen.

Splenic localization of pneumococcal polysaccharides

Control rats and CVF-treated rats were i.v. immunized with a mixture of serotype 4, 6B, 9V, 14, 18C, 19F and 23F pneumococcal polysaccharides (PPS). The *in vivo* splenic localization of pneumococcal polysaccharides in control rats was similar as described earlier [13]. Fifteen minutes after vaccination, PS19F was detected in the splenic marginal zone (Figure 2a). This marginal zone localization decreased in time and was followed by increased localization in the primary follicle corona (Figure 2b) at 7 hours after injection. After 5 days, polysaccharides were predominantly localized in the border of the lymphocyte corona and the germinal centers (Figure 2c).

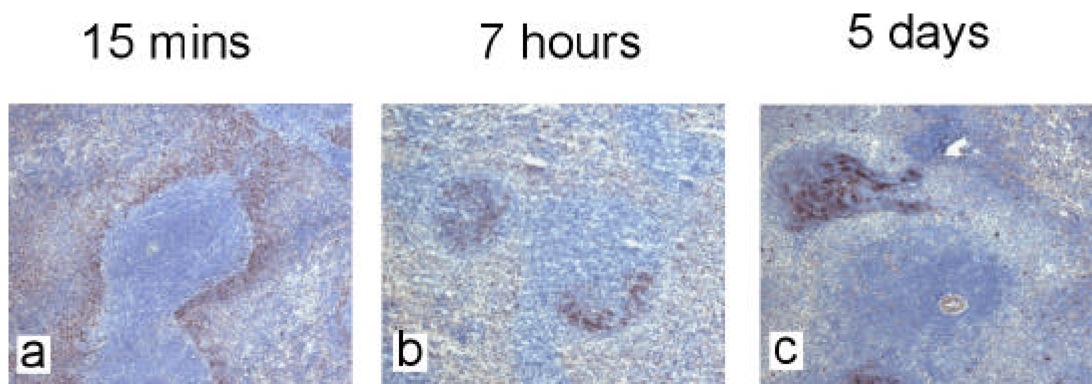
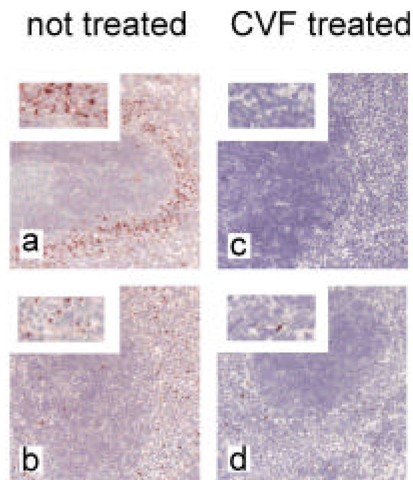


Figure 2.
Splenic localization of pneumococcal polysaccharide 19F after pneumococcal polysaccharide vaccination

2 μg of the pneumococcal polysaccharides (PPS) 4, 6B, 9V, 14, 18C, 19F and 23F were injected i.v. in control rats (panels a-c). Spleens were removed 15 minutes (a), 7 hours (b) and 5 days (c) after i.v. injection. Peumococcal polysaccharides were detected immunohistochemically on 4 μm cryostat sections, using serotype 19F-specific polyclonal rabbit antiserum followed by a peroxidase reaction (brown). Original magnification 10x.

For pneumococcal polysaccharides of serotypes 4, 6B, 9V and 23F, a similar localization pattern was observed, while PS14 did not localize in the spleen (data not shown). In

complement-depleted rats, PPS were undetectable in the spleen at all three time-points. We therefore repeated the localization experiments with a ten-fold higher dose (20 µg) PS19F (Figure 3). Even with this dose, no splenic localization was observed after CVF-treatment (Figure 3c).



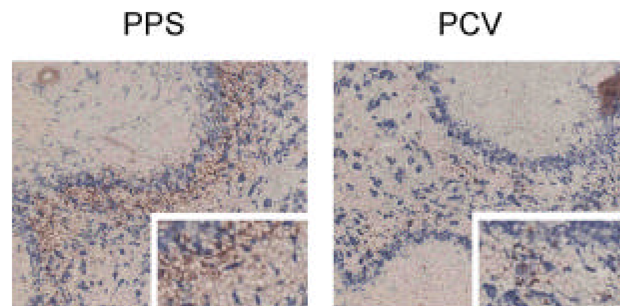


Figure 3.(left hand side)
Complement dependency of splenic localization of pneumococcal polysaccharide 19F after pneumococcal polyssacharide and conjugate vaccination

20 µg of PS 19F was injected i.v. in control (panel a) and in CVF-treated (panel c) rats. A heptavalent pneumococcal conjugate vaccine (PCV, containing 2 µg of the pneumococcal serotypes 4, 9V, 14, 18C, 19F and 23F and 4 µg of serotype 6B) was injected i.v. in control rats (panel b) and in CVF-treated rats (panel d). Spleens were removed 15 minutes after i.v. injection. Peumococcal polysaccharide was detected immunohistochemically on 4 µm cryostat sections, using serotype 19F-specific polyclonal rabbit antiserum followed by a peroxidase reaction (brown). Insets show transition from marginal zone (upper part) to germinal center (lower part). Original magnification 10x; inset 25x.

Figure 4. (right hand side)
Splenic localization of pneumococcal polysaccharide 19F after PPS and PCV vaccination, with simultaneous immunohistochemical characterization of splenic cells

15 minutes after i.v. injection of PPS (a) or PCV (b), pneumococcal polysaccharides were detected immunohistochemically, using serotype 19F-specific polyclonal rabbit anti-pneumococcal polysaccharide antibodies followed by a peroxidase reaction (brown). Splenic marginal zone macrophages are stained blue by MoAb ED3. Insets show the marginal zone. 25x original magnification, inset 40x.

In complement-sufficient rats, staining for the different polysaccharide serotypes 15 minutes after injection of pneumococcal conjugate vaccine showed a particulate polysaccharide staining pattern in the outer marginal zone (Figure 3b). After 7 hours, polysaccharides were detected in the corona; no polysaccharide was detectable after 5 days (data not shown). In complement-depleted animals, 15 minutes after PCV injection a same localization pattern (Figure 3d) of polysaccharide 19F was seen as in untreated animals, although the staining was less pronounced. In the splenic marginal zone, polysaccharides localized in association with

marginal zone B cells (Figure 4a). PCV localized partly in proximity of ED3-positive cells (Figure 4b).

Detection of pneumococcal polysaccharide 19F in rat serum

Because in decomplemented rats i.v. injected PPS does not localize in the spleen nor in the liver (data not shown), we investigated whether complement depletion resulted in prolonged circulation of PPS in blood. In serum of three complement-depleted rats, the concentration of circulating PS19F was approximately 5 times higher than in the three control rats at T=7 hours (Figure 5), indicating prolonged circulation after complement-depletion. The presence of CVF itself did not interfere with the binding of PS19F to nitrocellulose, nor did it interfere with detection by the anti-pneumococcal antiserum used (data not shown).

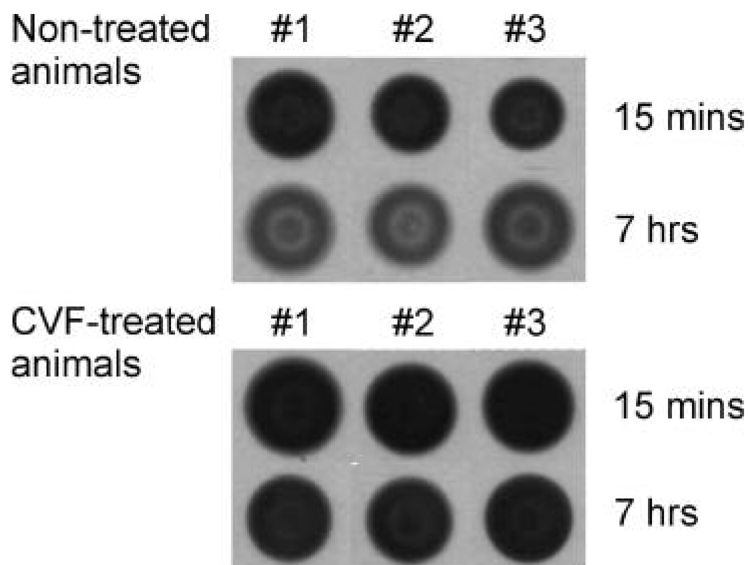


Figure 5. Detection of pneumococcal polysaccharide19F in serum

10 µl of serum drawn 15 minutes or 7 hours after i.v PPS vaccination from 3 rats treated with CVF (lower panel) and from 3 control rats (upper panel) was spotted on nitrocellulose filter. PS19F was detected using serotype 19F-specific polyclonal rabbit antiserum. After treatment with ECL™ (Amersham Pharmacia) for 1 min, a photofilm was developed for 1 min.

Anti-pneumococcal polysaccharide antibody responses.

On the base of localization data obtained earlier in this rat model (partly described in [13]), IgM and IgG antibodies against three (PS6B, PS19F and PS14) of the seven capsular polysaccharide serotypes present in both vaccines were determined.

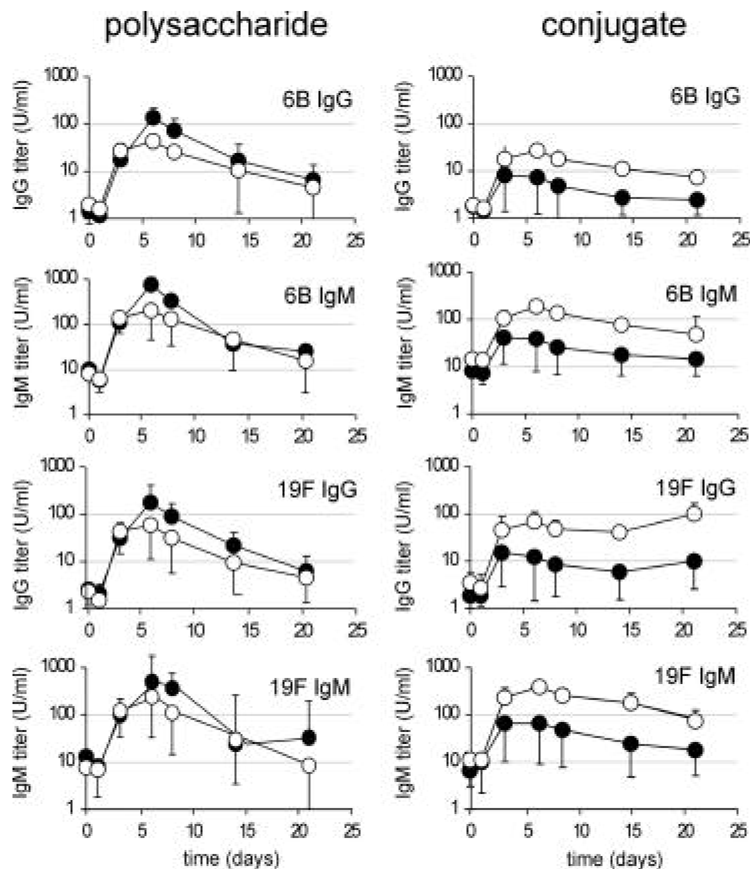


Figure 6. Serotype specific IgG and IgM anti-polysaccharide antibody response

Sera from rats immunized with PPS (lefthand panel) or PCV (righthand panel) were drawn at T= 0, 1, 3, 6, 8, 14, and 21 days after i.v. vaccination. IgG and IgM antibody concentrations against PS 6B and PS 19F were measured by ELISA. Antibody titers are expressed in U/ml. A rat hyperimmune serum pool used as a standard was assigned 100 U/ml for each serotype. Open symbols: CVF-treated animals; closed symbols: non-treated animals. Each data point represents the mean x/÷ SEM of 5 animals.

Immunization of control rats with PCV resulted in rises in IgG and IgM antibody titers for serotypes 6B and 19F, with an early peak at 4-6 days, followed by a slow decrease in antibody titers. Conjugating the poorly immunogenic PS14 to the protein carrier did not result in enhancement of immunogenicity in these control rats (data not shown).

In complement-sufficient rats, maximum antibody titers for IgG- and IgM-anti-6B and PS19F were reached 6 days after vaccination with 20 µg PPS (Figure 6). These titers dropped quickly, and were only slightly above baseline level 21 days after vaccination. After complement-depletion, antibody concentrations were significantly lower (p=0.043). Anti-PS14 antibody titers were not detectable after PPS vaccination (data not shown).

In contrast to the decreased response to polysaccharides after complement-depletion, immunization with PCV after CVF-treatment resulted in hígher IgG and IgM antibody titers than those obtained in control rats; and IgG and IgM anti-PS 14 antibodies were now detectable. Especially IgG anti-PS19F antibodies persisted longer after decomplementation. These differences did not reach significance.

Discussion

The initiation of the antibody response to polysaccharides is thought to be dependent on polysaccharide-C3d-complex localization on marginal zone B cells by interaction with complement receptor 2 (CD21). This model is based on the indirect argument that marginal zone B cells acquire high levels of CD21 only relatively late during ontogeny: a time frame that coincides with the acquisition of responsiveness to polysaccharides [10]. Direct evidence comes from the recent findings of C3 and CRI/II dependent localization of a synthetic TI-2 antigen, TNP-Ficoll, on murine marginal zone B cells [17].
In this study, we showed that antibody responses to pneumococcal polysaccharide-protein conjugates can be induced after complement-depletion. This finding is compatible with the observed immunogenicity of conjugate vaccines in infants and young children. Apparently no complement-dependent localization on marginal zone B cells is required for the initiation of an anti-polysaccharide antibody response by conjugated polysaccharides. This hypothesis is supported by our observations that conjugated polysaccharides localize in the splenic marginal zone without obvious relation to marginal zone B cells. A similar localization also is observed after complement-depletion, suggesting that it indeed is complement-independent.

The localization of unconjugated polysaccharides described in this study is consistent with data reported earlier [13]. At an early time point after immunization, specific localization is seen in the splenic marginal zone, in a staining pattern consistent with surface localization on marginal zone B cells. This supports the importance of the marginal zone B cell subset in the initial response to TI-2 antigens. In time, marginal zone localization decreases, concurrent with an increasing concentration first in the outer follicular mantle and later in a dendritic pattern in the complete follicle and follicle corona. Subsequently, in the center of the follicles, transfer of polysaccharides to the surface of follicular dendritic cells (FDC) coincides with the appearance of small germinal centers.

It has been previously shown [13], that *in vitro* localization of pneumococcal polysaccharides in the rat splenic marginal zone and follicle is complement-dependent. We are now able to extend this *in vitro* finding to the *in vivo* situation: in CVF-treated rats, i.e. in the absence of complement, no splenic localization of polysaccharides is observed. This *in vivo* finding extends the arguments derived from human *in vitro* studies [25] in favour of the current hypothesis that the initiation of an anti-polysaccharide antibody response is dependent on the presence of complement (presumably C3d) in combination with the presence of a functionally intact spleen. When these requirements are fulfilled, polysaccharide-complement complexes are formed and localize at the surface of the marginal zone B cells by interaction with the complement C3d receptor.

Several lines of evidence point towards a major role for marginal zone B cells in the immune response against TI-2 antigens [9;10]. Our data strongly indicate that the splenic marginal zone plays a major role in the immune response against native polysaccharides, but that the response to conjugated polysaccharides is not dependent on this specific B cell compartment. Consequently, an adequate response to conjugates may even be independent of the presence of a functioning spleen.

All pneumococcal polysaccharide serotypes tested localized clearly in the splenic marginal zone, except PS14. In humans, the localization patterns of all pneumococcal serotypes including serotype 14 are very similar [25]. PS14 is the only neutrally charged pneumococcal capsular polysaccharide, which may lead to trapping in extrasplenic tissue because of effective interaction with glycosyl receptors in rat [26]. It is tempting to correlate this aberrant pattern of PS14 localization with poor antibody responses to this serotype in this animal species.

We speculate that in the absence of complement, the initiation of the anti-polysaccharide antibody response is impaired because of insufficient formation of polysaccharide-C3d complexes. Consequently decreased interaction with complement receptors on splenic marginal zone B cells and thus less polysaccharide localization in the marginal zone leads to prolonged circulation of polysaccharides in blood. The higher levels of PS19F circulating in the bloodstream after CVF treatment are compatible with this hypothesis. The fact that the antibody response is not inhibited completely after CVF-treatment may be due to incomplete inactivation of complement. The interaction of C3d with the complement receptor is so

efficient that even low levels of complement still present after CVF treatment could be sufficient to induce a humoral immune response, even though splenic polysaccharide localization is no longer visible.
In the absence of complement, the antibody response to conjugated polysaccharide tends to be higher than in the presence of it. We speculate that this is due to the fact that complement components other than C3d (possibly C3b) play a role in the binding of the protein portion of the conjugate to receptors in e.g. the liver, resulting in lower levels of conjugate being available for a specific humoral immune response.

In conclusion, both our data on antigen localization as well as antibody levels show that the immune response to conjugated polysaccharides is distinctly different from that to native polysaccharides. Splenic localization of polysaccharides, and consequently the initiation of an anti-polysaccharide antibody response, is complement-dependent. The initiation of an anti-polysaccharide antibody response is impaired in absence of complement (this study, [17]) and/or B cells with adequate expression of CD21 [10;17], reflecting this reduced splenic localization. Coupling of polysaccharide to a protein carrier overcomes this C3d-CD21 dependency. These basic findings help to understand the success of conjugated vaccines in groups that are traditionally at risk for infections with encapsulated bacteria, especially infants and young children. This may open the way for application of conjugated vaccines in patient groups with inadequate anti-polysaccharide responses because of either decreased levels of complement or impaired splenic function.

Acknowledgements.

Financial support for this study was kindly given by the Groningen Foundation for Pediatric Oncology Research and the J.K. de Cock foundation.Wyeth Lederle Vaccines and Pediatrics, Rochester, NY, generously provided the heptavalent pneumococcal conjugate vaccine for these studies. Moabs CD45R, IgM, IgD, and HIS57 were a generous gift of Dr. Frans Kroese (Department of Histology and Cell Biology, University of Groningen, Groningen, The Netherlands).

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Chapter 3

Pneumococcal conjugate vaccine overcomes the splenic dependency of the antibody response to pneumococcal polysaccharides

Mijke A. Breukels[*], Andre Zandvoort[§], Germie P.J.M. van den Dobbelsteen[¶], Adrie van den Muijsenberg[*], Monique E. Lodewijk[§], Michel Beurret[¶], Pieter A. Klok[§], Wim Timens[§], Ger T. Rijkers[*]

[*]Dept. of Pediatric Immunology, Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands.

[§]Dept. of Pathology, University Hospital Groningen, Groningen, The Netherlands.

[¶]Laboratory for Vaccine Research, National Institute of Public Health and the Environment, Bilthoven, The Netherlands.

Abstract

Protection against infections with *Streptococcus pneumoniae* depends on the presence of antibodies against capsular polysaccharides that facilitate phagocytosis. Asplenic patients are at increased risk for pneumococcal infections, since both phagocytosis as well as the initiation of the antibody response to polysaccharides take place in the spleen. Therefore, vaccination with pneumococcal polysaccharide vaccines is recommended prior to splenectomy, which, as in case of trauma, is not always feasible.

We show that in rats after splenectomy vaccination with pneumococcal conjugate vaccine can induce good antibody responses, particularly after a second dose. The spleen remains necessary for a fast, primary response to (blood-borne) polysaccharides, even if presented in conjugated form. The impaired antibody response to pneumococcal polysaccharides after splenectomy can not be improved by co-administration of conjugates.

We conclude that pneumococcal conjugate vaccines can be of value in protecting asplenic or hyposplenic patients against pneumococcal infections.

Introduction

Protection against infections with encapsulated bacteria such as *Streptococcus pneumoniae* depends on the presence of antibodies against capsular polysaccharides. These serotype-specific antibodies facilitate phagocytosis, the main mode of host defence against *S. pneumoniae*. The spleen is an important organ in the immune response to *S. pneumoniae*, since it contains both antibody-producing B cells and phagocytes [1;2]. Asplenic patients are therefore at increased risk for invasive infections with *S. pneumoniae* [3]. Although most infections occur within the first few years after splenectomy, the risk of overwhelming postsplenectomy infections is lifelong [4;5]. Therefore vaccination against *S. pneumoniae* is indicated in this group. At present, the vaccine available for this aim is the 23-valent pneumococcal polysaccharide vaccine [6].
In a number of trials the immunogenicity of pneumococcal polysaccharide (PPS) vaccine in splenectomized patients has been assessed. Some studies have shown effectiveness of vaccination in inducing protective concentrations of specific antibodies [7-10], while others have shown limited serological responses [11]. As the initiation of the antibody response to polysaccharides seems to depend on the presence of splenic tissue, and in particular a functional marginal zone B-cel compartment [12-16], it may be expected that polysaccharide vaccines are of limited use in asplenic patients. In analogy with infants and young children, asplenic patients might profit from the recently developed pneumococcal conjugate vaccines.

Physical coupling of polysaccharides to carrier protein greatly improves the immunogenicity of the polysaccharide, a principle first described in 1929 [17]; and conjugated polysaccharides overcome the anti-polysaccharide unresponsiveness in early life [18;19]. This inability to generate antibodies to polysaccharide antigens is found until the age of 18-24 months, and is thought to be due to a still immature splenic marginal zone B cell compartment [19]. The induction of anti-polysaccharide antibodies by conjugate vaccines suggests that conjugates might initiate the anti-polysaccharide antibody response in absence of a functional splenic marginal zone B cell compartment, and thus be of value in the management of hypo- and asplenic patients.

To test this hypothesis of relative splenic independency of the anti-polysaccharide antibody response after conjugate vaccination, we studied the immunogenicity of a tetravalent

pneumococcal conjugate vaccine (PCV) after splenectomy. We used a rat model in which we have previously shown that splenectomy had a clearly negative effect on the anti-polysaccharide antibody response [20]. Because PCV contain only a limited number (4-11) of pneumococcal serotypes compared with the 23-valent PPS, we studied the potential benefit of admixture of additional polysaccharides with the PCV. We also studied the effects of booster-immunization.

Since the spleen is a major lymphoid organ, containing 25% of all white blood cells, mainly lymphocytes, it plays an important role in primary and secondary immune responses to all antigens, irrespective of way of presentation. Furthermore, the spleen is the only site of specific antibody production very early after exposure to blood-borne antigens. We therefore studied the effects of splenectomy on the antibody responses after immunization with PPS or PCV via two different routes, subcutaneously as well as intravenously, or only subcutaneously.

Materials and Methods

Animals, splenectomy and immunization

Young adult male Wistar rats (n=60, Harlan, Horst, the Netherlands), of approximately 200 g, were housed under standard laboratory conditions on a 12 h light-dark cycle. They were fed standard laboratory rat food (Hope Farms, Inc., Woerden, the Netherlands) and tap water ad libitum. Splenectomy was performed on 30 rats via an upper midline incision, under clean, but not sterile, conditions [21]. Rats were allowed to recover from this surgical procedure for 6 weeks. At T=0 and at T=28 days, both the splenectomized rats and control rats were immunized with one of the vaccines described below. In a first series, animals were simultaneously immunized with 0.5 ml vaccine subcutaneously (s.c) as well as 0.5 ml intravenously (i.v.) in the tail vein. In a second series of experiments the vaccine (0.5 ml) was administered s.c. only in order to study the kinetics of the antibody response and the importance of the spleen in this circumstance. Each experimental group consisted of five rats. All rats were marked individually, to allow antibody titers to be analyzed longitudinally for single rats.

At T=0, and at days 5, 28, 33 (5 days after second vaccination) and 56 (28 days after second vaccination), blood was obtained from the tail vein (timepoints were based on previous experience (Breukels et al., manuscript in preparation). Blood was allowed to clot on ice for two hours. Serum samples were stored at -20°C until analysis.

Vaccines

Pneumococcal polysaccharide serotypes 4, 6B, 9V, 14, 19F and 23F (American Type Culture Collection (ATCC), Rockville, MD) were dissolved in saline to a concentration of 5 µg/ml for each serotype. Each 0.5 ml dose of this vaccine (PPS) compares to one tenth of the human dose.

A tetravalent pneumococcal conjugate vaccine (National Institute of Public Health and the Environment (RIVM), Bilthoven, the Netherlands), consisting of 50 kD polysaccharides of pneumococcal serotypes 6B, 14, 19F and 23F conjugated to tetanus toxoid carrier protein via a cysteamine linker [22] containing aluminum phosphate as an adjuvant was used, mixed with unconjugated polysaccharide serotypes 4 and 9V(ATCC). Each 0.5 ml dose of this vaccine (PCV) contained 0.36 µg polysaccharides of the serotypes 14, 19F and 23F, and 1.08 µg of serotype 6B, and 2.5 µg of the unconjugated pneumococcal polysaccharide serotypes 4 and 9V.

Type-specific anti-pneumococcal antibody determinations.

IgG serum antibody levels against all six polysaccharides included in the two vaccines (serotypes 4, 6B, 9V, 14, 19F and 23) were measured by ELISA. All serum samples were preincubated overnight with excess free common cell wall polysaccharide (CPS) to neutralize anti-CPS antibodies [23;24].

Microtiter plates (Greiner Labortechnik, Langerthal, Germany) were coated with pneumococcal capsular polysaccharides (ATCC, 10 µg/ml in saline solution) at 37°C, overnight. Subsequently, plates were washed with phosphate buffered saline (PBS), Tween-20 0.05% (vol/vol) and incubated with serial dilutions of serum samples in PBS, 0.05% Tween-20, 1% bovine serum albumin (BSA, vol/vol). After washing (PBS-0.05 % Tween-20), the plates were incubated for two hours (37°C) with peroxidase labelled goat anti-rat IgG antibodies (Southern Biotechnology Associates, Birmingham, AL). After washing and incubation with enzyme substrate for 20 min at room temperature, absorbance was read at 450 nm on a Milennia ELISA reader (Flow Laboratories, Irvine, CA).

Serum samples obtained pre-vaccination and at the different time points after vaccination from individual rats were analyzed simultaneously. The antibody concentrations in the serum samples were calculated by comparison with a rat hyperimmune serum pool, generated from sera of rats vaccinated with a heptavalent pneumococcal conjugate vaccine (Prevnar®; Wyeth Lederle Vaccines and Pediatrics, Rochester, NY; containing the serotypes 4, 6B, 9F, 14, 18C, 19F and 23F) or a 23-valent pneumococcal polysaccharide vaccine (Pneumovax®, Merck, Westpoint, PA). This pool was included in every ELISA-run as a standard. The antibody concentrations in this hyperimmune pool were assigned 100 U/ml (100%) for each serotype. Based on comparison of OD readings, type-specific antibody concentrations in this hyperimmune standard were highest for serotypes 4, 9F, and 19F, and lower for serotypes 6B and 23F. Both the hyperimmune serum pool as well as individual post-immunization samples showed low OD readings for serotype 14. No reliable anti-PS14 antibody titers could therefore be assigned to sera after vaccination, only trends in antibody titers could be documented.

Statistical Analysis

Serological data from rats of the different vaccine groups, 5 rats per group, were analyzed and compared for statistically significant differences by using the Mann-Whitney *U* test. P-values < 0.05 were considered significant.

Results

Splenectomy impairs the antibody response to pneumococcal polysaccharides

In control rats, vaccination with the 6-valent pneumococcal polysaccharide mixture induced a clear IgG antibody response to all serotypes except serotype 14 (Figure 1, left-hand side, data for serotype 14 are not shown). Maximal geometric mean antibody titers, obtained at day 5, ranged from 21 to 57 U/ml. In splenectomized rats maximum geometric mean antibody titers, also reached at day 5, were significantly ($p<0.01$) lower, and remained <10 U/ml for the five serotypes (range 2-10 U/ml).

Revaccination of control rats with PPS -after 4 weeks- resulted in significantly lower IgG antibody titers after 5 days than after the first dose of PPS, with geometric mean serotype-

specific antibody titers ranging from 4-12 U/ml ($p<0.05$). Also in splenectomized rats, revaccination with PPS did not improve the IgG-type antibody response.

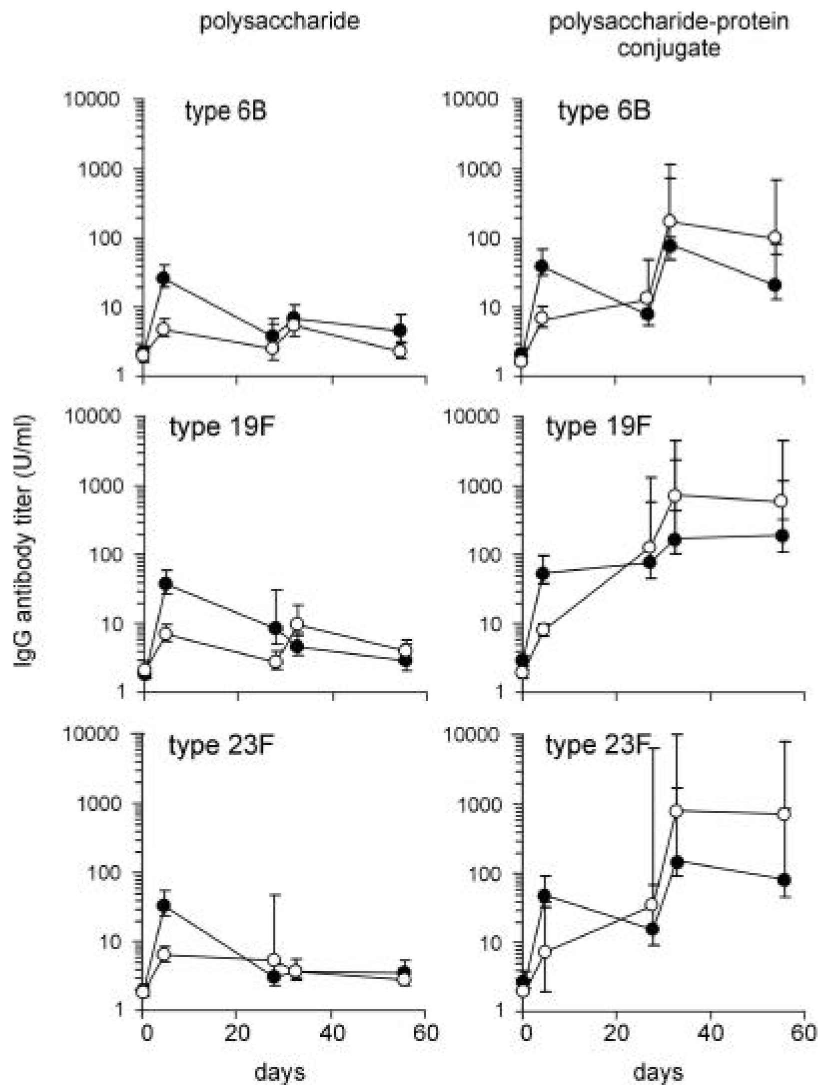

Figure 1. IgG antibody responses to vaccination with pneumococcal polysaccharides and pneumococcal polysaccharide-protein conjugates in control and splenectomized rats.

Control rats (closed symbols) and splenectomized rats (open symbols) were vaccinated i.v. and s.c. at day 0 and at day 28 with a) 2.5 µg of each of the pneumococcal polysaccharide (PPS) serotypes 4, 6B, 9V, 14, 19F and 23F or with b) a tetravalent pneumococcal conjugate vaccine (PCV) consisting of polysaccharides of pneumococcal serotypes 6B, 14, 19F and 23F conjugated to tetanus toxoid. Each 0.5 ml dose of PCV contained 0.36 µg polysaccharides of the serotypes 14, 19F and 23F, and 1.08 µg of serotype 6B. IgG anti-pneumococcal antibody titers were determined by ELISA in serum samples drawn at day 0, 5, 28, 33 and 56. Antibody titers are expressed in U/ml. A rat hyperimmune serum pool used as a standard was assigned 100 U/ml for each serotype. Shown are geometric mean antibody titers x: SEM.
Right panels show IgG antibody responses after vaccination with PPS. Antibody responses to serotypes 4 and 9V are not shown, but displayed a similar course as serotype 6B. Left panels show IgG antibody responses after vaccination with PCV.

Antibody response to pneumococcal conjugate vaccine

In control rats, the primary response after vaccination with PCV resulted in serotype-specific antibody titers at day 5 that were comparable with or slightly higher than those acquired after one dose of PPS (geometric mean antibody titers 27-83 U/ml) (Figure 1, right-hand side). Antibody titers to PS14 were also slightly higher than after vaccination with PPS (data not shown). Antibody titers reached a maximum after 5 days and remained at plateau level or decreased only slightly.

After splenectomy, IgG antibody titers at day 5 were significantly lower ($p< 0.01$) than those in controls 5 days after vaccination with PCV. However, in the period between day 5 and day 28, IgG antibody titers increased steadily and reached levels comparable with those in control rats at day 28. These antibody titers were higher than the maximal antibody titers reached by vaccination of splenectomized rats with PPS.

Revaccination with PCV 4 weeks after the first PCV dose resulted in a further increase in antibody concentration in control rats. In splenectomized rats, the increase in IgG after secondary vaccination with PCV was even more pronounced. The response to the second dose of PCV did not differ in kinetics between the control and splenectomy groups: in both groups already 5 days after vaccination high antibody titers were observed. These levels persisted until at least 28 days after the second PCV vaccination in both groups. In the splenectomy group these titers were significantly higher than maximum titers obtained after PPS vaccination ($p< 0.05$).

It thus can be concluded that a pneumococcal conjugate vaccine can overcome polysaccharide unresponsiveness in splenectomized rats.

Combined i.v. and s.c. vaccination compared with s.c. vaccination only.

S.c. injection of PPS 4 and 9V in control rats resulted in a lower antibody responses compared with combined i.v. and s.c. injection ($p< 0.05$), although still a rise in antibody titer was observed with a maximum at day 5 (range 2-13 U/ml). A second s.c dose of PPS after 4 weeks did not result in an increase in antibodies (Figure 2). S.c injection of PPS4 and 9V in splenectomized rats did but slowly induce an antibody response, reaching a maximum 5 days after a second injection.

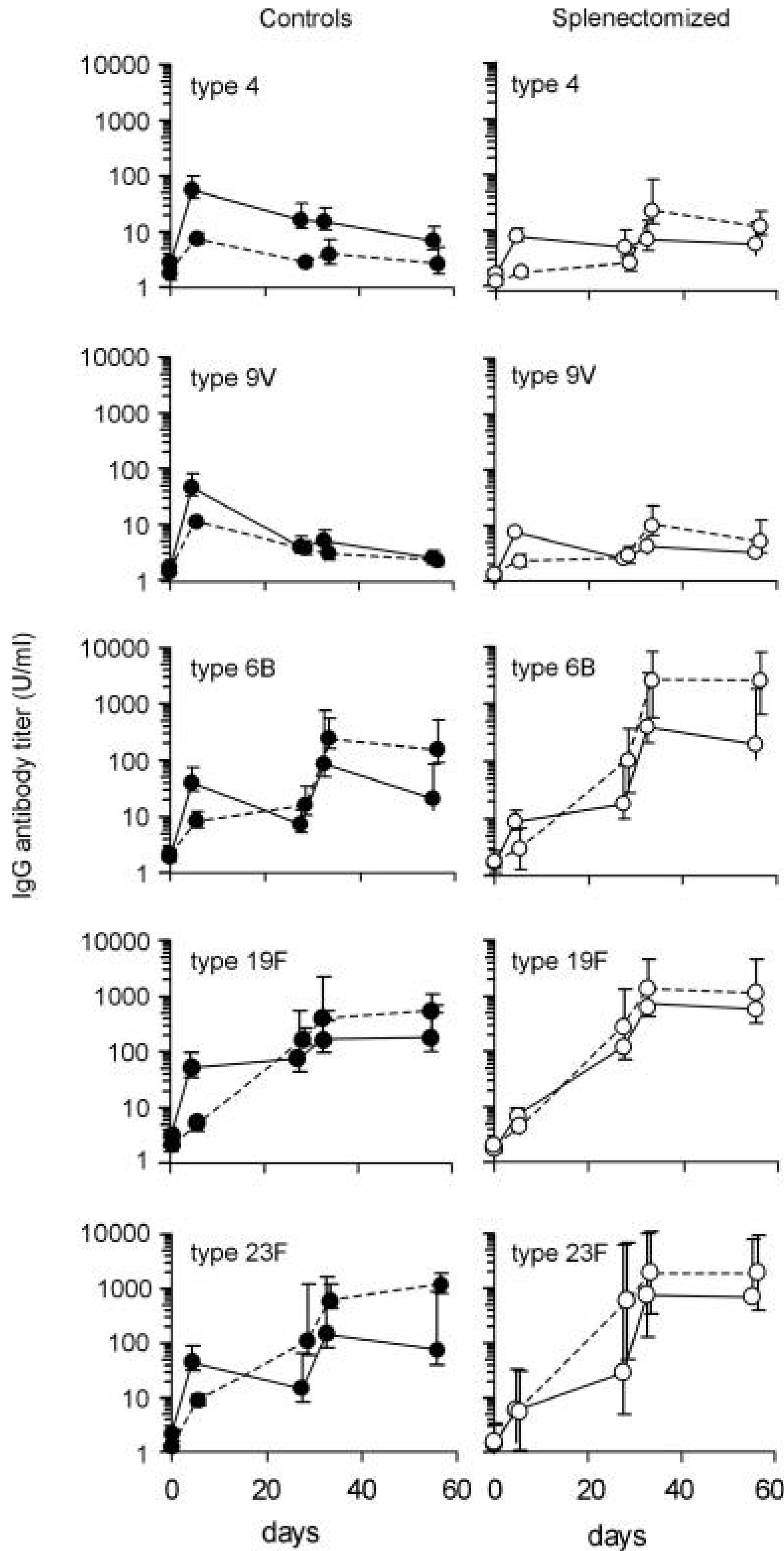

Figure 2. IgG anti-pneumococcal antibody response after subcutaneous (s.c.) vaccination compared with combined subcutaneous as well as intravenous (i.v.) vaccination with PPS or PCV.

Control rats (closed symbols) and splenectomized rats (open symbols) were vaccinated s.c. (broken lines) or i.v. as well as s.c. (drawn lines) at day 0 and at day 28 with a tetravalent (6B, 14, 19F, 23F) pneumococcal conjugate vaccine mixed with 2.5 µg of the unconjugated polysaccharide serotypes 4 and 9V. Anti-pneumococcal antibody titers were determined by ELISA. See legend Figure 1 for further details.

After s.c. injection of PCV in control rats, the specific antibody titers to serotypes 6B, 19F and 23F rose gradually over the first 4 weeks, to reach equal or even higher titers than the maximal titers achieved already at day 5 after combined i.v. and s.c injection (Figure 2).

A second dose of PCV resulted in a further rise in IgG antibody titer, which was maintained longer than after combined i.v. and s.c injection. S.c. vaccination of splenectomized rats with PCV resulted in an identical (both in magnitude and kinetics) IgG antibody response as in control rats.

Co-administration of non-conjugated polysaccharide with PCV.
Above data indicate that in splenectomized rats antibody responses to PCV were higher, and were maintained for longer periods, than antibody responses to PPS. We administered PPS together with PCV to see whether admixture of PPS with PCV would improve the antibody

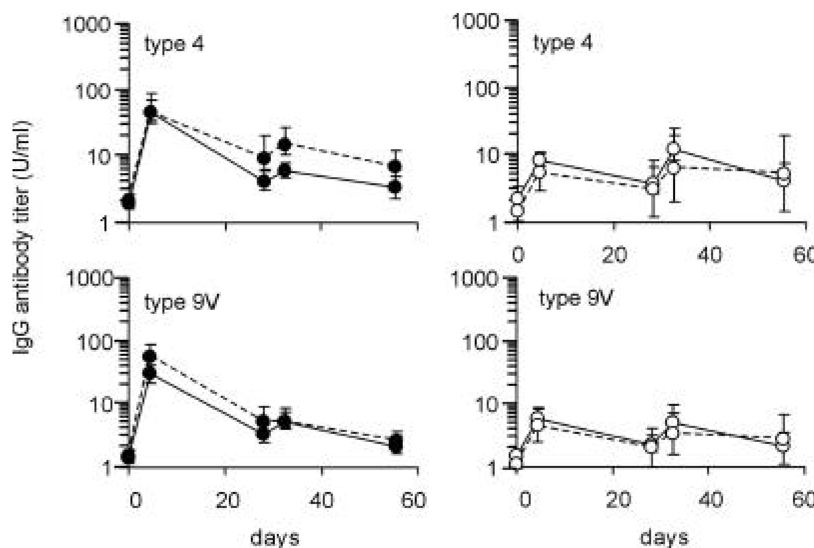

Figure 3. Antibody response to serotype 4 and 9V polysaccharides admixtured with native or conjugated polysaccharides.

Control rats (closed symbols) and splenectomized rats (open symbols) were i.v. and s.c. vaccinated on day 0 and on day 28 with 2.5 µg of the pneumococcal polysaccharide serotypes 4 and 9V mixed with conjugated (broken lines) or native polysaccharides (drawn lines) of the serotypes 6B, 14, 19F and 23F. See legend Figure 1 for further details.

response to PPS and thus increase the limited serotype coverage of PCV. In Figure 3 the anti-PPS 4 and anti-PPS 9V IgM- and IgG antibody responses are depicted. No differences in antibody responses are seen after admixture of PPS 4 and 9V with the 4-valent PCV

compared with unconjugated forms of the PPS 6B, 14, 19F and 23F; this holds true for both polysaccharides in control as well as in splenectomized rats. The poor immunogenicity of non-conjugated polysaccharides in splenectomized rats therefore can not be improved by concommitantly immunization with conjugate vaccine.

Booster vaccination with PPS or PCV after a first vaccination with PCV

In control rats, booster vaccination with PPS as well as PCV after primary PCV results in an increase in serotype-specific IgG antibody titers for all serotypes. In splenectomized rats, PCV booster vaccination clearly results in a better anti-polysaccharide antibody response as PPS.

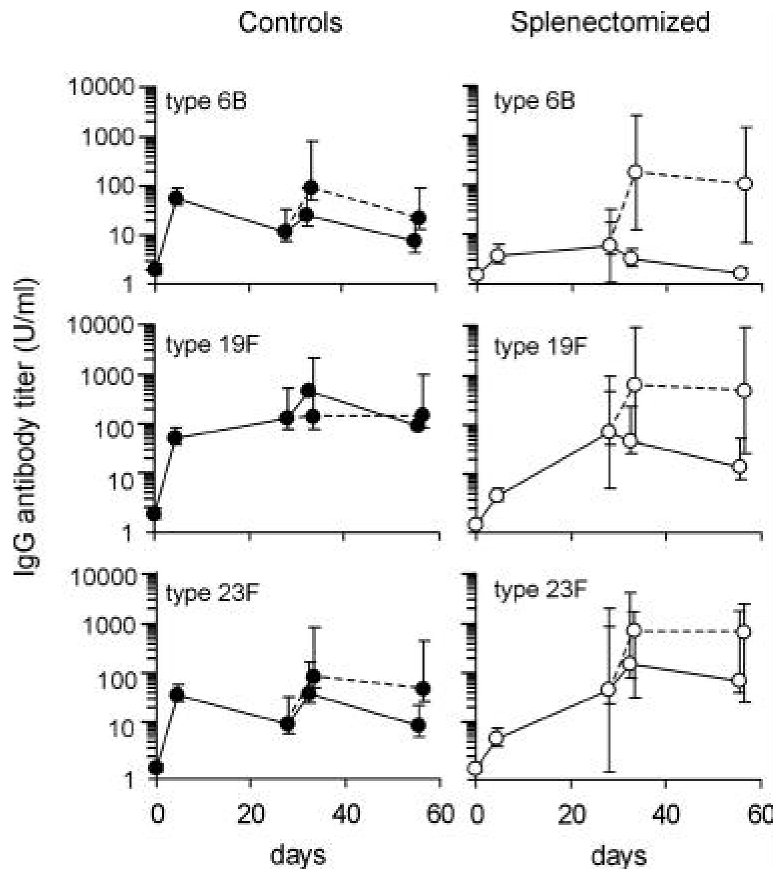

Figure 4. Booster vaccination with conjugated versus native polysaccharides.

Control rats (closed symbols) and splenectomized rats (open symbols) were i.v. and s.c. vaccinated on day 0 with a tetravalent pneumococcal conjugate vaccine consisting of polysaccharides of pneumococcal serotypes 6B, 14, 19F and 23F conjugated to tetanus toxoid. At day 28, animals were booster vaccinated with either pneumococcal polysaccharide serotypes 4, 6B, 9V, 14, 19F and 23F (drawn lines) or the tetravalent conjugate vaccine (broken lines). IgG anti-pneumococcal antibody titers were determined by ELISA. See legend Figure 1 for further details.

After splenectomy, PPS booster induces no increase in IgG anti-19F and 6B antibody titers, whereas boosting with PCV results in a rise in antibody titer for both serotypes (PCV-PPS versus PCV-PCV $p<0.05$ for IgG anti-6B and $p=0.07$ for IgG anti-19F). For serotype 23F both vaccination schedules do result in a comparable rise in antibody titer.

Discussion

Despite the wide range of antibiotics available, invasive pneumococcal infections remain a substantial cause of morbidity and mortality [25-27]. Patients with anatomical or functional asplenia are particularly at risk [3;28], because the spleen is the most important site of phagocytosis of poorly opsonized antigens in the early phase of bacterial invasion, before sufficient amounts of specific antibodies have been produced [2]. After splenectomy, the liver will partially take over the phagocytic function of the spleen. However, the liver needs a higher level of opsonization because of the relatively high blood flow [29]. Because of the splenic dependency of the anti-polysaccharide antibody response, the opsonization of encapsulated bacteria may be suboptimal after splenectomy [1]. Therefore, immunization with pneumococcal polysaccharide vaccine is recommended prior to elective splenectomy [6], although efficacy data are scarce [30]. However, vaccination prior to splenectomy is not always feasible, as after trauma.

Pneumococcal conjugate vaccines (PCV) are developed to enhance the immune response to pneumococcal polysaccharides. Potential target groups include, in addition to young children, several risk groups for pneumococcal infections where the immunogenicity of the pneumococcal polysaccharide vaccine (PPS) is suboptimal. Asplenic or hyposplenic patients might benefit from vaccination with PCV, because polysaccharide-protein conjugates can initiate an antibody response to the polysaccharide without the need for a functional spleen [31-33]. We therefore tested the immunogenicity of PCV in splenectomized rats and showed that a series of two doses of PCV given 6 weeks after splenectomy results in antibody titers that are at least as high as in control rats, whereas the antibody response to PPS is impaired by splenectomy (Figure 1). This indicates that the immunogenicity of PCV, in contrast with that of PPS, is not impaired by splenectomy.

When antibody titers start to decline after primary vaccination of control rats with PCV, a second vaccination with PCV results in a rise again. However, the decline in antibody titers after vaccination with PPS could not be influenced significantly by revaccination with PPS. This phenomenon is also observed after meningococcal A and C polysaccharide vaccination in infants [34]. It is unlikely that this is due to high levels of circulating antibody, since antibody titers at day 28 are identical in splenectomized and control animals, and in splenectomized animals no significant difference in antibody titers is observed after a second PPS dose compared with the first.
We have used rather high doses of PPS for immunization: 0.1 human dose i.v and 0.1 human dose s.c.. Therefore the inability of the second dose to cause an increase in anti-polysaccharide antibodies may be due to high-dose-tolerance, caused by in vivo persistence of polysaccharides. In our study, the persistence of PPS did not seem to affect the antibody response to PCV administered 4 weeks after PPS (data not shown). This finding provides an extra argument in favor of the hypothesis that PCV induce anti-polysaccharide antibody responses by a different mechanism of antigen presentation or at a different localization than PPS.

The spleen is not only of particular importance for phagocytosis of poorly opsonized foreign particles and for the antibody response to TI-2 antigens, as is indicated above, but it is also the site of antibody generation very early after exposure to all blood-borne antigens. We show that the spleen produces the fast primary antibody response to PCV injected in the bloodstream. After i.v. vaccination of control animals with PCV, maximum antibody titers are reached already at day 5 (figures 1 and 2). However, in the absence of splenic tissue, the antibody response to i.v. injected PCV is delayed, antibody titers rise gradually to reach a maximum after 28 days. These data indicate that extrasplenic lymphoid tissues (lymph nodes) are able to generate a primary antibody response to PCV. However, this response is slower than the splenic response. The primary antibody responses to PCV of both splenic and other lymphoid tissue are qualitatively comparable: ultimately equally high antibody titers are reached. The antibody response to a second dose of PCV is similar in the presence or absence of splenic tissue, and results in high antibody titers after 5 days.
When antigens are not introduced via the bloodstream, the fast primary splenic antibody response does not take place. Subcutaneously administrated PCV results in a comparable antibody response pattern as i.v. administration of PCV after splenectomy; the response is probably generated in extra-splenic tissue. Subcutaneously administered PPS ultimately

reaches the marginal zone of the spleen, but slower and more diffuse than after i.v. vaccination, resulting in a blunted antibody response.

PCV, as we show, have the advantage that they can induce antibody responses in the absence of a spleen, but have as major drawback the limitation of the number of serotypes that can be included. The licenced heptavalent pneumococcal conjugate vaccine (Prevnar®, Wyeth Lederle Vaccines and Pediatrics, Rochester, NY) includes seven serotypes. Although 11-valent PCV are under evaluation, the same coverage of serotypes as the 23-valent PPS will not be reached. Theoretically, admixture of PPS with PCV could be an approach to broaden the coverage. Conjugation of polysaccharide to protein changes the nature of the anti-polysaccharide antibody response, from a T cell independent to a T cell dependent response [35;36]. Antigen-presenting B cells probably take up the conjugated polysaccharide–protein molecule and internalize it via membrane immunoglobulins and present the peptides of the protein to T helper cells in association with major histocompatibility complex class II molecules on their surfaces. This induces T helper cells to stimulate polysaccharide-specific B cells to mature into antibody-producing plasma cells or into memory cells [37-39]. According to this model, activation of polysaccharide-specific B cells could take place either via direct physical interaction with peptide-specific T helper cells, or via soluble factors secreted by peptide-specific T cells [40]. Our experimental data indicate that responses to PPS 4 and 9V do not improve by admixture with 4-valent PCV containing serotypes 6B, 14, 19F and 23F (figure 5). Distinct differences in localization may explain this finding: in the splenic marginal zone PPS specifically localize on marginal zone B cells, whereas for PCV this localization on marginal zone B cells is far less pronounced (Breukels et al., manuscript in preparation). Physical interaction of serotype 4 and 9V specific B cells with peptide-specific T cells may not take place, and soluble factors secreted by peptide-specific T cells may be short-range acting cytokines unable to stimulate more distantly located B cells.

An alternative way to extend the coverage of PCV to more serotypes could be booster vaccination with the 23-valent PPS. In infants and children, priming with PCV leads to induction of memory: significant booster responses to serotypes included in the PCV are observed after additional doses of either PCV or polysaccharide vaccine [41-43]. In control rats, after priming with PCV, we indeed observed secondary responses to PPS and PCV that were higher than primary responses. But in splenectomized rats booster responses were

observed only after a second dose of PCV, and not after PPS. So even after priming with PCV, the response to PPS appears to be spleen-dependent.

It should be noted that both the capacity to respond to bacterial polysaccharide antigens, and the unique capacity of the spleen to phagocytose poorly opsonized bacteria are lost by splenectomy. Spleen-saving procedures or splenic autotransplantation at the time of splenectomy may potentially preserve or restore at least part of the splenic immune function, and should be pursued. Protection provided by conjugate vaccines is limited to the antigens present in the vaccine. The clinical efficacy of pneumococcal conjugate vaccines for asplenic and splenectomized patients in preventing overwhelming postsplenectomy infections therefore remains to be established, but this type of vaccines, extended with more vaccine antigens, seems at present to be the best option for patients with (functional) asplenia.

Our data indicate that the spleen is of prime importance in the antibody response to PPS. In the absence of splenic tissue, the ability to mount a primary antibody response to PPS is lost. This may explain the greatly increased risk of invasive infections with encapsulated bacteria like *S. pneumoniae* after splenectomy. We show that antibody response to PCV is independent of the spleen: in control as well as in splenectomized rats and after i.v as well as s.c. PCV vaccination, antibody responses are generated. We therefore conclude that PCV may be of value in the management of anatomic as well as functional asplenic patients.

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Chapter 4

Regulatory T cells in the antibody response to *Haemophilus influenzae* type b polysaccharide

Mijke A. Breukels, Ger T. Rijkers, Marleen M. Voorhorst-Ogink, Ben J.M. Zegers

Department of Pediatric Immunology, Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands.

Infection and Immunity (1999). *67*:789-793.

Abstract

An *in vitro* culture system for the induction of an anti-polysaccharide response is used to study the cellular interactions which determine the magnitude and nature of this B lymphocyte response. Healthy adult volunteers were vaccinated with the *Haemophilus influenzae* type b polysaccharide (PRP)-tetanus toxoid (TT) conjugate vaccine. Optimal *in vitro* anti-PRP and anti-TT antibody responses were obtained when B cells were cultured with equal amounts of T cells. The *in vitro* response is antigen dependent and antigen specific. Culturing with PRP mixed with TT in the presence of T cells induces the highest number of anti-PRP antibody secreting cells (ASC) (128.4 x/: 15.9 (geometric mean x/: SD) IgM anti-PRP ASC/10^6 cells, 9.3 x/: 7.6 IgG anti-PRP ASC/10^6 cells). Culturing without T cells induced no anti-PRP ASC; culturing with only PRP, in the presence of T cells, yielded low numbers of anti-PRP ASC (3.7 x/: 5.2 IgM anti-PRP ASC/10^6 cells and 1.2 x/: 2.2 IgG anti-PRP ASC/10^6 cells). Transwell studies show that the requirements for the antibody response against the polysaccharide are different from those of an anti-protein response. Cytokines formed as a consequence of contact between protein-specific B and T cells are on itself not sufficient to activate TT specific B cells (3.9 x/: 4.6 anti-TT ASC/10^6 cells); direct T-B cell contact appeared to be an absolute requirement. However, physical contact between B and T cells in one compartment of the Transwell system, results in the release of soluble factors able to stimulate B cells in the other compartment to secrete anti-polysaccharide antibodies (36.3 x/: 8.6 anti-PRP ASC/10^6 cells).

Introduction

The defence against infections with encapsulated bacteria such as *Haemophilus influenzae* type b and *Streptococcus pneumoniae* depends primarily on the ability to produce antibodies against the capsular polysaccharides of these microorganisms. The immune response against these antigens (that are categorised as so-called T cell independent type 2 (TI-2) antigens) has several characteristics. There is no memory formation [1], the isotypes used are preferentially IgM and IgG2, and idiotype use of anti-TI-2 antibodies is restricted [2]. Furthermore, responsivity to TI-2 antigens develops relatively late in life [1;3;4], implying that children up to the age of 18-24 months, in general, are less able to produce anti-polysaccharide antibodies. They (hence) do have an increased susceptibility for infections with these encapsulated bacteria. Polysaccharide based vaccines are not effective in this age group [1;5].
Coupling of polysaccharides to carrier proteins converts the anti-polysaccharide response into a response with a T cell dependent character. Polysaccharide-protein conjugate vaccines are able to induce anti-polysaccharide antibodies in 2-3 months old children. Moreover, *H. influenzae* type b polysaccharide (polyribosyl ribitol phosphate, PRP) protein conjugate vaccines have proven to be clinically effective during infancy and virtually eliminated invasive *H. influenzae* type b disease [6-8]. The mechanisms by which these conjugate vaccines induce T and B lymphocyte activation, still remains a matter of debate.

T-cell dependent (TD) protein antigens are bound and internalized by the antigen receptor on B cells (mIg), and re-expressed as processed peptides in MHC class II molecules. The peptide-MHC class II complex on B cells is then able to activate specific T cells. In this interaction, CD40-CD40L functions as an essential ligand-receptor pair which provides a second activation signal [9]. Because polysaccharide processing does not occur [10], this model is not valid for TI-2 antigens.
In vivo, the first step in B cell activation by polysaccharides occurs via ligation and cross-linking of mIg. A second activation signal is probably provided by co-ligation of complement receptor 2 (CR2, CD21). Polysaccharide-C3d complexes, formed by complement activation through the alternative pathway, have the ability to bind to CD21 [11]. The mechanism of co-ligation of mIg and CD21 may account for the fact that antigen-specific T cells are not strictly required for induction of an anti-polysaccharide B cell response.

While the *in vitro* B cell response against TI-2 antigens can be induced in the absence of T cells, the presence of T cells does augment the magnitude of the response [12]. The T cells that mediate this function have been termed "amplifier" cells, to distinguish them from helper T cells in the T cell dependent (TD) antibody response to protein antigens [13]. The *in vivo* anti-polysaccharide antibody response induced by polysaccharide-protein conjugates bears the characteristics of a TD antibody response. In such an antibody response, the role of T helper cells and the specificity of these cells is still unclear.

In order to investigate the cellular interactions which determine the magnitude and nature of the anti-polysaccharide antibody response, we used the previously described *in vitro* culture system for restimulation of *in vivo* primed human B lymphocytes [14]. Using this system we showed that an *in vitro* anti-PRP antibody response can be induced in human B cells, derived from *in vivo* primed individuals. Vaccination with conjugated polysaccharide (PRPTT, *Haemophilus influenzae type b* polysaccharide covalently linked to tetanus toxoid) is required to obtain a positive *in vitro* anti-PRP antibody response [14]. The *in vitro* generation of anti-PRP antibody secreting cells (ASC) was shown to be T cell dependent, antigen dependent and antigen specific. In the present paper we further investigated the cellular interactions between B and T cells which determine the magnitude and nature of the anti-polysaccharide antibody response. We therefore modified the culture system, using the Transwell system to physically separate the cell populations. The results show that T- and B cell derived soluble factors are able to stimulate antigen primed B cells to secrete anti-polysaccharide antibodies and that physical contact between T- and B cells is not absolutely required for anti-polysaccharide B cell differentiation.

Materials and Methods

Immunization

Healthy adult volunteers were given an intramuscular immunization with a full dose of PRPTT (Act-Hib®, Pasteur Mérieux, Lyon, France) which contains 10 µg of the *Haemophilus influenzae* type b polysaccharide covalently linked to tetanus toxoid (± 24 µg). Blood samples were collected 3 to 4 weeks after immunization.

In the Netherlands, children are routinely immunized at the age of 3, 4, 5 and 12 months with diphtheria, pertussis, tetanus and poliomyelitis vaccine. Booster immunizations are given at the

age of 4 and 9 years with diphtheria, tetanus and poliomyelitis vaccine. All adult donors used in this study were vaccinated according to this schedule during childhood.

Lymphocyte preparations

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood (100 ml) from healthy adult donors by density gradient centrifugation on Ficoll Isopaque (Pharmacia, Uppsala, Sweden; 1.077 g/cm^3) at 1000g for 20 minutes. The cell suspension was washed twice with MEM-Tris (Tris buffered minimal essential medium, Gibco, Grand Island, NY). T cells were separated from non-T cells by rosetting with 2-aminoethylisothiouroniumbromide (Sigma Chemical Co, St. Louis, MO) treated sheep erythrocytes (SRBC). The non-T fraction was then depleted of monocytes by treatment with iron carbonyl. After these procedures the monocyte-depleted non-T cell fraction contained an average of 40% B cells (25-72%) and less than 1% T cells. A fixed concentration of 5% monocytes was used in all experiments by mixing appropriate numbers of monocyte depleted non-T cells and non-T cells not depleted for monocytes.

Culture conditions

Cultures were set up according to a 1:1 T/non-T cell composition and each culture was supplemented with 5% monocytes [14]. A total of 2 x 10^6 cells in a volume of 2 ml was cultured at 37°C, 100% humidity and 5% CO_2. The medium consisted of RPMI-1640 (Flow Laboratories, Irvine, UK) supplemented with 200 µg/ml glutamine, 100 IU/ml penicilline, 100 µg/ml streptomycine, 10^{-5} M 2-mercaptoethanol and 10% heat inactivated pooled (5 donors) human AB serum. After culturing for 6 days, cells were washed once in MEM-Tris supplemented with 1% bovine serum albumin (BSA, Organon Technika, Oss, Netherlands). Cells were then resuspended in 1.0 ml RPMI-1640 supplemented with 200 µg/ml glutamine, 100 IU/ml penicilline, 100 µg/ml streptomycine, and 10% heat inactivated fetal calf serum (FCS, Flow Laboratories, Irvine, UK).

In some experiments the monocyte depleted non-T cell fraction was physically separated from the T cell plus 5% monocyte fraction by the use of the Transwell system wells (Costar®, Cambridge MA), 6.5 mm wide with pore size of 0.4 µm. In preliminary experiments it was demonstrated that the Transwell system allowed for transfer of biologically active cytokines across the membrane (data not shown). After 6 days of culture, cells from the various compartments were analysed separately for specific antibody secreting cells (ASC).

Determination of anti-TT and anti-PRP specific antibody secreting cells

B cells secreting anti-tetanus toxoid (TT) or anti-PRP IgG or IgM antibody were enumerated by a modification of the ELISA-SPOT test described by Sedgwick and Holt [15]. Polyvinyl chloride 96-well microtiter plates (Flow) were coated overnight at 4°C with 100 µl of tyramine coupled PRP (5 µg/ml) in 0.9% NaCl or TT (1 µg/ml) in bicarbonate buffer pH 9.6. Plates were also prepared for the detection of all IgG or IgM secreting lymphocytes by coating with goat anti-human IgG or IgM (Tago Inc, Birmingham, AL) at a 1 in 1,000 dilution in bicarbonate buffer pH 9.6 overnight at 4°C. All plates were then blocked following three washes with phosphate buffered saline (PBS) with 1% BSA in MEM for 30 minutes at 37°C. The cultured cells were added to the coated wells in serial dilution starting with 200,000 cells per well. The plates were subsequently incubated for 3-4 hours at 37°C, 100% humidity and 5% CO_2. The wells were then washed with PBS to remove all cells and incubated with 100 µl alkaline phosphatase (AP) conjugated goat anti-human IgM or IgG (Tago) at a dilution of 1 in 1,000 in PBS plus 1% BSA for 2 hours at 37°C. After extensive washing with PBS plus 0.05% Tween-20 (w/v), 100 µl of the AP substrate 5-Bromo-4-chloro-3-indolylphosphate (0.1 mg/ml) in 1 M 2-amino-2-methyl-1-propanol buffer pH 10.25 (Sigma), containing 5 mM $MgCl_2$, 0.01% Triton X405 (Sigma), 0.01% sodium azide and 0.6% 36°C gelling agarose (Sigma) was added to each well. Blue spots representing antibody producing cells were counted after overnight incubation at room temperature using an inverted microscope (Zeiss, Oberkochen, Germany). Antibody secreting cells (ASC) were expressed as the number per 1 x 10^6 input cells.

The specificity of the ASC was assessed by addition of excess soluble antigen (50 µg/ml) during incubation of cultured cells on antigen coated plates. This procedure resulted in a >80% reduction of the number of spots, whereas remaining spots were significantly reduced in size as compared to the non-inhibited situation.

Antigens

The following antigens were used for *in vitro* cultures: polyribosyl ribitol phosphate (PRP, Pasteur Mérieux, Lyon, France) final concentration 5 ng/ml; Tetanus toxoid (TT; National Institute of Public Health and Environment, Bilthoven, The Netherlands) 1.5 µg/ml; PRPTT conjugate at a final PRP concentration of 0.5 µg/ml and TT concentration of 1.2 µg/ml. Two TT-peptides encompassing T helper epitopes) were synthesized [16]: 156 (corresponding to TT sequences 830-843, QYIKANSKFIGITE) and 158 (corresponding to TT sequences 947-967, FNNFTVSFWLRVPKVSASHLE). These peptides were used in a concentration of 7.5 µg/ml.

Results

In vitro induction of the anti-PRP (and anti-TT) antibody response

Adult volunteers were given a single dose of the PRPTT vaccine (Act-Hib®, Pasteur Mérieux, Lyon, France). 3-4 weeks following vaccination peripheral blood mononuclear cells (PBMC) were isolated and purified B cells, mixed with equal numbers of T lymphocytes were cultured and stimulated with various forms of PRP (Figure 1).

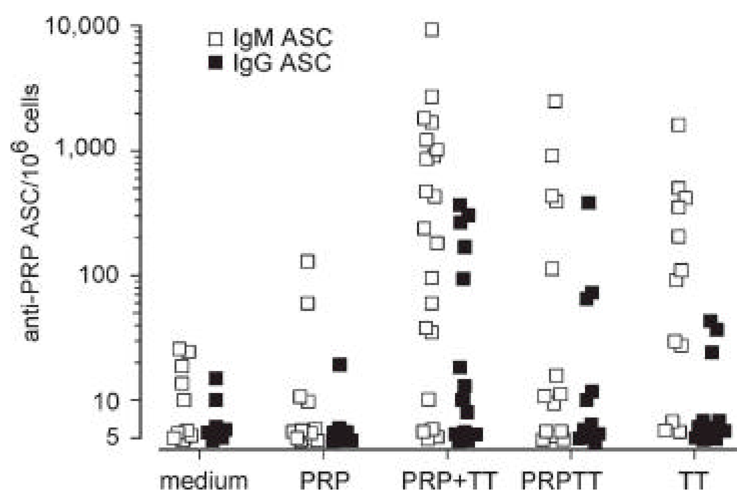

Figure 1. *In vitro* anti-PRP response of blood B cells.

In vitro anti-PRP response of blood B cells obtained 3-4 weeks after vaccination with PRPTT. B cells (0.5 x 10^6/ml) were cultured with an equal number of irradiated T cells and 5% monocytes in medium containing 10% heat inactivated pooled human AB serum and various antigens as indicated. After 6 days of culture, IgG and IgM anti-PRP (antibody secreting cells (ASC) were determined with a spot-forming cell assay and are expressed per 10^6 lymphocytes. PRP, final concentration 5 ng/ml; TT, 1.5 µg/ml; PRPTT, 0.5 µg PRP/ml, 1.2 µg TT/ml. The data are from 21 independent experiments, with different donors. Not all antigens were included in all experiments.

In vitro culturing with PRP only, induced low numbers of IgM (geometric mean 3.7 x/: 5.2) and IgG (1.2 x/: 2.2) anti-PRP ASC/10^6 cells, comparable to ASC numbers detected in cells cultured in medium only (IgM anti-PRP 2.8 x/: 3.8, IgG anti-PRP 2.0 x/: 2.7). Restimulation with PRPTT induced 23.3 x/: 13.9 IgM anti-PRP ASC/10^6 cells, and 4.9 x/: 7.3 IgG anti-PRP ASC/10^6 cells. Comparable ASC responses were obtained in cultures stimulated with PRP+TT (TT in equal amounts as present in the conjugate) (128.4 x/: 15.9 IgM anti-PRP ASC/10^6 cells, 9.3 x/: 7.6 IgG anti-PRP ASC/10^6 cells)(Figure 1). From cells cultured with TT only, variable numbers of IgM anti-PRP ASC were obtained, and less IgG anti-PRP ASC (2.4 x/: 4.9 anti-PRP ASC/10^6 cells).

Anti-TT ASC were obtained only after culturing with TT, either free or mixed with PRP or conjugated to PRP. The response to TT consisted predominantly of IgG ASC (Figure 2).

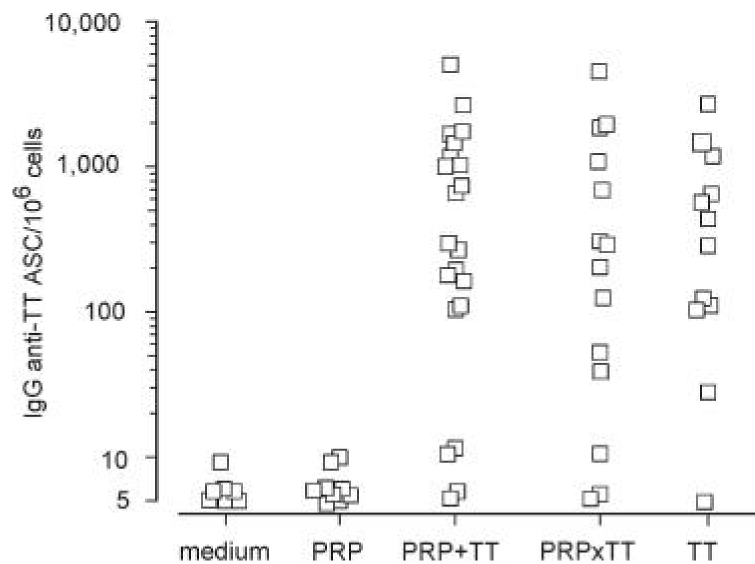

Figure 2. *In vitro* IgG anti-TT response of blood B cells.

In vitro IgG anti-TT response of blood B cells obtained 3-4 weeks after vaccination with PRPTT. See legend Figure 1 for details.

It thus can be concluded that an optimal *in vitro* antibody response to PRP also requires stimulation with TT. There is, however, no absolute need for polysaccharide and protein to be physically coupled. Next to PRP and TT, T cells are required for an optimal *in vitro* anti-PRP B cell response [14](see also Figure 3).

Relation between the in vitro induced anti-PRP response and T cell proliferation

PRPTT vaccination in adults actually is a booster vaccination with respect to the TT component. The *in vitro* proliferative T cell response induced by TT (Table I) is in magnitude comparable to that induced by a T cell mitogen such as poke weed mitogen (PWM). Because of the magnitude of this T cell response, the question arised whether the T cells that augment the anti-PRP B cell response would be antigen (TT) specific, or that the T cell proliferation was due to an aspecific activation process. To address this question, two TT peptides, 156 and 158, encompassing two different universal T helper epitopes, were synthesized [16]. Although these peptides are claimed to be universally antigenic irrespective of MHC haplotype, both peptides induced T cell proliferation above background values in only 50% of unvaccinated donors tested (Table I).

Donor	1	2	3	4	5	6	7	8	9
Medium	0.6*	0.2	1.2	0.3	0.5	0.4	0.3	0.3	0.5
Pokeweed mitogen	21	42	22	15	24	35	49	14	57
Peptide 156	9.7	0.5	2.0	3.1	0.6	0.8	0.3	0.3	4.2
Peptide 158	1.8	6.2	1.8	2.8	0.7	1.1	2.5	0.3	28
Tetanus toxoid	61	57	43	70	25	101	82	12	78

Table I. T cell proliferative response upon stimulation with tetanus toxoid and tetanus toxoid peptides.

T cells, obtained from 9 healthy human volunteers (not vaccinated with PRPTT), were reconstituted with autologous irradiated non-T cells and cultured at a density of 0.5 x 10^6/ml with different antigens. Cells were cultured for 6 days, the last 16 hrs in the presence of 1 mCi [^{3}H]thymidine. *Data are expressed as mean [^{3}H]thymidine incorporation (x 1000 cpm) of quadruplicate cultures. Antigen concentrations used: tetanus toxoid: 1.5 µg/ml; tetanus toxoid peptides 156 and 158: 7.5 µg/ml. Pokeweed mitogen: 5 µg/ml.

An association was found between the ability of a given TT peptide to induce T cell proliferation and to support the *in vitro* anti-PRP antibody response (Table II). Note that because the peptides encompass T helper epitopes, they are unable to induce an anti-TT B cell response.

These data show that TT peptide specific T cell activation is able to support the anti-PRP B cell response.

	ASC/10^6 cells		T cell proliferation
	anti-PRP	anti-TT	(cpm × 1000)
Medium	5	<5	1.8*
PRP	<5	<5	<1
PRPTT	88	316	13.5
PRP + tetanus toxoid	165	497	48.5
PRP + peptide 156	114	<5	23
PRP + peptide 158	<5	<5	<1
tetanus toxoid	221	1340	58

Table II. Relation between the *in vitro* anti-PRP response of blood B cells obtained 3-4 weeks after vaccination with PRPTT and the *in vitro* induced T cell proliferation.

B cells (0.5 x 10^6/ml) were cultured with an equal number of irradiated T cells and 5% monocytes in medium containing 10% heat inactivated pooled human AB serum and different antigens. After 6 days of culture, anti-PRP as well as anti-TT ASC were determined with a spot-forming cell assay and are expressed per 10^6 lymphocytes. T cells were reconstituted with autologous irradiated non-T cells and cultured at a density of 0.5 x 10^6/ml with different antigens. Cells were cultured for 6 days, the last 16 hrs in the presence of 1 mCi [^{3}H]thymidine. *Data are expressed as mean [^{3}H]thymidine incorporation (x 1000 cpm) of quadruplicate cultures. Antigen concentrations used: TT, 1.5 µg/ml; PRPTT, 0.5 µg PRP/ml, 1.2 µg TT /ml, TT-peptides 156 and 158: 7.5 µg/ml. Data from one representative donor out of 4 independent experiments.

Interaction between T and B lymphocytes in the anti-PRP antibody response

In order to characterize in more detail the T cell help necessary for an anti-PRP B cell response, Transwell studies were set up. In this system it is possible to physically separate different cell populations, while diffusion of antigen and soluble mediators remains possible.

Culturing of B lymphocytes with only PRP induces no or minimal IgM anti-PRP ASC (data not shown). Also no anti-PRP antibody response is seen when the B cells (lower compartment) are cultured with PRP plus TT in the absence of T cells in the upper compartment (Figure 3 a). Culturing of B and T lymphocytes together with 5% antigen presenting cells in one compartment, with only PRP as antigen, induces a few IgM anti-PRP ASC (Figure 1).

In vitro culture of B lymphocytes and T lymphocytes in the lower compartment with PRP plus TT as antigens induces both anti-PRP (geometric mean 151.9 x/: 18.4 ASC/10^6 cells) and anti-TT ASC (110.1 x/: 17.0) (Figure 3 b). Culturing the B cells physically separated from the T cells and the monocytes, however, elicits no anti-TT ASC (3.0 x/: 5.2) and hardly any anti-PRP ASC (21.7 x/: 5.3) (Figure 3 c). When B and T cells are cultured together with 5% monocytes in the lower compartment, and B cells only in the upper compartment of the Transwell system, quite remarkable results are obtained. The lower compartment shows results as expected: both anti-PRP (140.8 x/: 19.6) and anti-TT ASC (105.0 x/: 13.8) (Figure 3 d). Assessing ASC from the upper (B cells only) compartment shows that anti-PRP ASC are generated (36.3 x/: 8.6), but no anti-TT ASC (3.9 x/: 4.6) (Figure 3 e). This result suggests that soluble mediators derived from T cells can support an anti-polysaccharide antibody response by B lymphocytes, while an anti-tetanus toxoid antibody response does not take place under these circumstances.It can be concluded that optimal *in vitro* B cell activation by polysaccharides requires T lymphocyte activation. However, there seems to be a distinct difference between the T cell help necessary for B cell activation by polysaccharides and the T cell help needed in an anti-protein response. In the latter situation physical interaction between T and B lymphocytes is required; for a polysaccharide response soluble mediators are sufficient.

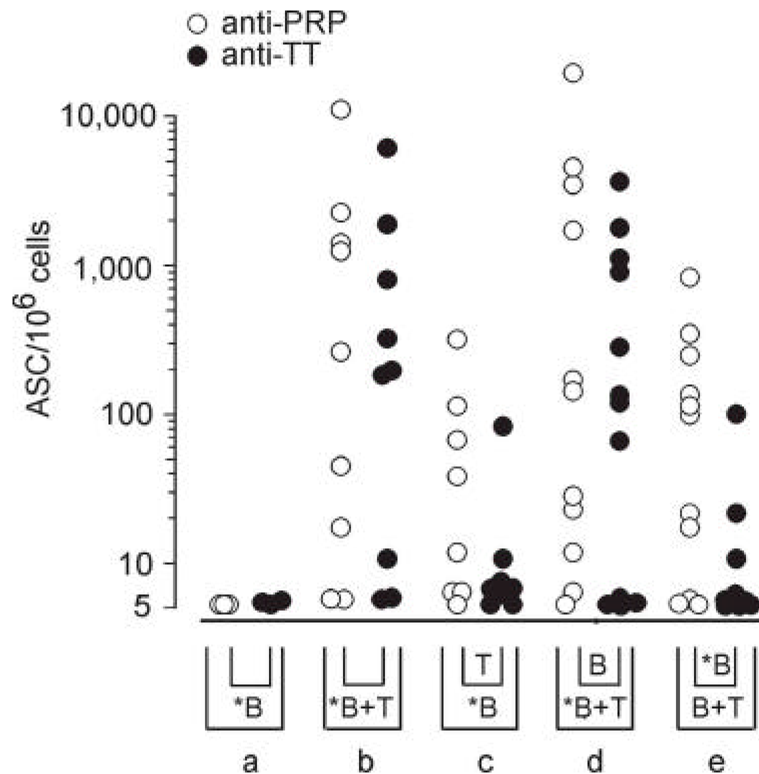

Figure 3. *In vitro* anti-PRP and anti-TT response of blood B cells under various culture conditions using the Transwell system.

In lane a, B cells (0.7 x 10^6) were cultured in medium containing 10% heat inactivated pooled human AB serum and PRP + TT. In the control experiment (lane b), B cells (0.7 x 10^6) were cultured with an equal number of irradiated T cells and 5% monocytes in medium containing 10% heat inactivated pooled human AB serum and PRP + TT. By means of a Transwell system, B cells (0.7 x 10^6) were also cultured separately from T cells (0.7 x 10^6) with 5% monocytes (lane c). The last two lanes (d and e) show the results of culturing B cells (0.7 x 10^6) separated by the Transwell system from the lower compartment containing B cells (0.7 x 10^6) and an equal number of irradiated T cells and 5% monocytes. After 6 days of culture, anti-PRP as well as anti-TT ASC were determined with a spot-forming cell assay and are expressed per 10^6 lymfocytes. All cultures were stimulated with PRP (5 ng/ml) + TT (1.5 µg/ml). * = compartment from which the ASC are depicted. The data represent 9 separately conducted experiments, with 9 different donors.

Discussion

The proposed cellular interaction mechanism for protein conjugated polysaccharides combines the models for TI-2 and TD B cell activation. The B cell receptor of a polysaccharide-specific B cell recognizes the polysaccharide component and binds and internalizes the polysaccharide-protein conjugate. Intracellularly, the protein is processed and peptide fragments are subsequently presented in MHC class II molecules. Peptide-specific T cells become activated and produce B cell stimulating cytokines. In this way, the polysaccharide-specific B cell receives

T cell help from the peptide- (protein-) specific T cell [17;18]. Whether it is sufficient to have activated (peptide-specific) T cells, producing B cell stimulatory cytokines, in the proximity of polysaccharide-specific B cells, or that physical contact between the two cell types is necessary, is unknown.

From our experiments it can be concluded that for the formation of anti-polysaccharide ASC, the presence of (TT-)activated T cells is needed. This is in accordance with the above described model for the working mechanism of conjugate vaccines: the activated TT-peptide-specific T cells, produce cytokines that stimulate polysaccharide-specific B cells.
As we have shown, this T cell help can be induced by culturing with PRP+TT, with PRPTT, as well as with PRP mixed with TT-peptides. The number of anti-PRP ASC generated correlates with the magnitude of the T cell proliferative response to these antigens (Table II).

The highest number of anti-PRP ASC are obtained after culturing with PRP mixed with TT or conjugated to TT (Figure 1). We do find IgM anti-PRP ASC when culturing with TT only, even although at the time of sampling (3-4 weeks after vaccination with PRPTT) no anti-PRP (nor anti-TT) ASC are found in a direct ASC assay (data not shown). We presume that at the time of sampling there are cells present in the peripheral blood of the vaccinees, that are committed to become anti-PRP ASC. They apparently only need activation provided by cytokines secreted by activated (anti-TT) T cells.

In vivo, admixture of polysaccharide and protein does not change the nature of the anti-polysaccharide antibody response. For an effective conjugate vaccine, the polysaccharide and the protein need to be physically coupled [19]. *In vitro*, however, mixtures of polysaccharide and protein are as effective as a conjugate in inducing B cell activation in donors immunized with the conjugate vaccine [20]. These data therefore are compatible with Mosier's postulate [21] that the function of a conjugate would merely be to ensure localization of protein antigens (required for T cell activation) at the site of polysaccharide homing. *In vitro*, protein and polysaccharide are present in close relationship with B as well as T cells, so physical coupling might not be necessary. Throughout all our experiments, PRP conjugated to TT induced anti-PRP responses equal to those seen after restimulation with mixture of PRP and TT. This confirms the earlier observations mentioned previously.

In our *in vitro* system, the presence of T cells is necessary for an optimal anti-protein as well as for an anti-polysaccharide ASC response (Figure 3). The requirements for the antibody response against the polysaccharide, however, are different from those in the response against proteins. In agreement with the described model for activation of protein-specific B cells in the introduction, direct physical contact between B cells and (activated) T cells is necessary for induction of an anti-protein antibody response (Figure 3).
When T cells and antigen presenting cells are physically separated from the B cells by the Transwell membrane, no anti-TT antibody secreting cells were found. Also, when contact between B and T cells was possible in one compartment of the Transwell system, the release of soluble factors due to T-B cell contact in that compartment did not result in an anti-TT antibody response in the other compartment. Apparently, cytokines formed as a consequence of contact between protein-specific B and T cells are in itself not sufficient to activate protein specific B cells; direct T-B cell contact is an absolute requirement.

The Transwell studies also indicate that contact between B and T cells is necessary to induce an optimal anti-PRP antibody response (Figure 3). When T cells and APC were physically separated from the B cells by the Transwell membrane, hardly any anti-PRP specific antibody secreting cells were found (Figure 3 c). The presence of low levels of anti-PRP ASC when the B cell fraction was cultured with PRP + TT may be explained by T cell activation in the upper compartment by TT. These activated T cells may secrete cytokines which augment differentiation of PRP-activated B cells in the lower compartment.

When contact between B and T cells was possible in one compartment of the Transwell system, this apparently resulted in the release of soluble factors able to stimulate B cells in the other compartment to secrete anti-PRP antibodies (Figure 3 e). Both in Figure 3 c and e, T lymphocytes are cultured with antigen presenting cells (monocytes in 3 c, B cells and monocytes in 3 e) and TT. These T lymphocytes can produce cytokines which can diffuse to the other compartment. Yet, this does not result in anti-PRP ASC formation in 3 c, while in 3 e anti-PRP ASC are generated. The difference between 3 c and e is the presence of B lymphocytes in 3 e. Our results suggest that cytokines are produced by B cells that can activate anti-polysaccharide-specific B cells to make antibodies.

These results are partly in accordance with the proposed model for the working mechanism of a conjugate vaccine: the polysaccharide-specific B cells internalize the conjugate, process the

protein part and express the peptides in their MHC to activate peptide-specific T cells. Direct physical contact between B and T cells is necessary in this part of the process. Activated T cells than secrete cytokines that activate the polysaccharide-specific B cells to become ASC. We now have shown that in this part of the process, the activated B cells may secrete cytokines that are able to activate other polysaccharide-specific B cells to become ASC. The nature of these soluble mediators is currently under investigation.

Acknowledgements

We thank Dr. J.T. Poolman (National Institute of Public Health and Environment, Bilthoven, The Netherlands; current address Smith Kline Beecham, Rixensart, Belgium) for the synthesis of the tetanus toxoid peptides.

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Chapter 5a

Pneumococcal conjugate vaccine primes for polysaccharide inducible IgG2 antibody response in children with recurrent Otitis Media

Mijke A. Breukels, Ger T. Rijkers, Marleen M. Voorhorst-Ogink, Ben J.M. Zegers, Elisabeth A.M. Sanders.

Department of Pediatric Immunology, Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands

Journal of Infectious Diseases. (1999). *179*: 1152-1156.

Abstract

Children with recurrent episodes of otitis media can have a deficient IgG2 antibody response to polysaccharide antigens. We have vaccinated 5 otitis-prone children with heptavalent pneumococcal conjugate vaccine. While the patients did show an IgG1 antibody response to all pneumococcal serotypes included in the conjugate vaccine, the IgG2 response, especially to serotypes 6B, 9V, 19F and 23F, was poor. However, vaccination with a 23-valent polysaccharide vaccine 6 months after conjugate vaccination did induce an 11.5-163 fold increase in IgG2 anti-polysaccharide antibody titers. It can be concluded that a specific IgG2 anti-polysaccharide antibody deficiency can be overcome by priming with a pneumococcal conjugate vaccine, followed by a booster with a polyvalent polysaccharide vaccine.

Introduction

Acute otitis media (AOM) is the most frequently occurring bacterial infection in infants and young children. By the age of three years, 71% of children have had one or more episodes of AOM [1]. *Streptococcus pneumoniae* is the main pathogen in bacterial AOM, involved in at least 50-60% of the cases. The defense against infections with these bacteria, characterized by their polysaccharide capsule, depends primarily on antibodies against the capsular polysaccharide.

The peak incidence of childhood AOM is between 6-18 months of age, which matches the period after maternally derived antibodies have disappeared and before onset of antibody formation to polysaccharide antigens. Up to 5% of all children are highly susceptible to infections with *Streptococcus pneumoniae,* manifested in frequently recurrent episodes of AOM. This condition, termed otitis prone, can persist beyond the age of 2 years. Otitis-prone children are suggested to be immunologically different from age matched healthy children in their inability to mount an adequate IgG2 antibody response to *S. pneumoniae* [2]. We previously described a group of children with frequent recurrent mucosal respiratory tract infections. These children showed low to absent IgG2 and/or IgA anti-pneumococcal polysaccharide antibody responses following vaccination with pneumococcal polysaccharide vaccine, despite normal serum IgG2 en IgA immunoglobulin levels [3]. Recently, D'Hooghe et al. observed in a group of patients with otitis media with effusion (OME), significantly lower IgG2 and/or IgA anti-pneumococcal polysaccharide antibody responses following vaccination with pneumococcal polysaccharide vaccine compared to age-matched, healthy controls (Dhooge et al, manuscript in preparation). In all these three studies, the IgG1 anti-polysaccharide antibody responses was normal. These observations suggest that IgG2 anti-polysaccharide antibodies are required for clinical protection against AOM.

Polysaccharide conjugate vaccines have been developed to change the nature of the anti-polysaccharide antibody response into a T cell dependent response and thus induce responsivity early in life. Pneumococcal conjugate vaccines (PCV) indeed have been shown to be immunogenic in infants [4-6] and to induce immunological memory [6]. PCV induce primarily an IgG1 type anti-polysaccharide antibody response [6]. It remains to be determined whether IgG1 antibodies only will be sufficient to prevent mucosal infections like AOM in an otitis prone childhood population. In view of above arguments, the induction of IgG2 or IgA anti-

pneumococcal polysaccharide antibodies may be necessary for optimal protection against mucosal respiratory tract infections.
Based on the results of a study of O'Brien [6] and our own -unpublished- observations, we hypothesized that priming with a conjugate vaccine, followed by vaccination with a pneumococcal polysaccharide vaccine might induce an increased IgG antibody response and favor a shift towards an IgG2 type antibody response. We here present the results of a preliminary study in five otitis-prone children vaccinated with a pneumococcal conjugate vaccine, followed by booster vaccination with a pneumococcal polysaccharide vaccine.

Methods

Patient population

The patients included in this study were referred to the Department of Immunology of our hospital by their pediatrician or otolaryngologist because of recurrent AOM with otorhea for at least 6-8 episodes per year. All five patients were Caucasian and older than two years of age (2.1 - 12.5 years at time of conjugate vaccination). The episodes of AOM were characterized by signs of pain, irritability, new otorhea and additional symptoms like fever (> 38.5 °C) and concomitant signs of upper respiratory tract infection. The diagnosis AOM was confirmed when patients also had otoscopic signs typical for AOM (Casselbrant,1992). None of the patients had anatomic abnormalities, nor suffered from recognized disease entities like cystic fibrosis or granulocytopenia. Total serum immunoglobulin levels (IgG and IgG subclasses, IgA and IgM) were normal for age. In two patients total IgE levels were increased (300 and 53 U/l, respectively). Complement-activity was normal as were antibody responses to tetanus and diphtheria toxoid. Two of the patients had previously (2 and 7.5 years respectively before conjugate) received a 23-valent pneumococcal polysaccharide (PnPS) vaccine (Pneumovax, Merck, Sharp and Dohme, Haarlem, The Netherlands), containing 25 µg of purified type-specific capsular polysaccharides of 23 pneumococcal serotypes (1, 2, 3, 4, 5, 6B, 8, 9N, 9V, 10A, 12F, 14, 15B, 17F, 18C, 19F, 20, 22F, 23F, 33F) to assess their capacity to respond to polysaccharide antigens. Both patients mounted an IgG1 and IgM anti-pneumococcal polysaccharide response; no IgG2 (post vaccination titers <20 U/ml for 6/8 and 8/8 tested serotypes respectively) and hardly any IgA anti-polysaccharide antibodies were induced.

Written parental consent was obtained before immunization intramuscularly with a heptavalent pneumococcal conjugate vaccine (PCV, containing pneumococcal polysaccharides serotypes 4, 6B, 9V, 14, 18C, 19F and 23F, conjugated either to CRM_{197} (Wyeth Lederle Vaccines and Pediatrics, Rochester, NY) or conjugated to meningococcal outer membrane protein (OMP; Merck Research Laboratories, Blue Bell, PA). Latter PCV was given in the context of a compassionate use protocol. A booster vaccination with 23-valent PnPS vaccine was given 6 months after the vaccination with PCV. Serum was drawn before and 2-4 weeks after each vaccination and stored at -80 °C until use.

Determination of type specific anti-polysaccharide antibodies.

IgG1-, IgG2-, IgA- and IgM-type serum antibody levels to the seven pneumococcal polysaccharides included in the conjugate vaccine and to two pneumococcal polysaccharides not included in the PCV (serotypes 1 and 12), were measured by ELISA as described previously [3]. Pre- and postvaccination serum samples were analyzed simultaneously. All serum samples were preincubated overnight with excess free common cell wall polysaccharide (CPS) to remove anti-CPS antibodies. A standard serum from a normal non-vaccinated adult was included in every ELISA-run as a control.

Antibody concentrations of patient samples were expressed relative to a reference adult hyperimmune plasma pool [7]. The latter was assigned 100 U/ml (100%) for each serotype.

Results

Anti-pneumococcal antibody responses following PCV

Vaccination with PCV induced a significant (2.7 - 43 fold) increase in IgG1 antibody levels for all pneumococcal serotypes included in the vaccine (4, 6B, 9V, 14, 18C, 19F and 23F; see Table 1). Mean IgG2-anti-pneumococcal polysaccharide antibody levels after vaccination with PCV remained <20 U/ml for all serotypes, except for serotype 14. The reason is that one patient mounted an extremely high IgG2 anti-PS14 antibody response (1968 U/ml). The IgA antibody response was >20 U/ml for 4 of the serotypes included in the PCV. As expected, antibody levels to pneumococcal serotypes not included in the PCV (serotypes 1 and 12) did not change (Table 1)

	pre-vaccination		post-vaccination		fold-increase	
	a	range	b	range		range
IgG1-PS4	6	(1-19)	116	(16-207)	43.0	(8.0-120)
IgG1-PS6B	151	(21-615)	295	(30-1115)	2.7	(1.4-5.2)
IgG1-PS9V	85	(1-396)	99	(3-442)	3.6	(1.0-13.0)
IgG1-PS14	26	(7-67)	267	(16-922)	6.0	(2.3-13.8)
IgG1-PS18	14	(7-32)	139	(70-252)	15.9	(5.3-36.0)
IgG1-PS19F	171	(13-675)	405	(32-1264)	4.2	(1.1-14.2)
IgG1-PS23F	55	(8-198)	117	(33-251)	5.3	(1.3-15.0)
IgG2-PS4	2	(1-6)	12	(1-28)	9.8	(1.0-28.0)
IgG2-PS6B	1	(1-3)	1	(1-2)	1.1	(0.7-2.0)
IgG2-PS9V	1	(1-1)	2	(1-2)	1.2	(1.0-2.0)
IgG2-PS14	8	(1-16)	522	(1-1968)	35.0	(1.0-123)
IgG2-PS18	2	(1-3)	19	(4-36)	12.8	(4.0-28.0)
IgG2-PS19F	1	(1-1)	1	(1-3)	1.4	(1.0-3.0)
IgG2-PS23F	1	(1-1)	2	(1-4)	1.6	(1.0-4.0)
IgA-PS4	3	(1-10)	65	(3-274)	32.0	(1.2-13.7)
IgA-PS6B	4	(1-9)	68	(2-295)	10.8	(1.4-33.0)
IgA-PS9V	3	(2-5)	5	(2-12)	1.5	(1.0-2.4)
IgA-PS14	9	(4-14)	235	(9-852)	4.4	(2.1-8.5)
IgA-PS18	136	(6-471)	142	(61-223)	6.2	(0.4-14.8)
IgA-PS19F	6	(3-10)	21	(7-69)	4.6	(0.9-17.0)
IgA-PS23F	7	(4-12)	16	(6-39)	2.4	(0.9-9.8)
IgG1-PS1	8	(3-13)	6	(5-8)	0.8	(0.6-1.1)
IgG1-PS12	45	(23-65)	34	(23-42)	0.8	(0.6-1.3)
IgG2-PS1	4	(1-15)	4	(1-13)	1.0	(0.9-1.0)
IgG2-PS12	6	(1-24)	4	(1-17)	0.9	(0.7-1.0)
IgA-PS1	14	(5-23)	10	(5-15)	0.8	(1.0-1.5)
IgA-PS12	10	(4-14)	10	(4-16)	1.1	(0.7-1.6)

Table 1. Serotype specific antibody response to pneumococcal conjugate vaccine in children with recurrent otitis media:

Mean antibody levels to pneumococcal serotypes included in the PCV (4, 6B, 9V, 14, 18, 19F and 23F) and 2 non-PCV serotypes (1 and 12) before and after vaccination with PCV. The range in antibody titers and fold increase in titer is indicated between brackets.

Antibody responses following pneumococcal polysaccharide vaccination

Six months after vaccination with the PCV, patients were booster vaccinated with a 23-valent PnPS vaccine. This booster vaccination with non-conjugated polysaccharides now induces a clear-cut IgG2 antibody response, illustrated for serotypes 9V and 23F in Figure 1. Among the 7 PCV serotypes, post PnPs IgG2 titers to 6B and 23F are the lowest, although for both serotypes the fold increase in IgG2 antibodies is >10 (Table 2).

	pre-vaccination		post-vaccination		fold-increase	
	a	range	b	range		range
IgG1-PS4	56	(16-180)	778	(38-2109)	23.8	(2.4-63.9)
IgG1-PS6B	66	(30-96)	2677	(284-5000)	40.2	(9.5-89.5)
IgG1-PS9V	11	(3-24)	568	(29-2058)	47.9	(9.7-137)
IgG1-PS14	126	(28-316)	1813	(489-5000)	31.3	(2.3-126)
IgG1-PS18	88	(12-314)	732	(49-1569)	25.1	(0.7-54.1)
IgG1-PS19F	58	(17-138)	3103	(93-5000)	76.2	(2.0-178)
IgG1-PS23F	49	(19-134)	246	(46-744)	10.3	(0.4-39.2)
IgG2-PS4	6	(1-12)	125	(18-230)	28.3	(1.5-138)
IgG2-PS6B	1	(1-7)	31	(2-54)	17.0	(2.0-25.5)
IgG2-PS9V	5	(1-17)	65	(27-143)	47.9	(1.6-143)
IgG2-PS14	92	(1-334)	273	(174-447)	48.8	(1.3-224)
IgG2-PS18	146	(2-334)	98	(36-221)	26.4	(0.2-49.5)
IgG2-PS19F	1	(1-3)	184	(17-558)	163.2	(17.0-558)
IgG2-PS23F	3	(1-9)	18	(10-30)	11.5	(1.4-28.0)
IgA-PS4	40	(5-135)	219	(13-488)	10.4	(1.9-25.7)
IgA-PS6B	31	(7-111)	165	(27-368)	14.0	(1.8-52.6)
IgA-PS9V	7	(4-13)	169	(14-485)	23.7	(3.5-38.8)
IgA-PS14	59	(19-172)	241	(49-635)	8.9	(1.2-33.4)
IgA-PS18	57	(19-122)	491	(39-1132)	8.7	(0.6-19.1)
IgA-PS19F	16	(7-23)	954	(56-1596)	55.7	(8.0-98.4)
IgA-PS23F	16	(7-26)	65	(20-174)	3.9	(1.4-6.8)
IgG1-PS1	6	(3-10)	82	(11-144)	10.0	(1.8-18.4)
IgG1-PS12	32	(19-46)	488	(36-1209)	13.2	(1.4-28.8)
IgG2-PS1	4	(1-13)	29	(6-74)	22.7	(1.2-74.0)
IgG2-PS12	4	(1-17)	15	(7-23)	8.2	(1.4-22)
IgA-PS1	12	(5-20)	58	(17-155)	3.8	(1.1-7.8)
IgA-PS12	11	(4-16)	40	(17-73)	3.5	(1.6-5.6)

Table 2. Antibody response to polyvalent pneumococcal polysaccharide vaccine in PCV primed patients.

Mean antibody levels to pneumococcal serotypes included in the PCV (4, 6B, 9V, 14, 18, 19F and 23F) and 2 non-PCV serotypes (1 and 12) before and after vaccination with PCV. The range in antibody titers and fold increase in titer is indicated between brackets.

Booster vaccination with the PnPS vaccine also induced a substantial IgA antibody response and a further increase in IgG1-anti-pneumococcal polysaccharide antibodies (Figure 1 and Table 2).he antibody titers reached after vaccination with PnPS against serotypes not included in the PCV (1 and 12) were considerably lower.

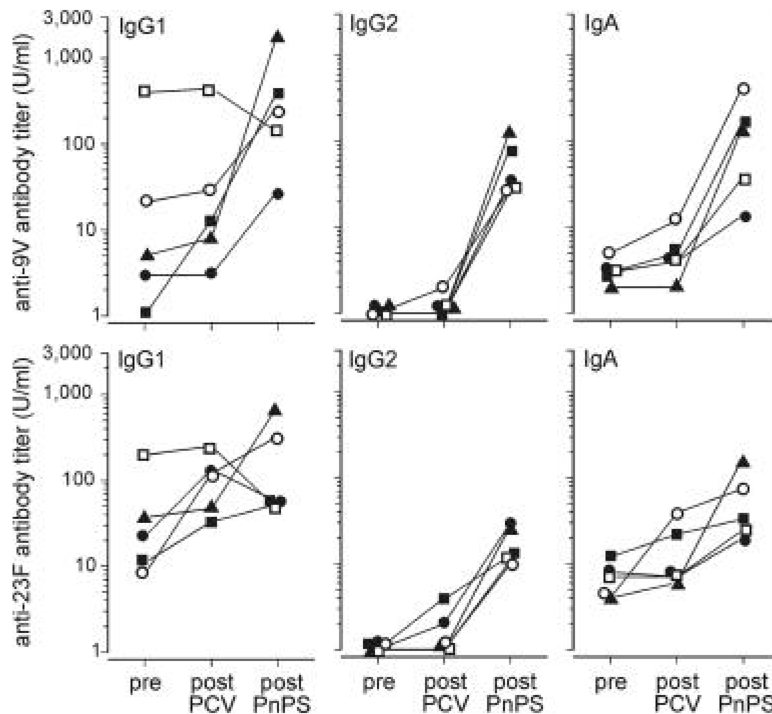

Figure 1. Antibody response to serotypes 9V (upper panels) and 23F S. pneumoniae polysaccharides (lower panels) in children with recurrent otitis media.

Open symbols represent patients who received the OMP pneumococcal conjugate vaccine (PCV), closed symbols are the patients vaccinated with the CRM PCV. Six months after PCV all patients received a 23-valent pneumococcal polysaccharide vaccine (PnPS).

Discussion

Polyvalent pneumococcal polysaccharide vaccines normally induce IgM, IgG1, IgG2 and IgA antibodies of which especially in adults and older children IgG2 is the predominant isotype. Although it has been demonstrated that anti-polysaccharide antibodies confer protection against infections with encapsulated bacteria, the actual contribution of the various isotypes to host defense is unknown. Patients with an IgA and/or IgG2 deficiency have an increased susceptibility for mucosal infections with encapsulated bacteria. We previously described a childhood population with recurrent mucosal upper respiratory tract infections, where encapsulated bacteria like *S. pneumoniae* are a major pathogen. Striking was the low to absent IgG2 and IgA antibody response: 43% of all children had defective IgG2 antibody responses and 22% also low IgA antibody responses following vaccination with the 23-valent PnPS vaccine [3]. Therefore, the inability to make IgG2- and IgA-type anti-pneumococcal polysaccharide

antibodies appears to define a clinical entity of high susceptibility for mucosal infections with bacteria like *S. pneumoniae*.

The frequent occurrence of infections with encapsulated bacteria in infants and young children reflects the ontogeny of the components of host defense [8;9]. The existing polysaccharide vaccines are of limited use in infants and young children because for many polysaccharide antigens, adequate antibody titers are not attained until the age of 18-24 months or later [10]. However, covalent conjugation of a protein carrier to bacterial capsular polysaccharides induces upon immunization a T cell dependent antibody response and priming for an anamnestic or secondary response [10-12]. Pneumococcal conjugate vaccines (PCV) have recently been shown to be safe and immunogenic in healthy infants and young children [13-15]. They are known to prime the vaccinees to respond to a nonconjugated polysaccharide vaccine [14;15]. Furthermore, secondary immunization with pneumococcal polysaccharide vaccine after priming with a conjugate vaccine, elicited in those healthy infants and young children an increase in both IgG1 and IgG2 subclasses [6].

In children with recurrent mucosal respiratory tract infections, an aberrant response to pneumococcal polysaccharide vaccines is frequently observed [3;16]. The data in this study indicate that the specific IgG2 anti-polysaccharide antibody deficiency in patients with recurrent respiratory tract infections, can be overcome by vaccination with PCV followed by booster vaccination with a polyvalent pneumococcal polysaccharide vaccine. Otitis prone children as well as other patient groups at risk for invasive infections with encapsulated bacteria therefore might benefit from such a vaccination schedule.
The mechanism of how a PCV can prime for a PnPS inducible IgG2 response is unknown. It would be desirable if priming would also occur for serotypes not included in the PCV. Our data on the PnPS booster vaccination induced IgG2 response to serotypes 1 and 12 are not conclusive in that respect. A larger group of patients, as well as a control group not primed with PCV, are required to address this issue.

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Chapter 5b

Pneumococcal conjugate vaccination in management of children with recurrent upper respiratory tract infections: appearance of IgG2 and IgA type antibodies after booster vaccination with pneumococcal polysaccharide vaccine

Mijke A. Breukels, Ger T. Rijkers, Adrie J.C. van de Muijsenberg,
Ben J.M. Zegers, Elisabeth A.M. Sanders.

Department of Pediatric Immunology, Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherland

Abstract

Despite good IgG1 responses, patients with recurrent bacterial respiratory tract infections often show poor IgG2- and IgA anti-pneumococcal antibody titers as compared with healthy age-matched controls, both before and after immunization with pneumococcal polysaccharide vaccines.

Also, children with a partial IgG2 and/or IgA deficiency have an increased susceptibility for mucosal infections with *Streptococcus pneumoniae.* In children, pneumococcal conjugate vaccines induce primarily IgG1 antibody responses. We show that in patients with recurrent respiratory tract infections, both with normal IgA and IgG2 serum immunoglobulin levels as well as in and in patients with partial IgG2 and/or IgA deficiency, the combination of pneumococcal conjugate vaccine followed by pneumococcal polysaccharide vaccination not only increases IgG1 type antibody responses, but also induces IgG2 and IgA anti-pneumococcal antibody responses. This vaccination stategy may be optimal to prevent recurrences of respiratory tract infection in susceptible subjects and has to be clinically investigated in these infection-prone children.

Introduction

Streptococcus pneumoniae is a major pathogen in respiratory tract infections (RTI) in children, like acute otitis media (AOM), sinusitis, bronchitis and community-acquired pneumonia [1-3]. In the past, two studies have shown a beneficial effect of vaccination with polyvalent pneumococcal polysaccharide vaccine in preventing *recurrent* attacks of AOM in children over 2 years of age. The duration of the protective effect was limited to one year [4;5]. However, in large efficacy trials, immunization with the polyvalent pneumococcal polysaccharide vaccines was not successful in the overall reduction of RTI including AOM, nor in reduction of antibiotic use in infants and preschool children [4;6-8;9].
This lack of efficacy is explained by the fact that most polysaccharide antigens are poorly immunogenic in children below 5 years of age, and by the fact that polysaccharide antigens do not induce immunological memory [10;11]. Furthermore, despite good IgG1 responses, children with recurrent bacterial RTI may show poor IgG2- and IgA anti-pneumococcal antibody titers as compared with non-otitis prone controls, both before and after immunization with polysaccharide vaccines [12;13] and some children fail to respond at all to vaccination with polysaccharide antigens [14-17]. There seems to be a correlation between impaired antibody responses to pneumococcal polysaccharide vaccine and recurrent attacks of pneumococcal AOM.

Pneumococcal conjugate vaccines (PCV) seem to overcome many of these major drawbacks in infants and children. In contrast to polysaccharide vaccines, both the heptavalent (PCV7) and nonavalent PCV (PCV9) induce good IgG antibody responses in infants when given as a separate injection at 2, 4, 6 and 12-15 months of age concomitantly with routine infant vaccinations. Induction of memory seems to occur, since significant booster responses are observed after additional doses of either PCV or pneumococcal polysaccharide vaccine (PPV) given at intervals of a few months to a year after the primary series [11;18;19]. Importantly, PCV is able to induce IgG antibody responses in otitis-prone children [20] and in patients with RTI who previously had failed to respond to the polysaccharide vaccine [21]. Furthermore, in highly otitis-prone children, PCV followed by PPV induced, apart from specific IgG1-, also IgG2- and IgA- anti-pneumococcal antibody responses [22].

PCV7 was shown to be highly effective in preventing invasive disease in healthy infants in the randomized efficacy trial by the Northern California Kaiser Permanente Vaccine Study Center Group. This study, including 37,830 infants, showed a 93.9% efficacy in preventing invasive disease (intent-to-treat analysis, 95% confidence interval (CI) 79.6 to 98.5% [23]). The effect of PCV7 on the incidence and severity of AOM was evaluated as a secondary end-point in this study. Total otitis media-related visits were decreased by 8.9% (95% CI 5.8-11.8%) in PCV7 vaccine recipients. A more pronounced reduction in AOM was observed among those children with frequent episodes of AOM: a 9.1% (95% CI 4.1-13.8%) decrease in those children with at least 3 episodes in 6 months or 4 episodes in one year, and a 22.8% decrease (95% CI 6.7%–36.2%) in those children with 5 episodes in 6 months or 6 episodes in a year. In the analysis of spontaneous draining ears, vaccine efficacy was 65% for AOM caused by vaccine-serotypes. In a Finnish trial, an estimated vaccine efficacy of 57% (95% CI 44%-67%) against culture-confirmed AOM, caused by vaccine serotypes was found. Efficacy against AOM, again in healthy infants, irrespective of etiology was 6% (95% CI 4%-16%) [24].

Data from the nineteen-eighties from Rosen and from Makela [4;5] with the 14-valent polysaccharide vaccine in children with previous attacks of AOM as well as the recent Black data [23] suggest that the children with *recurrent* AOM or *recurrent* respiratory tract infections may be the ones to benefit from pneumococcal vaccination, both with the conjugate and polysaccharide vaccine. The optimal vaccination strategy to achieve not only adequate IgG responses and memory at an early age and in non-responders, but preferably also IgG2- and IgA responses in children with frequent recurrent AOM or RTI has to be determined.
In this study we analyzed antibody responses after pneumococcal vaccination of children with frequent RTI. The patients had either normal serum immunoglobulin concentrations or partial IgG2 and/or IgA deficiency, which is frequently observed in these infection-prone children. We compared antibody responses after vaccination with PCV7 followed by a PPV booster with responses obtained after vaccination with PPV only. In younger (12-18 months old) otitis-prone children we evaluated the immunogenicity of two doses of PCV7.

Methods

Subjects.

The protocol was approved by the local research ethics committees.

A total of 65 patients (1-6 years old) were included in this study. They were referred to the Department of Pediatric Immunology of the University Hospital Utrecht by pediatricians or otolaryngologists because of recurrent and often prolonged upper respiratory tract infections. All patients suffered from recurrent acute otitis media or sinubronchitis episodes for which they needed antibiotics (at least 3 episodes in 6 months, or at least 4 in the previous year). Patients with underlying chronic or progressive disease, craniofacial abnormalities, asplenia, Down syndrome, immunodeficiencies other than partial serum IgA- and IgG2 deficiencies, as well as patients with a history of seizures or previous pneumococcal vaccination were excluded. Written informed consent was obtained from the parents or legal guardian before enrollment.

Patients were recruited between April 1996 and September 1999. Before January 1998, all patients included were over 2 years of age and were immunized intramuscularly with 23-valent pneumococcal polysaccharide vaccine (PPV23), containing 25 μg of purified 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. After January 1998, patients were included from the age of 12 months. They were vaccinated with the investigational heptavalent pneumococcal conjugate vaccine (PCV7), containing the polysaccharides of the pneumococcal serotypes 4, 9V, 14, 19F and 23F and an oligosaccharide of serotype 18C (2 μg each) and polysaccharide type 6B (4 μg), conjugated to CRM_{197} carrier protein (Wyeth Lederle Vaccines and Pediatrics, Rochester, NY). Children over 24 months of age receiving PCV7 were boosted with PPV23 six months after vaccination with PCV7. Children 12-23 months of age received two doses of PCV7, at least 6-8 weeks apart. Serum was drawn before and 2-4 weeks after each vaccination and stored at -20ºC until analysis.

Determination of serum immunoglobulins and antibody response to protein antigens

Concentrations of IgM, IgG and IgG subclasses, and IgA were determined on 2 or more occasions by radial immunodiffusion. A range of 2SD below to 2SD above the age-adjusted mean was considered normal [25;26]. Diphtheria and tetanus-toxoid antibodies were

determined as described [27]. A titer of less than 0.1 U/ml for a fully vaccinated child was considered below normal.

Determination of specific anti-pneumococcal polysaccharide antibodies

IgG1-, IgG2-, and IgA-type serum antibody levels to four pneumococcal polysaccharides included in both the conjugate vaccine and polysaccharide vaccine (serotypes 4, 6B, 9V and 19F) were measured by ELISA as described previously [17]. All serum samples were preincubated overnight with excess free common cell wall polysaccharide (CPS) to remove anti-CPS antibodies [28;29]. A standard serum from a normal non-vaccinated adult was included in every ELISA-run as a control. Pre- and post-vaccination serum samples from individual patients were analyzed simultaneously. Antibody concentrations of patient samples were calculated using a reference adult hyperimmune plasma pool [30]. The latter was assigned 100 U/ml (100%) for each serotype. Weight-based antibody concentrations were assessed in comparison with the anti-pneumococcal standard serum lot 89-S [31;32].

Statistical analysis

Comparability of groups regarding to age and comparisons of anti-pneumococcal antibody responses between groups were made by two-sided t-tests. P-values <0.05 were considered statistically significant. Log-transformed values were used in calculations of means of responses to each pneumococcal serotype, expressed as geometric mean antibody titers.

Results

Patients

Before April 1998, 27 children with normal concentrations of serum immunoglobulins (Tables 1a and 2a) and 13 with partial IgA and/or IgG2 deficiency (Tables 1b and 2b) were vaccinated i.m. with a single dose of PPV23.

After April 1998, 25 children were vaccinated i.m. with PCV7; 16 of those 25 had normal serum immunoglobulin concentrations (Tables 1a and 2a) while 9 patients had a partial IgA and/or IgG2 deficiency (Tables 1b and 2b). Eight of the 25 patients were younger than 2 years; they received a second vaccination with PCV after 6-8 weeks. Of the 17 patients older than 2 years, 12 received a booster with PPV23 at least 6 months after vaccination with

PCV7. The antibody responses to diphtheria and tetanus toxoid were adequate for all 65 patients (data not shown). The groups of children over 2 years of age, both with normal immunoglobulins or with partial IgA and/or IgG2 deficiency, receiving either PCV7-PPV23 or PPV23, did not differ in age (Tables 1 and 2).

Anti-pneumococcal antibody responses in children over 2 years of age.
In children over two years of age with recurrent respiratory infections, including those with partial serum-IgA and/or IgG2-deficiency, one dose of PCV induced an IgG1 antibody responses against all serotypes tested. The IgG1 type responses to 6B and 19F were significantly higher than after vaccination with PPV23 ($p < 0.05$). However, after one dose of PCV7, no increase in specific IgG2 nor in IgA- antibodies was observed for any serotype, despite the fact that they were at least 24 months of age. After boosting with PPS23 of children with normal serum immunoglobulin concentrations not only further increases in the IgG1 antibody responses were observed, but now also IgG2 and IgA-anti-pneumococcal polysaccharide responses were induced, reaching significance for IgG2 and IgA anti-6B and -19F ($p<0.01$), and IgA anti-9V ($p<0.05$) as compared with vaccination with PPV23 only. Importantly, even in children with partial serum IgG2- and IgA-deficiency, boosting with PPV23 resulted in a rise in IgG2- and IgA-type anti-pneumococcal antibody titers, reaching significance for IgG2 anti-6B ($p<0.05$) and IgG2- and IgA- anti-19F ($p<0.01$) as compared with a single dose of PPV23.

Anti-pneumococcal antibody responses in children 12-18 months of age
In children 12-18 months old, a single dose of PCV did not induce high antibody titers, but primed for high IgG1 responses after the second dose, although anti-6B antibody production remained poor in the children with normal serum immunoglobulin concentrations. This may be due to their younger age. Two doses of PCV did not induce significant amounts of IgG2- and IgA antibodies at this early age.

Discussion

S. pneumoniae is an important cause of bacterial RTI and the most common cause of AOM and sinusitis among young children [33]. Otitis media is the most frequent reason for pediatric

office visits in the United States, resulting in more than 15 million visits per year [34]. Although serious complications are rare nowadays, direct and indirect economic costs of otitis media were estimated at $3.8 billion a year in 1995 [35]. AOM is also the leading reason for prescribing antibiotics during childhood, and the use of antibiotics for treatment of RTI contributes substantially to increased antimicrobial resistance [36-38]. It is established that acquisition and carriage of *S. pneumoniae* in the nasopharynx is associated with the occurrence of AOM [39-41].
The lack of efficacy of pneumococcal polysaccharide vaccines (PPV) in preventing AOM [4;7;8] may be explained by their poor immunogenicity in children under 5 years of age [10;11], and their inability to reduce nasopharyngeal carriage [42]. In contrast, pneumococcal conjugate vaccines (PCV) are immunogenic even in infants and toddlers [18;43;44] and reduce nasopharyngeal carriage of *S. pneumoniae* of the vaccine serotypes [45-48]. The reduction of nasopharyngeal carriage of vaccine serotypes suggests that immunoprophylaxis by pneumococcal conjugate vaccination might result in reduction of RTI caused by *S. pneumoniae*. Data from two large efficacy trials in healthy infants indeed show a 5-10% reduction in the occurrence of AOM [23;24]. Most importantly, in otitis-prone children the efficacy is higher [23].

Otitis-prone children differ from healthy age-matched controls in their inability to produce IgG2 and IgA type anti-pneumococcal antibodies after immunization with polysaccharide vaccines [12;13]. In this study we show that one dose of PCV induces good IgG1 antibody responses in most of these children over 2 years of age, even in those with partial serum IgA and/or IgG2 deficiency. However, only low IgA- or IgG2 responses are obtained. After priming with PCV, booster vaccination with PPV23 induces not only a further rise in IgG1-type anti-pneumococcal antibodies, but also the IgA- and IgG2 anti-pneumococcal antibodies which might be of importance in the defense against mucosal infections by encapsulated bacteria. This shows that after priming with conjugate, polysaccharides are able to induce isotype switching to IgG2 and IgA. In children 12-18 months of age, two doses of PCV induce good IgG1-type anti-pneumococcal antibody concentrations, but IgG2- and IgA levels remain low, even in the group with normal total serum IgA-and IgG2 antibody concentrations. Booster vaccination with PCV after primary PCV vaccination apparently does not induce isotype switching in this age group. This may be due to the fact that these children were below two years of age. An alternative explanation could be that the high (25 μg) amount of polysaccharides in the 23-valent polysaccharide vaccine (re)activates polysaccharide-specific B

cells in a way that causes isotype switching, whereas the lower (2-4 μg) amount of polysaccharide coupled to proteins in the PCV, does not. To obtain IgG2- and IgA anti-pneumococcal antibodies, we therefore advocate booster vaccination with PPV23 after completion of primary vaccination schedules with PCV from the age of 24 months. Further study is needed to see whether 23-valent PPV can induce IgA and IgG2 after priming also in children younger than 24 months. In children over two years, one dose of PCV followed by a booster vaccination with 23-PPV results in both IgG1, IgG2 and IgA anti-pneumococcal antibodies. The 23- PPV might also offer the advantage of expanded serotype coverage.

In conclusion, this immunogenicity study shows that the combination of PCV7 followed by 23-valent PPV not only induces IgG1 type anti-pneumococcal antibody responses, but also IgG2 and IgA type responses. Taken into account the results of the clinical efficacy studies that showed a beneficial effect of PCV7 [23] and 14-valent PPV [4] in children with recurrent AOM, we suggest that the sequential use of PCV7 and PPV23 may contribute significantly to the prevention of frequent recurrence of AOM. Such a vaccination schedule could be used in patients with recurrent upper respiratory tract infections both with normal serum immunoglobulin concentrations and in patients with partial IgG2 and/or IgA deficiency. In the Netherlands, a large double-blinded randomized clinical trial measuring the efficacy of this vaccination schedule in preventing recurrent episodes of AOM in otitis-prone children (OMAVAX-study) is currently ongoing.

Ad. Tables 1 and 2

[1]Dysimmunoglobulinemia was expressed as follows:

Patients vaccinated with PPV: 8 patients with partial IgG2 deficiency, 3 patients with partial IgA deficiency and 2 patients with combined partial IgG2- and IgA deficiency.
Patients vaccinated with PCV followed by PPV: 1 patient with partial IgG2 deficiency, 2 patients with partial IgA deficiency and 2 patients with combined partial IgG2- and IgA deficiency.
Patients vaccinated with doses of PCV: 2 patients with partial IgG2 deficiency, 1 patient with partial IgA deficiency and 1 patient with combined partial IgG2- and IgA deficiency.

		PPV		PCV		PCV+PPV	
Number of patients		27		12		8	
Age (months)		43	(24-71)	44	(24-70)	48	(24-70)
Serotype	Isotype	geo	range	geo	range	geo	range
4	IgG1	0.22	(0.01-4.00)	0.23	(0.02-1.23)	0.21	(0.08-1.33)
4	IgG2	0.43	(0.03-9.88)	0.68	(0.10-5.20)	1.53	(0.60-9.22)
4	IgA	0.26	(0.02-1.83)	0.19	(0.05-0.82)	0.20	(0.12-0.54)
6	IgG1	0.27	(0.02-6.02)	§ 0.39	(0.02-5.13)	** 1.00	(0.02-32.20)
6	IgG2	0.46	(0.23-2.06)	0.91	(0.23-10.05)	** 2.97	(0.23-52.52)
6	IgA	0.14	(0.01-0.65)	0.07	(0.01-1.24)	0.13	(0.01-1.31)
9	IgG1	1.30	(0.03-12.27)	0.56	(0.13-6.01)	1.07	(0.35-3.36)
9	IgG2	0.72	(0.04-25.17)	0.81	(0.04-62.30)	* 1.89	(0.04-334.0)
9	IgA	0.67	(0.16-3.65)	0.48	(0.14-4.42)	0.93	(0.16-4.60)
19	IgG1	0.32	(0.03-1.14)	§ 0.62	(0.22-1.27)	** 1.12	(0.28-10.36)
19	IgG2	0.14	(0.05-7.19)	1.74	(0.05-126.3)	** 5.40	(1.50-18.74)
19	IgA	0.56	(0.06-4.31)	0.35	(0.06-1.78)	0.93	(0.23-2.15)

		post PCV1		post PCV2	
Number of patients		4		4	
Age (months)		13	(12-16)	13	(12-16)
Serotype	Isotype	geo	range	geo	range
4	IgG1	0.09	(0.00-0.60)	0.18	(0.01-0.72)
4	IgG2	0.20	(0.03-0.68)	0.57	(0.15-7.24)
4	IgA	0.03	(0.01-0.14)	0.03	(0.01-0.08)
6	IgG1	0.07	(0.02-0.40)	0.05	(0.02-1.86)
6	IgG2	0.23	(0.23-0.46)	0.91	(0.23-18.95)
6	IgA	0.02	(0.01-0.07)	0.06	(0.01-2.53)
9	IgG1	0.10	(0.05-0.19)	1.48	(0.30-20.45)
9	IgG2	0.45	(0.09-2.34)	0.58	(0.13-2.65)
9	IgA	0.08	(0.03-0.32)	0.34	(0.05-3.47)
19	IgG1	0.14	(0.01-0.90)	1.04	(0.03-29.52)
19	IgG2	0.19	(0.05-0.33)	5.97	(1.22-94.93)
19	IgA	0.02	(0.02-0.02)	0.43	(0.02-10.43)

Table 1a. Geometric mean (geo) antibody responses, (μg/ml) to pneumococcal vaccination in children with recurrent bacterial respiratory tract infections and normal serum immunoglobulin levels.

§ indicates a significant difference in antibody concentration after vaccination with PCV compared with PPV; § $p<0.05$.

* indicates a significant difference in antibody concentration after vaccination with PCV followed by booster vaccination with PPV(PCV+PPV), compared with PPV only; * $p<0.05$, ** $p<0.01$.

		PPV		PCV		PCV+PPV	
Number of patients		13		5		5	
Age (months)		50	(34-72)	52	(40-71)	52	(40-71)
Serotype	Isotype	geo	range	geo	range	geo	range
4	IgG1	0.13	(0.00-0.40)	0.13	(0.02-0.32)	0.22	(0.04-0.45)
4	IgG2	0.23	(0.03-2.14)	0.23	(0.03-0.75)	0.50	(0.03-1.08)
4	IgA	0.10	(0.01-0.36)	0.13	(0.07-0.46)	0.10	(0.02-0.30)
6	IgG1	0.46	(0.02-5.48)	§ 2.13	(0.13-24.41)	** 4.91	(0.08-34.51)
6	IgG2	0.69	(0.23-12.33)	1.83	(0.23-59.83)	* 2.74	(0.23-24.44)
6	IgA	0.10	(0.01-0.55)	0.19	(0.01-0.86)	0.17	(0.01-0.82)
9	IgG1	1.06	(0.01-4.70)	0.47	(0.14-1.29)	1.48	(0.18-8.49)
9	IgG2	0.49	(0.04-17.84)	0.45	(0.09-18.52)	1.75	(0.13-78.03)
9	IgA	0.39	(0.02-4.35)	0.24	(0.08-1.38)	0.87	(0.24-4.68)
19	IgG1	0.27	(0.02-3.50)	§ 1.48	(0.21-4.03)	** 6.53	(0.70-41.90)
19	IgG2	0.19	(0.05-7.05)	1.41	(0.28-13.29)	** 16.86	(4.27-72.38)
19	IgA	0.41	(0.02-2.73)	1.34	(0.91-2.00)	** 3.06	(0.35-16.31)

		post PCV1		post PCV2	
Number of patients		4		4	
Age (months)		16	(13-18)	16	(13-18)
Serotype	Isotype	geo	range	geo	range
4	IgG1	0.18	(0.06-0.37)	0.33	(0.16-0.62)
4	IgG2	0.17	(0.03-6.31)	0.80	(0.13-7.89)
4	IgA	0.06	(0.03-0.12)	0.05	(0.03-0.10)
6	IgG1	0.76	(0.12-5.04)	8.42	(0.30-126.0)
6	IgG2	1.14	(0.23-68.05)	1.60	(0.23-67.14)
6	IgA	0.04	(0.01-0.66)	0.13	(0.01-0.67)
9	IgG1	0.16	(0.13-0.21)	0.45	(0.09-2.28)
9	IgG2	0.36	(0.09-3.73)	0.72	(0.04-12.50)
9	IgA	0.10	(0.03-0.31)	0.10	(0.03-0.22)
19	IgG1	0.32	(0.04-9.97)	1.49	(0.04-6.73)
19	IgG2	0.33	(0.23-0.80)	0.70	(0.14-11.79)
19	IgA	0.08	(0.02-0.41)	0.19	(0.02-0.85)

Table 1b. Geometric mean (geo) antibody responses (expressed in µg/ml) to pneumococcal vaccination in children with dysimmunoglobulinemia[1] and recurrent bacterial respiratory tract infections.

§ indicates a significant difference in antibody concentration after vaccination with PCV compared with PPV; § $p<0.05$.

* indicates a significant difference in antibody concentration after vaccination with PCV followed by booster vaccination with PPV(PCV+PPV), compared with PPV only; * $p<0.05$, ** $p<0.01$.

		PPV		PCV		PCV+PPV	
Number of patients		27		12		8	
Age (months)		43	(24-71)	44	(24-70)	48	(24-70)
Serotype	Isotype	geo	range	geo	range	geo	range
4	IgG1	130	(5-2313)	133	(11-713)	119	(46-771)
4	IgG2	17	(1-393)	27	(4-207)	61	(24-367)
4	IgA	27	(2-191)	20	(5-85)	21	(12-56)
6	IgG1	16	(1-357)	§23	(1-304)	**59	(1-1909)
6	IgG2	2	(1-9)	4	(1-44)	**13	(1-230)
6	IgA	12	(1-54)	6	(1-103)	11	(1-109)
9	IgG1	190	(5-1787)	82	(19-876)	156	(51-489)
9	IgG2	16	(1-560)	18	(1-1386)	*42	(1-7430)
9	IgA	42	(10-227)	30	(9-275)	58	(10-286)
19	IgG1	53	(5-190)	§103	(37-212)	**187	(46-1730)
19	IgG2	3	(1-153)	37	(1-2689)	**115	(32-399)
19	IgA	29	(3-222)	18	(3-92)	48	(12-111)

		post PCV1		post PCV2	
Number of patients		4		4	
Age (months)		13	(12-16)	13	(12-16)
Serotype	Isotype	geo	range	geo	range
4	IgG1	54	(1-346)	104	(7-416)
4	IgG2	8	(1-27)	23	(6-288)
4	IgA	3	(1-15)	3	(1-8)
6	IgG1	4	(1-24)	3	(1-110)
6	IgG2	1	(1-2)	4	(1-83)
6	IgA	2	(1-6)	5	(1-211)
9	IgG1	14	(7-27)	216	(43-2979)
9	IgG2	10	(2-52)	13	(3-59)
9	IgA	5	(2-20)	21	(3-216)
19	IgG1	24	(2-150)	173	(5-4932)
19	IgG2	4	(1-7)	127	(26-2021)
19	IgA	1	(1-1)	22	(1-538)

Table 2a. Geometric mean antibody responses (expressed in U/ml) to pneumococcal vaccination in children with recurrent bacterial respiratory tract infections and normal serum immunoglobulin levels.

§ indicates a significant difference in antibody concentration after vaccination with PCV compared with PPV; § $p<0.05$.

* indicates a significant difference in antibody concentration after vaccination with PCV followed by booster vaccination with PPV(PCV+PPV), compared with PPV only; * $p<0.05$, ** $p<0.01$.

		PPV		PCV		PCV+PPV	
Number of patients		13		5		5	
age (months)		50	(34-72)	52	(40-71)	52	(40-71)
Serotype	Isotype	geo	range	geo	range	geo	range
4	IgG1	77	(1-229)	77	(11-184)	130	(25-262)
4	IgG2	9	(1-85)	9	(1-30)	20	(11-43)
4	IgA	10	(1-38)	14	(7-48)	10	(2-31)
6	IgG1	27	(1-325)	§126	(8-1447)	**291	(5-2046)
6	IgG2	3	(1-54)	8	(1-266)	*12	(1-107)
6	IgA	8	(1-46)	16	(1-72)	14	(1-68)
9	IgG1	154	(1-684)	68	(20-188)	215	(26-1237)
9	IgG2	11	(1-397)	10	(2-412)	39	(3-1736)
9	IgA	24	(1-271)	15	(5-86)	54	(15-291)
19	IgG1	45	(3-585)	§247	(35-674)	**1091	(117-7000)
19	IgG2	4	(1-150)	30	(6-283)	**359	(91-1541)
19	IgA	21	(1-141)	69	(47-103)	**158	(18-841)

		post PCV1		post PCV2	
Number of patients		4		4	
age (months)		16	(13-18)	16	(13-18)
Serotype	Isotype	geo	range	geo	range
4	IgG1	102	(35-215)	190	(94-359)
4	IgG2	7	(1-251)	32	(5-314)
4	IgA	6	(3-13)	5	(3-10)
6	IgG1	45	(7-299)	499	(18-7471)
6	IgG2	5	(1-298)	7	(1-294)
6	IgA	3	(1-55)	11	(1-56)
9	IgG1	23	(19-31)	65	(13-332)
9	IgG2	8	(2-83)	16	(1-278)
9	IgA	6	(2-19)	6	(2-14)
19	IgG1	54	(6-1666)	249	(7-1124)
19	IgG2	7	(5-17)	15	(3-251)
19	IgA	4	(1-21)	10	(1-44)

Table 2b. Geometric mean (geo) antibody response (expressed in U/ml) to pneumococcal vaccination in children with dysimmunoglobulinemia[1] and recurrent bacterial respiratory tract infections.

§ indicates a significant difference in antibody concentration after vaccination with PCV compared with PPV; § $p<0.05$.
* indicates a significant difference in antibody concentration after vaccination with PCV followed by booster vaccination with PPV(PCV+PPV), compared with PPV only; * $p<0.05$, ** $p<0.01$.

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Chapter 6

Fcg-receptor polymorphisms determine the magnitude of in vitro phagococytosis of *Streptococcus pneumoniae* mediated by pneumococcal conjugate sera.

Wouter T.M. Jansen[1], Mijke A. Breukels[2], Harm Snippe[1],
Lieke AM Sanders[2], André F.M. Verheul[1] and Ger T. Rijkers[2]

[1]Eijkman-Winkler Institute for Microbiology, Infectious Diseases and Inflammation, Section Vaccines, University Medical Center, Utrecht, The Netherlands.
[2]Department of Pediatric Immunology, Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands

Journal of Infectious Diseases (1999). *180*:888-891.

Abstract

Fcγ receptors show two genetically determined polymorphisms: the biallelic FcγRIIa-R131 and -H131 polymorphism, and the NA1/NA2 FcγIIIb polymorphism. Using 10 pre- and postconjugate vaccination antisera from adults, we analyzed in vitro phagocytic capacities of three different combinations of polymorphonuclear leukocyte FcγR allotypes: those homozygous for the H131 and Na1 allotype, those homozygous for the R131 and Na2 allotype, and those heterozygous for both receptors. For pre- and post vaccination sera, mean phagocytosis levels for the homozygous H131/Na1 allotype were 4-fold higher than for the homozygous R131/Na2 allotype. There was a strong and significant correlation between IgG2 ELISA antibody titers and phagocytosis levels for the homozygous H131/Na1 Fcγ receptor allotype and the heterozygous allotype, but not for the homozygous R131/Na2 allotype. There was no relation between IgG1 ELISA titer and phagocytosis level. Apparently the IgG2 antibodies induced are functionally the most important. This may explain the large impact of Fcγ receptor polymorphisms on the in vitro phagocytosis of pneumococci mediated by conjugate antisera.

Introduction

Pneumococcal conjugate vaccines (many of which are being evaluated in phase III trials) may be able to protect risk groups that are not covered by polyvalent polysaccharide vaccines. Ultimately, the parameter for evaluation of the efficacy of these vaccines is the incidence of pneumococcal disease in the vaccinated group versus that in the nonvaccinated group. Because clinical protection against infection with encapsulated bacteria depends on antibodies against capsular polysaccharides, pneumococcal antibody levels after vaccination can be used as a surrogate marker for vaccine efficacy. Since the crucial defense mechanism against *S. pneumoniae* consists of antibody and complement-mediated phagocytosis, the assessment of functional antibody levels may give a better indication of vaccine efficacy. We have developed a fast, standardized phagocytosis assay based on flow cytometry [1]. This assay is serotype specific and exhibits, in general, highly significant correlations with ELISA and classical killing assay (W.T.M. Jansen et al., unpublished data). All functional components of the assay are of human origin (e.g. human complement and human polymorphonuclear leukocytes [PMNLs]).

PMNLs constitutively express two different receptors for IgG: FcγRIIa and FcγRIIIb. These receptors show two genetically determined polymorphisms: the biallelic FcγRIIa-R131 and -H131 polymorphism, and the NA1/NA2 FcγIIIb polymorphism. In humans, only FcγRIIa-H131 can efficiently interact with IgG2. FcγRIIIb NA1 homozygotes have a higher phagocytosing capacity than the NA2 homozygotes for IgG1 and IgG3 opsonized particles [2]. Of the general Caucasian population, about 25% is FcγRIIa-RR, 50% FcγRIIa-RH and 25% FcγRIIa-HH [3]. Genotype frequencies for the Na1 and Na2 FcγIIIb polymorphism are ~ 0.37 and ~ 0.67, respectively [4].

In infants, children, and the elderly, pneumococcal conjugate vaccines elicit a different antibody response than polysaccharide vaccines in induction of memory and a higher immunogenicity. Polysaccharide conjugates induce a shift in IgG isotype from IgG2 towards IgG1 [5]. Since only the FcγRIIa-H131 can efficiently interact with IgG2, Fcγ-receptor polymorphisms should mainly influence phagocytosis mediated by antibodies of the IgG2 isotype. Therefore, we studied whether PMNL Fcγ-receptor polymorphism has an effect on in vitro phagocytosis of *S. pneumoniae* mediated by antibodies induced by a pneumococcal conjugate vaccine.

Methods

Human antisera

Healthy adult volunteers (n=10) were immunized intramuscularly with heptavalent pneumococcal conjugate vaccine (PCV; Wyeth Lederle Vaccines and Pediatrics Rochester, NY). PCV includes polysaccharides of serotypes 4, 6B, 9V, 14, 19F and 23 F conjugated to CRM197 carrier protein, and an oligosaccharide conjugate of serotype 18C. Each dose contains 2 μg saccharides of pneumococcal serotypes 4, 9V, 14, 18C, 19F and 23F, and 4 μg of serotype 6B. Serum was drawn before and 3-4 weeks after vaccination and stored at -80°C until use.

Determination of type specific anti-polysaccharide antibodies

IgG-, IgG1- and IgG2- serum antibody levels to pneumococcal serotype 23F polysaccharide were measured by ELISA as described previously [6]. Pre- and postvaccination serum samples were analyzed simultaneously. All serum samples were preincubated overnight with excess free common cell wall polysaccharide (CPS) to remove anti-CPS antibodies. A serum sample from a normal non-vaccinated adult was included in every ELISA as a control. Antibody concentrations were expressed relative to a reference adult hyper immune plasma pool [7]. The latter was assigned 100 U/ml (100%) for each isotype.

Typing for FcγRIIa and FcγRIIIb polymorphisms

The allotypes of the FcγRIIa and FcγRIIIb receptors in white Dutch Caucasian donors were determined by nested polymerase chain reaction (PCR) as described [8]. To determine the FcγRIIa-RR/HH polymorphism, a 1100 bp fragment containing the polymorphic region was amplified. This PCR product was used in two allele-specific-PCRs, one with a histidine (H) primer and one with the arginine (R) primer. To determine FcγRIIIb-NA1/NA2 polymorphism a 506 bp fragment was amplified. This PCR product was used in two separate PCRs with specific primers for NA1 and NA, which require different annealing temperatures. All products were run on a 1.5% agarose gel and photographed.

Phagocytosis assay

The assay was performed as described previously [1]. A pneumococcal strain, serotype 23F, was grown three times to log phase to ensure high encapsulation, then heat

inactivated and labeled with fluorescein isothiocyanate (FITC). Human pooled serum was depleted of IgG by protein G affinity chromatography and used as complement source. Human PMNLs were isolated from the blood of healthy, Fcγ receptor-typed volunteers by a ficoll-histopaque gradient.
Serial dilutions of heat-inactivated serum samples and a fixed amount of complement (2% vol/vol) were made in bovine serum albumin-Hanks' balanced salt solution (BSA-HBSS) and pipetted into round-bottomed micro-titer plates. Samples of 2.5 H 10^6 pneumococci were added to each well and incubated for 30 min at 37°C with shaking on a micro-titer plate agitator. Next, plates were placed on ice and 2.5 H 10^5 PMNLs in 50µl BSA-HBSS were added to each well. Phagocytosis was done at 37°C with shaking. After washing with ice-cold BSA-HBBS, the cells were fixed with 2% paraformaldehyde in phosphate buffered saline and analyzed by flow cytometry (FACScan, Becton Dickinson, San José, CA). The percentage of FITC positive PMNLs was used as a measure for the phagocytic activity of serum. Results are expressed as 25% phagocytosis titers (serum dilution resulting in 25% FITC-positive PMNLs).

Statistical analysis
Correlations between ELISA and FACS phagocytosis titers were analyzed by linear regression tests. Statistical significance of correlations was assessed by Pearson correlation analysis. Statistically significant differences between pre- and post vaccination phagocytosis titers for a given PMNL allotype, as well as differences in phagocytosis titers between different PMNL allotypes, were assessed with a Wilcoxon signed rank test. $P < 0.05$ was considered statistically significant.

Results

To study the effect of PMN Fcγ receptor polymorphism on the phagocytosis of *S. pneumoniae*, we compared three different allotype combinations: PMNL that were homozygous for both receptors (HH11), which were expected to give the highest phagocytosis; PMNL that were heterozygous for both receptors (HR12), which were expected to give an intermediate phagocytosis, and PMNL that were homozygous for both receptors (RR22), which were expected to give the lowest phagocytosis.

Different allotypes were distinguished by allele specific PCR as shown in Figure 1.

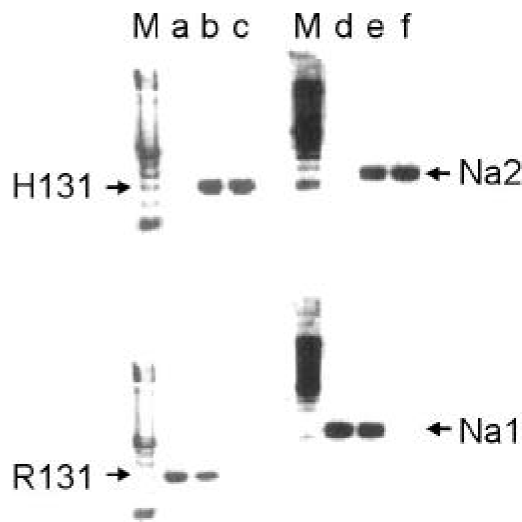

Figure 1. Polymerase chain reaction analysis of FcγRIIa and FcγRIIIb genotype.

DNA was amplificated with FcγRIIa-H131 or -R131 specific primers (upper and lower left panels, respectively) or using FcγRIIIb-Na2 or -Na1 specific primers (upper and lower right panels respectively). Amplified products were analyzed by agarose gel electrophoresis and EtBr staining. The figure shows the three possible allele combinations for FcγRIIa (homozygous R131, lane a; R/H131, b; H/H131, c) and for FcγRIIIb (Na1/Na1, d; Na1/Na2, e; Na2/Na2, f). M denotes bp ladder.

Vaccination with a heptavalent pneumococcal conjugate results in an increase in functional antibody titers for all three PMNL allotypes, as measured by the in vitro phagocytosis of type 23F S. pneumoniae ($p < 0.01$, all allotypes). The degree of phagocytosis depends on the allotype of FcγRIIa and FcγRIIIb. The highest fold-increase (FI) in phagocytosis as well as geometric mean phagocytosis titer (GMPT) of the 10 postvaccination sera tested was obtained with HH11 PMNLs (FI, 17.2; GMPT, 38; Figure 2). Using RR22 PMNLs, vaccination induced the smallest increase in phagocytosis (FI, 4.7; GMPT, 9.4); and with heterozygous PMNLs (HR12), we observed intermediate values (FI, 13.3; GMPT, 20.6).

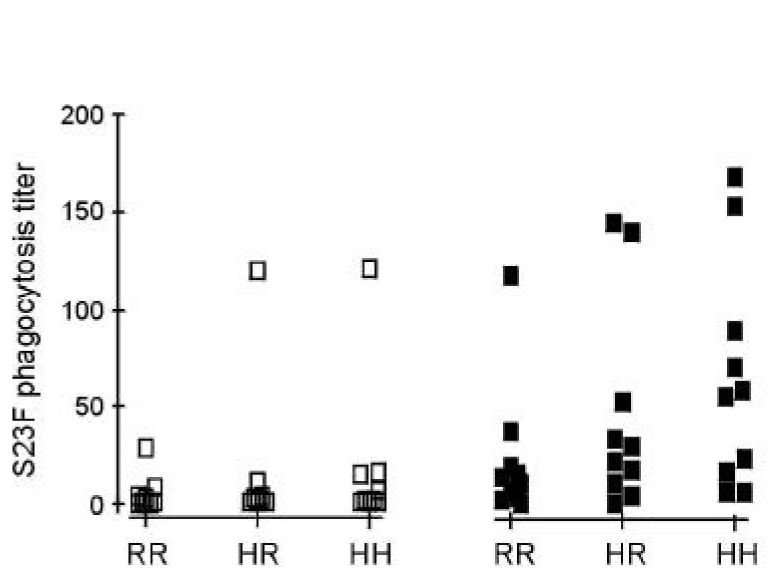

Figure 2. S23F phagocytosis titers pre (open squares) and post (closed squares) immunization with a heptavalent conjugate vaccine for three different PMN allotypes.

For the pre vaccination sera, p-values for the difference in phagocytosis titers between the allotypes were: HH11 versus RR22 $p < 0.05$; HR12 versus RR22 $p < 0.05$. For the post-vaccination antisera, all the observed differences in phagocytosis titers were significant: HHII PMN versus HR12 PMN $p < 0.005$; HR12 PMN versus RR22 PMN $p < 0.05$; HHII PMN versus RR22 $p < 0.005$. (RR: FcγRIIa RR / FcγRIIIb NA2NA2, HR: FcγRIIa HR / FcγRIIIb NA1NA2 and HH: FcγRIIa HH / FcγRIIIb NA1NA1).
The pre-immune serum sample causing the high pre-immune phagocytosis titer corresponds to the post-immune serum sample with the highest phagocytosis titer with the three allotypes.

Since FcγRIIa H131 and R131 differ in their ability to interact with IgG2 antibodies, we next measured subclasses of IgG anti-23F antibodies (Table 1) and studied their relation to phagocytosis (Table 2). For both total IgG and IgG2, there was a strong and significant correlation between phagocytosis of 23F *S. pneumoniae* by HH11 PMNLs and post-vaccination ELISA titer. A significant correlation was also observed for HR12 PMNLs but not for RR22 PMNLs. Surprisingly, ELISA IgG1 titers did not correlate at all with phagocytosis, independent of the PMN allotype.

Discussion

Our data clearly demonstrate that FcγR polymorphisms affect the magnitude of in vitro phagocytosis of pneumococci mediated by pneumococcal conjugate antisera. Both phagocytosis titer as well as fold increase in titer obtained with PMNLs homozygous for

histidine and NA1, are 4-fold higher than those obtained with PMNLs that are homozygous for arginine and NA2. These data have important consequences for the usage and interpretation of in vitro assays measuring vaccine efficacy.

serum	IgG		IgG1		IgG2	
no.	pre	post	pre	post	pre	post
1	39	1060	12	43	6	5
2	52	1143	33	4992	438	6693
3	67	1900	1	318	50	4951
4	210	3597	57	248	14	1932
5	5	25	3	23	82	11
6	9	23	19	213	33	1093
7	49	2498	57	125	21	62
8	29	1162	21	513	15	170
9	138	7067	13	62	833	10000
10	56	439	28	76	642	10000

Table 1. IgG, IgG1 and IgG2 antibody levels (U/ml)against serotype 23F *S. pneumoniae* before (pre) and 3-4 weeks after (post) vaccination with heptavalent pneumococcal conjugate vaccine.

PMNL allotype	IgG	IgG1	IgG2
RR22	0.25	0.05	0.19
HR12	0.46*	0.04	0.48*
HH11	0.58*	0.0	0.64**

Table 2. Correlation (r^2) between postvaccination anti-23F antibody levels and phagocytosis titers of polymorphonuclear leucocyte (PMNL) FcγR allotypes.

PMNL FcγR allotype combinations used were as follows: RR22: FcγRIIa RR/FcγRIIIb NA2NA2; HR12:FcγRIIa HR/FcγRIIIb NA1NA2; and HH11FcγRIIa HH/FcγRIIIb NA1NA1. *$p<0.05$; **$p<0.01$.

There is no consensus about what level of antibodies defines a vaccine responder. Most studies define a responder as a vaccinated person who shows a 2- or 4-fold increase in

antibody level after immunization: however, other studies specify different levels of postimmunization antibody as thresholds [9]. Nor is there agreement on quantitative protective antibody levels. The same uncertainties also apply to our functional antibody assay. Indeed, the data reveal that FcγR allotype combination of the vaccinee is another important variable that may determine conjugate vaccine efficacy. Clearly, the definition of protective functional antibody levels awaits the outcome of ongoing vaccine efficacy trials.

Our data suggest that individuals with the most unfavorable FcγR allotype combination would have to produce four times more antibodies than persons with the optimal allotype combination to reach equivalent antibody titers. This finding not only underscores the importance of FcγR polymorphism for phagocytosis of *S. pneumoniae*, but also has impact for the design of in vitro assays for functional antibodies. Clearly, the FcγR allotype combination of donor PMNLs must be known. Whether an optimal (FcγRIIa HH131, FcγRIIIbNa1/Na1) or a less favorable combination of FcγR should be used in functional antibody assays remains to be determined.

Although the effect of Fcγ receptor polymorphism has been studied extensively at the molecular level, little is known about the clinical relevance of these different allotypes in the normal population. Sanders et al. [3] showed that of 48 children with frequent bacterial respiratory tract infections and normal IgG2 titers, a smaller proportion were of the FcγRIIa-HH phenotype than in a large healthy white control population. Platonov et al. [10] showed that FcγRIIa polymorphisms are associated with susceptibility to and severity of meningococcal disease.

The use of protein conjugated polysaccharide vaccines results in an anti-polysaccharide antibody response with characteristics of a T cell dependent response, including IgG1 antibodies. Yet, our data indicate that the level of phagocytosis correlates with IgG2 ELISA titers and not with IgG1 titers. This suggests that in both polysaccharide and protein-conjugated polysaccharide vaccines, the functionally most active antibodies are of the IgG2 isotype. The concept that IgG2 anti-polysaccharide antibodies are important for the protection against encapsulated bacteria seems to be supported by clinical observations and experimental data. Rodriguez et al. [11] showed that FcγRIIa and IgG2 are crucial in phagocytosis of pneumococci. Patients with IgG2 deficiency are prone to infections with *S. Pneumoniae* and *Haemophilus influenzae* type b [12]. Otitis-prone children tend to have similar IgG1 anti-pneumococcal antibody levels compared to age-matched controls, but lower IgG2 levels [13].

The same isotype distribution of anti-pneumococcal antibodies is observed in children with otitis media with effusion (I.J. Dhooge et al., unpublished data). Moreover, since the polymorphism in FcγRIIa determines the capacity to interact with IgG2 antibodies, but binding of IgG1 does not differ between the H131 and R131 isoforms, the dominance of IgG2 antibodies in phagocytosis would explain the effect of FcγR polymorphisms in this study. Clearly, functional experiments with purified IgG1 and IgG2 anti-polysaccharide antibodies (induced by either conjugated or non-conjugated polysaccharide vaccines) are required in order to substantiate this conclusion.

Acknowledgments

We thank J. Van der Winkel and N. Westerdaal, Department of Immunology, University of Utrecht, for providing the facilities and help for typing donors for FcγIIa and FcγIIIb allotypes, and Wyeth-Lederle Vaccines and Pediatrics, Rochester NY, USA for providing the heptavalent pneumococcal conjugate vaccine.

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Chapter 7a

Immunological characterization of conjugated *Haemophilus influenzae* type b vaccine failures after infant vaccination.

Mijke A. Breukels [1], Lodewijk Spanjaard [2], Lieke A.M. Sanders [1], Ger T. Rijkers [1].

[1]Department of Pediatric Immunology, Wilhelmina Children's Hospital, University Medical Center, Utrecht, the Netherlands
[2]Netherlands Reference Laboratory for Bacterial Meningitis (AMC/RIVM), Department of Medical Microbiology, Academic Medical Center Amsterdam, the Netherlands

Accepted for publication, Clinical Infectious Diseases (2001).

Abstract

Infant vaccination with conjugated *Haemophilus influenzae* type b (Hib) vaccine is highly effective in protecting against invasive Hib infections, but vaccine failures do occur. Twenty-one vaccine failures are reported since the introduction of the Hib conjugate vaccine in the Netherlands. Of the 14 evaluable patients, 6 children had an absent antibody response (< 1.0 μg/mL IgG anti-Hib) to Hib polysaccharide in convalescent serum, including one child with hypogammaglobulinaemia and one child with IgG2 deficiency. After revaccination almost all children developed anti-Hib antibodies.
In case of Hib vaccine failure, case investigation, including measurement of serum immunoglobulin concentrations as well as specific anti-Hib antibodies should be performed. Invasive Hib disease after infant conjugate Hib vaccination may be the presentation of an underlying immunodeficiency, but more often only a decreased antibody response to Hib is found; revaccination with conjugated Hib vaccine is advised.

Introduction

Infant vaccination with conjugated *Haemophilus influenzae* type b (Hib) vaccine is highly effective in protecting against invasive Hib infections [1;2]. In the Netherlands, vaccination with conjugate Hib vaccine has been incorporated into the National Vaccination Program for children born after April 1, 1993. At the time of introduction, the Hib vaccine was given at the ages of 3, 4, 5, and 11 months, concomitantly with the DTP and poliomyelitis vaccinations. Unlike the policy of several other countries, no catch-up vaccination did take place for children under the age of five years. From January 1999 onwards, infants are vaccinated at 2, 3, 4 and 11 months of age.

In the years following the introduction of Hib vaccination, the number of cases of invasive Hib disease dropped impressively [3;4]. But invasive Hib disease did not disappear completely. This might be expected, since cases of invasive Hib disease still occur in not yet fully vaccinated infants, and vaccine coverage in the Netherlands, although high (95,5% of all infants received at least three doses of Hib vaccine; January 1997), is not complete. But also true vaccine failures did occur in children who were completely (3 or 4 times) vaccinated with the conjugate vaccine. We here report the clinical and immunological follow-up of the 19 children who developed invasive Hib disease despite having received at least three vaccinations as an infant.

Methods

Surveillance of invasive Hib infections

Since 1975, invasive Hib isolates and surveillance data on Hib meningitis in the Netherlands are collected by the Netherlands Reference Laboratory for Bacterial Meningitis (RBM) in Amsterdam. Since 1994, *Haemophilus influenzae* isolates from all normally sterile sites are sent to the RBM. These isolates are identified and typed for capsular polysaccharide serotype using coagglutination [5]. Capsular polysaccharide serotyping was controlled by PHLS Haemophilus Reference Unit, Oxford, UK

Collection and testing of serologic data

The laboratory of the department of Immunology, Wilhelmina Children's Hospital, University Medical Center Utrecht, is a central facility for anti-Hib antibody determinations. Sera from patients with invasive Hib infections are sent in by clinicians. Since 1999, active immunological follow-up of patients with invasive Hib infections despite vaccination is realized by combining the serological data with the RBM data system.

IgG, IgG1 and IgG2 (and IgM, data not shown) anti-Hib antibody titers were determined by ELISA as described, and calibrated using a standard anti-Hib hyperimmune serum, containing 27.2 μg/mL IgG-anti-Hib, 4.05 μg/mL IgG1-anti-Hib and 9.48 μg/mL IgG2-anti-Hib [6]. Total serum concentrations of IgG, IgA and IgM, as well as serum concentrations of IgG subclasses were determined by radial immunodiffusion in serum samples obtained at onset of illness, except for patients d, g, i, and n, for whom the serum samples obtained at convalescence were used.

Vaccine failures

Cases of invasive Hib infections are defined as vaccine failures when they occur despite at least three vaccinations with the conjugated Hib vaccine (or after one vaccination in a child > 1 year). In the Netherlands the Hib vaccine used is the PRP-T vaccine (polyribosylribitolphosphate conjugated to tetanus toxoid, Pasteur Mérieux Sérums et Vaccins, Lyon, France)

Subjects

We obtained additional clinical information and evaluated the immune status of patients who developed invasive Hib disease despite three or four vaccinations. The invasive Hib infection was proven by culturing Hib from blood and/or cerebrospinal fluid. Vaccination records were checked. Serum samples were taken at the time of disease and after reconvalescence (3-4 weeks after the onset of the disease). For patients with IgG anti-Hib antibody concentrations < 1.0 μg/mL in sera obtained 3-4 weeks after the onset of the disease, revaccination with the Hib vaccine was recommended. Booster vaccination was also given when anti-Hib antibodies were slightly above this level. In both cases informed consent was obtained. Three to four weeks after revaccination, IgG, IgG1, IgG2 and IgM anti-Hib antibody concentrations were determined again. Vaccination was repeated in case of persistently low antibody concentrations.

Results

Hib failures

In the 7 years after the introduction of the Hib vaccination program for infants in the Netherlands, a total number of 1.13 million children received at least three doses of Hib vaccine. 21 true vaccine failures were reported: 17 (81%) cases of Hib meningitis, one (5%) Hib cellulitis, and 3 (19%) cases of Hib epiglottitis. Bacterial isolates of 19 (90%) of these patients were directly sent to the RBM. From the two (10%) additional (epiglottitis-) patients, bacterial isolates from blood were typed at the local clinical microbiology laboratories, and sera of these patients were sent to the laboratory of the department of Immunology, University Medical Center Utrecht. Checking of vaccination data confirmed that these two cases were vaccine failures, too. The mean age of the meningitis patients was 26 (9-62, median 25) months; the mean age of the epiglottitis patients was 38 (25-56, median 33) months. Clinical information was obtained in 18 (86%) of the 21 children, 11 boys and 7 girls. One of them (patient c) experienced another episode of meningitis -of unknown cause- at the age of 3 months. After the second (Hib) meningitis at the age of 10 months, he received antibiotic prophylaxis till the age of 33 months. Patient a did not experience other invasive infections before the Hib meningitis. She received antibiotic prophylaxis after the diagnosis of hypogammaglobulinaemia. A third patient (patient t) was known with trisomy 16p. Apart from a 2½ years old girl (patient o), who died from Hib meningitis, all children recovered without major sequelae. Importantly, they experienced no further serious infections during the follow-up period. All but one patient (patient f) were of Caucasian origin. Height and weights of all children were within the normal range (± 2 SD) for age according the Dutch standard curves.

Serological investigations

Serum samples were available in 14 (67%) cases. Results of the analysis of humoral immunity are shown in Table 1. Serum immunoglobulin concentrations were within the normal range (±2 SD) for age in 12 (86%) of the 14 patients. In patient f, the serum concentration of IgG2 was below minus 2 SD the mean concentration for his age. Patient a was shown to have hypogammaglobulinaemia, with low concentrations of IgA and IgG, as well as low concentrations of IgG2.

Patient	Sex	Age, m	Disease	IgA	IgG	IgM	IgG1	IgG2	IgG3	IgG4
a	F	24	meningitis	0.04	3.3	0.73	2.8	0.15	0.25	<0.1
b	M	13	meningitis	0.65	9.9	0.9	8.2	0.9	0.55	0.15
c	M	10	meningitis	0.17	5.5	1.37	4.0	0.3	0.2	<0.1
d	M	27	meningitis	0.83	9.5	1.16	5.9	1.65	0.6	0.55
e	F	36	meningitis	1.23	7.7	1.5	7.3	1.05	0.1	0.1
f	M	11	meningitis	0.36	6.4	0.94	5.1	<0.1	0.45	<0.1
g	M	33	epiglottitis	0.75	6.0	1.5	5.7	0.9	0.4	<0.1
h	F	28	meningitis	0.45	11.7	0.95	9.4	1.3	0.6	0.3
i	M	62	meningitis	1.11	11.7	1.84	9.8	0.9	0.5	<0.1
j	F	18	meningitis	0.78	9.9	2.83	8.2	1.41	0.74	<0.05
k	M	56	epiglottitis	0.91	9.0	0.96	5.3	1.65	0.25	0.65
l	M	38	meningitis	1.00	9.8	1.65	7.3	1.5	0.2	0.1
m	M	25	epiglottitis	0.60	9.3	1.3	9.1	1.5	0.2	0.4
n	M	42	meningitis	3.62	12.8	1.5	10.4	2.61	0.27	0.25
o	F	28	meningitis							
p	F	33	meningitis							
q	F	9	meningitis							
r	M	25	meningitis							
s	F	13	cellulitis							
t	M	22	meningitis							
u	M	8	meningitis							

Table 1. Clinical characteristics and serum immunoglobulin concentrations (g/L)

Patient c experienced a first meningitis (unknown cause) at the age of 3 months; he received antibiotical prophylaxis from 10-33 months of age.
Patient o died from complications of Hib meningitis.
Patient t had trisomy 16p.
Serum samples used for determination of immunoglobulin concentrations were obtained at onset of disease for all patients except patients d, g, i, and n, for which convalescence serum samples were used.
Serological data were not available for patients o-u.

Underlined values are below the normal range for age.
Normal values serum immunoglobulin concentrations:
15 months - 4 years: IgA 0,30-0,90 g/L; IgG 4,6-7,8 g/L
12-24 months: IgG1 2,0-6,5 g/L; IgG2 0,35-2,2 g/L
6-12 months: IgG2 0,10-0,50 g/LTable 2. Anti-Hib antibody concentrations during the acute phase (0), after convalescence (I), after revaccination (II and III) for patients a-n.

Specific anti-Hib antibody concentrations in acute serum samples, in convalescent serum samples and antibody concentrations after revaccination are shown in Table 2. Most of the

patients had low (or unknown) anti-Hib antibody titers at the time of disease, with the exception of patient m, who had antibody concentrations well above the level considered to be protective. On the base of analysis of convalescent serum samples, two patient groups could be distinguished: I) a group with persistently low anti-Hib antibody titers after invasive infection (patients a-f) and II) a group of patients with an IgG anti-Hib antibody response > 1 µg/mL following the invasive Hib infection (patients g-n). The mean age of patients of group I was significantly lower than that of group II (20 ± 10 versus 38 ± 15 months; t-test p<0.05).

Patient	IgG anti-Hib (µg/mL)				IgG1 anti-Hib (µg/mL)				IgG2 anti-Hib (µg/mL)			
	0	I	II	III	0	I	II	III	0	I	II	III
a	1,5	<			2.25	<			<	<		
b	1.2	<	9.1	96	0.90	<	10.4	215.8	<	<	<	4.44
c	<	<	65		<	<	75.5		<	<	2.73	
d		<				1.35				<		
e	<	<	7.3		1.35	<	4.5		<	<	16.7	
f	<	<	<		1.80	0.9	0.9		<	<	<	
g		1.35	2.7	18		1.8	1.8	59.3		<	<	11.3
h	<	1.5	27		<	0.9			<	<		
i		3.3				2.25				<		
j	<	9			<	4.05			<	5.12		
k	<	22	6.4		0.90	29.7	12.1		<	4.78	3.41	
l	<	23			<	23.8			<	1.02		
m	23	27			12.6	13.9			1.37	0.68		
n		127				28.8				4.44		

Table 2. Anti-Hib antibody concentrations during the acute phase (0), after convalescence (I), after revaccination (II and III) for patients a-n.

The lower level of detection of IgG anti-Hib antibody was 0.9 µg/mL, of IgG1 anti-Hib antibody was 0.45 µg/mL, and of IgG2 anti-Hib antibody was 0.34 µg/mL. Antibody concentrations below these values are shown as ‘<’.

Mean IgG anti-Hib antibody concentration after three vaccinations in a control group (aged 9-10 months, mean 9.6, median 10, n=83) was 2.8 µg/mL (0.05-6.8), after four vaccinations in a control group (aged 24-43 months, mean 31, median 31, n=221) was 3.2 µg/mL (0.05-64.1).

Two (33%) of the 6 patients in group I were subsequently lost for serological follow-up. The 4 other patients (67%) were revaccinated. The three younger patients (patients b, c and f) received their regular fourth Hib vaccination; the other patient (patient e), who had completed her series of four Hib vaccinations two years before the onset of disease, received a fifth vaccination with PRP-T. Three patients in group II were revaccinated, two of them (patients g and h) because of the fact that their anti-Hib antibody titers had only marginally risen above the minimal protective level. All but one (patient f) of the evaluable12 patients (92%) ultimately reached IgG anti-Hib antibody concentrations > 1.0 µg/mL. The patient that did not respond was the youngest in this series.
Most of the patients who responded with high antibody titers against Hib after their Hib infection (group II), developed predominantly IgG1 anti-Hib antibodies; only patient j produced IgG1 and IgG2 in equal amounts. The revaccination with PRP-T after invasive infection with Hib resulted in patients b and c in an IgG1 anti-Hib response. Both patients were rather young at the time of disease and revaccination. In the older patients, relatively more IgG2 was produced after booster vaccination, although only one patient (patient e) showed an IgG2-dominated antibody response.

Discussion

Invasive *H. influenzae* type b (Hib) infections such as meningitis and epiglottitis are primarily diseases of early childhood, occurring predominantly in children less than 2 years of age, although considerable morbidity is seen until 5 years of age [7]. This age-related susceptibility to Hib disease correlates with the absence of serum bactericidal antibodies to Hib polysaccharide. Natural antibodies specific for Hib polysaccharide begin to appear at approximately 2 years of age and reach adult levels by about 5 years. Hib polysaccharide by itself does not function as an effective vaccine in the primary target population [8]. Covalent conjugation of protein carriers to Hib polysaccharide converts it into an effective immunogen, capable of inducing both IgG antibody production and memory in infants [9;10]. Vaccination with conjugated Hib vaccine has resulted in protection against Hib disease and a rapid decline of the number of cases of invasive Hib disease [11].

In the Netherlands, the Hib conjugate vaccine also proved to be successfull, with an efficacy of 99,4 % [4]. Before the introduction of the conjugate Hib vaccine in the National Vaccination Program of the Netherlands, yearly a number of 700 invasive Hib infections was estimated, of which 90% occurred in children < 5 years of age. About 50% of these invasive Hib infections concerned meningitis, 15-30% epiglottitis [12]. The highest incidence of Hib meningitis was found in children aged 6-12 months, whereas Hib epiglottitis concerned mainly children 2-3 years of age. Following the introduction of Hib vaccination in 1993, the number of invasive Hib infections decreased steadily and sharply: in 1994 106 cases were reported, in 1995 37, in 1996 18 and in 1997 7 cases. Only the minority of these cases were true vaccine failures, with most of the invasive Hib infections occurring in children that were not vaccinated, -either because of religious reasons or because they were not covered by the health care system- , or not yet fully vaccinated because of their age.

We here report the 21 real vaccine failures that have occurred in the Netherlands since the introduction of the Hib conjugate vaccine. Their average age was higher than the age in the previous unvaccinated population, namely 26 months for the meningitis patients and 38 months for epiglottitis patients. The surveillance data obtained during the first three years after the introduction of the Hib vaccine showed an average age of the cases of invasive Hib infections of 7.4 months [4]. The surveillance data obtained 1997- July 2000 however, showed an increase in average age of the Hib patients to 15.5 months (unpublished data RBM). The age shift is most likely due to herd immunity because of reduced circulation of *H. influenzae* type b after the introduction of Hib vaccination [13;14].

In literature [15], 35-40% of children who developed invasive Hib disease after a single dose of conjugated Hib vaccine at the age of >15 months were described to have subnormal concentrations of serum immunoglobulins. Specifically, a high prevalence of subnormal concentrations of IgG2 was observed [15;16]. In contrast, reports on Hib polysaccharide vaccine failures in children older than 18 months of age, showed a low incidence of immunodeficiency: almost all of these children had normal serum concentrations of immunoglobulins, including IgG2 [17]. These data suggested immunological differences between populations of children with polysaccharide and conjugate vaccination failure [16]. However, these results were obtained in children vaccinated at > 15 months of age. In our series of Hib conjugate vaccine failures after infant immunization, one of the 14 evaluable children had hypogammaglobulinaemia, and one had low serum IgG2 concentrations. Although there are only a few patients, this suggests that the

frequency of humoral immunodeficiency in infant Hib conjugate vaccination failure (14% in our series) is not different from the conjugate vaccination failures in children vaccinated at > 15 months of age or in polysaccharide vaccine failures (10%) after 18 months of age [16].

We evaluated the specific anti-Hib antibody production in our patients. An important question is whether vaccination failure resulted from insufficient levels of circulating antibody after vaccination or from a qualitatively different and suboptimally protective antibody response. In 9 of 10 patients from who data were available we found low levels of specific IgG anti-Hib antibodies at the time of disease. These data suggest that these children, similar to children with polysaccharide vaccination failure [16] are low antibody responders to vaccination. However, low levels of anti-Hib antibodies are more frequently observed in this age group ([18] and own unpublished data). In considering the correlation of disease susceptibility with antibody concentrations after Hib conjugate vaccination, account must also be made of the capacity of conjugate vaccines to induce immunological memory [19]. Even infants who fail to show a serum antibody response to immunization with conjugate vaccine may be protected from disease as a result of immunologic priming and the ability to develop a rapid increase in anti-Hib antibody. However, immunologic priming by conjugate Hib vaccine might not always be sufficient to confer protection against invasive Hib disease: some children show high antibody responses to the disease (patient n and [1;15]), and thus were primed, but nevertheless had acquired the invasive Hib infection.

Evidence of priming by the conjugate vaccination was absent in the 6 patients of group I: the two patients with the immunoglobulin deficiency (patients a and f), and 4 out of the 12 children with normal serum immunoglobulin concentrations. They had low anti-Hib antibody concentrations in convalescent serum samples after invasive Hib disease. The failure to see an antibody response in younger children after infection (convalesence) is consistent with impaired capacity of young children to generate antibodies to unconjugated polysaccharides. A defective antibody response to Hib infection is also found in children with Hib polysaccharide vaccine failure. In contrast, children with conjugate vaccination failure after vaccination at the age of > 15 months appear to have normal or even increased antibody responses [16], more alike the children of group II.

The serum antibody concentration sufficient to confer protection is not known with certainty, but based on epidemiologic and animal studies it is estimated to be between 0.1 and 1.0 µg/ml [20-

23]. Apart from the antibody concentration, isotype distribution, avidity and complement mediated bactericidal capacity all may contribute to the 'protective' antibody concentration [24-26].

The ability to mount an antibody response after Hib-vaccination or disease is in part genetically determined. This could play a role in the susceptibility to invasive Hib-infections [27-29]. However, ultimately almost all our patients developed anti-Hib antibodies. Several ethnic populations do respond upon vaccination with Hib conjugate vaccines, but need several extra booster vaccinations [27]. In Navajo-Indians a variant A2 V_k light chain gene (a gene which is used in 60% of anti-Hib antibodies) is found, which may explain their aberrant pattern of anti-Hib antibody development [30]. In patient b and his father (who failed to mount an IgG anti-Hib antibody titer >1.0 μg/mL after a single Hib conjugate vaccination), molecular analysis did not reveal this variant light chain gene, nor another abnormality in the A2 V_k light chain gene (data not shown).

The causes of Hib conjugate vaccine failure are probably multiple and complex. Inadequate delivery of the vaccine might be a major reason. In most children, vaccination failure does not appear to be the result of the presence of hypogammaglobulineamia or absence of certain V-region genes. Certain immunoglobulin allotypes are associated with susceptibility to polysaccharide encapsulated bacteria [31] and risks of vaccination failure [32]. Risk of infection with encapsulated bacteria is also increased in patients with the unfavourable Fc gamma receptor IIa polymorphism [33;34]. The number of vaccine failures in our series for whom we had DNA available was to small to make firm conclusions with respect to a possible relationship of Hib vaccination failure with immunoglobulin allotypes or Fc receptor polymorphisms.

In our series of 21 vaccine failures, of which in 14 patients immunological evaluation could be performed, for one patient a recognized immunodeficiency, namely hypogammaglobulinaemia, was shown to be underlying the vaccine failure. In the others, the Hib disease apparently was an isolated event. Many of them failed to mount antibody responses to Hib, and were therefore at an increased risk of developing second episodes of the disease. Revaccination was able to overcome this impaired antibody response. We conclude that it is worthwhile to perform case investigation, including measurement of serum immunoglobulin levels as well as specific anti-Hib antibodies in case of Hib vaccine failure. Booster vaccination may be necessary.

Acknowledgements

We thank Dr. M.A.E. Conyn-van Spaendonck, Department of Infectious Disaeses Epidemiology, National Institute of Public Health and the Environment, Bilthoven, The Netherlands, and collaborating pediatricians, for providing clinical information.
We thank Dr. Ann J. Feeney, Department of Immunology, The Scripps Research Institute, La Jolla, California, for performing the molecular analysis of the A2 V_k light chain gene for patient b and his father.

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Chapter 7b

Concentration and Avidity of anti-Hib antibodies in sera of Hib-vaccination failures

Mijke A. Breukels[1], Els M. Jol-van der Zijde[2], Maarten J.D. van Tol[2]
Ger T. Rijkers[1]

[1]Department of Pediatric Immunology, Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands
[2]Department of Pediatrics, Leiden University Medical Center, Leiden, The Netherlands

Abstract

Haemophilus influenzae type b (Hib) conjugate vaccines are extremely efficacious in protecting infants and children from invasive Hib infections. However, vaccine failures do occur. We studied the anti-Hib antibody production both quantitatively and qualitatively in 12 Hib failure patients, all with normal serum immunoglobulin concentrations and without clinical risk factors for invasive Hib disease. Both anti-Hib antibody concentration and (IgG2) anti-Hib antibody avidity were significantly lower in Hib failure patients, at onset of disease as well as after reconvalescence, compared with controls. This finding suggests that the patients who developed invasive Hib disease despite three or four Hib conjugate vaccinations were inadequately primed by these vaccinations.

Introduction

Haemophilus influenzae type b (Hib) conjugate vaccines have proven to be extremely efficacious in protecting infants and children from invasive Hib infections [1;2]. However, a very few children still develop invasive Hib disease despite vaccination and are therefore considered vaccine failures. In the Netherlands, vaccination with Hib conjugate vaccine has been incorporated into the National Vaccination Program for all children born after April 1, 1993. In the 7 years since the introduction of the vaccine, 21 vaccine failures have been reported; 14 of these patients have been immunologically characterized (Breukels CID, 2001). Serum immunoglobulin concentrations, including IgG subclasses, were within the normal range for age in 12 (86%) of these children; in one patient the serum concentration of IgG2 was below 2 SD of the mean concentration for age, and one patient had a hypogammaglobulinaemia. Therefore, in the majority of the cases, no recognized humoral immunodeficiency was identified as the underlying cause of the Hib vaccination failure.
We hypothesized that quantitative and/or qualitative defects might specifically exist in the production of anti-Hib antibodies in cases of Hib vaccination failure. Considerable evidence points towards the importance of antibody avidity as a determinant of protective efficacy [3-6]. Most likely, avidity plays a role in determining the ability of antibodies to neutralize Hib when it is encountered in the bloodstream; at low serum concentrations, antibodies of high avidity are more effective than antibodies of lower avidity. We therefore studied Hib antibody avidities in sera obtained from the Hib vaccine failures at onset of disease, at reconvalescence and after revaccination. Children with low antibody titers to Hib after the regular four conjugate Hib vaccinations without clinical manifestations of Hib disease, who were booster vaccinated to evaluate their capacity to produce anti-Hib antibodies, served as controls.

Patients and Methods

Vaccine failures

Cases of invasive Hib infections are defined as vaccine failures when they occur in children despite at least three infant vaccinations with the Hib conjugate vaccine (or after a single vaccination in a child over 1 year of age). In the Netherlands, the Hib vaccine used is the

PRP-T vaccine (polyribosylribitolphosphate (PRP) conjugated to tetanus toxoid, Pasteur Mérieux Sérums et Vaccins, Lyon, France).

Subjects

The 21 true vaccine failures reported in the seven years since introduction of the conjugate Hib vaccine in the National Vaccination Program have been described previously (Breukels, CID 2001). One of these 21 patients had a chromosomal abnormality as clinical risk factor. Serum samples were drawn from 14 patients at the onset of disease and/or after reconvalescence (3-4 weeks after the onset of the disease), and after revaccination in case of persistently low Hib antibody concentrations. In one of these 14 patients the serum concentration of IgG2 was below 2 SD of the mean concentration for age, and one patient had hypogamma-globulinaemia. These 2 patients with abnormal serum immunoglobulin concentrations were excluded from this study, so in total 12 patients were evaluated.

As controls we used sera from 13 children with recurrent respiratory tract infections submitted to our laboratory in the same time period for immunological evaluation. These children had received the regular Hib vaccinations (at 3, 4, 5 and 11 months of age) but appeared to have low Hib antibodies at the time of analysis; they had normal serum immunoglobulin concentrations. In order to evaluate their immune status, these children were booster vaccinated with the Hib conjugate vaccine, and Hib antibodies were determined 2-3 weeks later.

Assays

Total serum concentrations of IgG, IgA and IgM, as well as serum concentrations of IgG subclasses were determined by radial immunodiffusion (Dade Behringer, Marburg, Germany and CLB, Amsterdam, The Netherlands).

IgG, IgG1 and IgG2 anti-Hib antibody titers were determined by ELISA as described [7], and calibrated using a standard anti-Hib hyperimmune serum (CBER standard, kindly provided by Dr. C. Frasch, FDA, Washington DC, USA). IgG1 anti-tetanus toxoid antibodies were quantified by an antibody-capture ELISA [8]. In short, the wells of a 96-well polystyrene microtiter plate were coated with tetanus toxoid (TT, 5 Lf/ml in 0.05 M carbonate buffer pH 9.6), blocked with bovine serum albumin, and incubated with two-fold serial dilutions of serum samples and standard sera. Anti-TT-antibodies in the IgG1 subclass were measured by successive incubation with IgG1-specific monoclonal antibodies (MH 161-1, CLB, Amsterdam, The Netherlands), followed by incubation with alkaline phosphatase-conjugated

rabbit anti-mouse Ig (Dakopatts, Glostrup, Denmark). After incubation with substrate (*p*-nitrophenylphosphate), the reaction was stopped with 3 M NaOH, and the optical density at 405 nm was recorded. The amount of IgG1 anti-TT antibodies in the sera of Hib patients and controls was calculated by comparison with standard sera containing known amounts of IgG1 anti-TT antibodies.

Avidity indices of IgG1 and IgG2 anti-Hib antibodies were measured by a modification of the sodium thiocyanate (NaSCN) elution ELISA described by Pullen [9]. Polystyrene microtiter plates were coated overnight at 37 °C with Hib polysaccharide (PRP) diluted to 1 µg/ml in PBS pH 7.2, and then blocked for 1 hour at 37 °C with PBS-1% bovine serum albumin. Two-fold serial dilutions of serum samples and standard serum were incubated for 2 hours at 37 °C. Sera were diluted in PBS-0.05% Tween 20- 1% bovine serum albumin to 50% of the amount of Hib antibodies which can maximally bind to the plate. After washing, the chaotropic agent NaSCN diluted in PBS was added in duplicate at concentrations ranging from 0.0 to 3.5 M. After 15 minutes at 37 °C, the wells were washed, and binding of remaining antibodies was then detected by subsequent incubation with IgG1- (MH 161-1, CLB, Amsterdam, The Netherlands) or IgG2- (35-1-27-2, TNO, Leiden, The Netherlands) specific monoclonal antibodies, followed by incubation with alkaline phosphatase-conjugated rabbit anti-mouse Ig (Dakopatts, Glostrup, Denmark). After incubation with substrate (*p*-nitrophenylphosphate), the reaction was stopped with 3 M NaOH, and the optical density at 405 nm was recorded. The relative avidity index (AI) is defined as the molarity of NaSCN at which 50% of the amount of IgG1 or IgG2 subclass antibodies bound to the coated antigen in the absence of NaSCN has been eluted from the antigen. This value represents an estimation of ‘average’ antibody avidity. The percentile elution contributed by each serial NaSCN concentration was calculated and these values were used to analyse the heterogeneity of avidity. Shannon’s coefficient (SC), which represents an index of this avidity heterogeneity, was calculated from these percentile elution intervals as described [10].
Avidity of IgG1 anti-TT antibodies was determined using a similar procedure as described above for anti-Hib antibodies. The concentrations of NaSCN applied ranged from 0 to 4.5 M.

Statistics
Antibody titers against Hib and TT, as well as antibody avidity parameters were compared for significant differences between the two groups by two-tailed Mann-Whitney U test. P-values <0.05 were considered statistically significant.

Results and discussion

Hib conjugate vaccines have excellent immunogenicity in healthy infants and children [11;12;13] as well as in children with underlying diseases like sickle cell disease and congenital asplenia [14;15]. These vaccines are highly efficacious in preventing disease, thus the rare vaccine failures warrant further investigation. Others [16;17] reported that a large proportion of children with Hib vaccination failure have associated clinical risk factors or humoral immunodeficiencies. In contrast, in our series (Breukels, CID, 2001) only 3 of the 21 children had such risk factors: one child had a chromosomal abnormality, one was IgG2 deficient and in one child hypogammaglobulinaemia was identified as the underlying cause of the Hib failure. In accordance with earlier studies [16;17], we found a large proportion of the children with Hib vaccine failure (6 out of 14) that showed low reconvalescent antibody responses to Hib. Booster vaccination with Hib conjugate vaccine ultimately resulted in IgG antibody levels above 1.0 µg/ml in all these children (Breukels, CID, 2001). Here we studied the specific anti-Hib and anti-TT antibody responses of Hib failure patients not only quantitatively but also qualitatively. Children with recurrent respiratory tract infections and with low Hib-antibody titers but no history of invasive Hib disease were used as a control group.

Antibody concentration

IgG, IgG1 and IgG2 antibody concentrations against Hib, and IgG1 antibody concentrations against TT, of patient and control sera are summarized in Table 1.

Serum samples obtained at onset of invasive Hib disease were available from 8 patients (0.8-4.7 years of age) with Hib vaccine failure. Anti-Hib concentrations were generally low (geometric mean IgG anti-Hib 1.6 µg/ml), and below 1.0 µg/ml in 4 of the patients. Among these 4 patients were two children that, because of their young age, had only received three vaccinations with Hib conjugate.

Hib conjugate vaccines prime infants and children for memory responses to subsequent encounter with Hib polysaccharide [18]. Theoretically, even individuals with low specific anti-Hib antibody titers will thus be protected from invasive Hib infection. To evaluate whether the patients with Hib failure had been primed for memory responses by Hib conjugate vaccination, we compared Hib antibody responses of the12 patients after Hib disease (0.9-5.2 years of age) with responses after Hib booster vaccination of 13 control

children (2.0-3.7 years of age) with low anti-Hib antibody concentrations (geometric mean IgG anti-Hib 0.36 µg/ml) after their regular four Hib vaccinations.
During reconvalescence, the children who developed invasive Hib disease despite having received at least three Hib vaccinations, mounted significantly lower ($p < 0.01$ for total IgG, IgG1 and IgG2) anti-Hib antibody responses than did control children after revaccination. This difference remained significant ($p<0.01$) after exclusion of the two patients who had only received three Hib vaccinations prior to developing invasive disease.
Apparently, immunological priming by conjugate vaccination was not sufficient to result in a vigorous memory response to the invading Hib bacteria, whereas the same priming enabled the control children to mount high antibody responses to a single booster vaccination. This suggests that the patients with Hib vaccination failure have an intrinsic defect in specific anti-Hib antibody production. Alternatively, the difference in memory responses between the two groups may be explained by differences in immunogenicity between Hib polysaccharide present in the capsule of living *H. influenzae* type b bacteria and Hib polysaccharide conjugated to a carrier protein.

					anti-Hib IgG (µg/ml)		anti-Hib IgG1 (µg/ml)		anti-Hib IgG2 (µg/ml)		anti-TT IgG1 (µg/ml)	
Patients	*n*	vaccina-tions	age (years)	range	geo	range	geo	range	geo	range	geo	range
A	*8*	3 or 4	2.4	(0.8- 4.7)	1.6	(0.6 -18)	1.0	(0.4 -11)	0.2	(0.1 -2.9)	2.5	(0.4 -16)
B	*12*	3 or 4	2.8	(0.9- 5.2)	6.6	(1.1 -29)	3.3	(0.5 -24)	1.2	(0.1 -15)	2.4	(0.7 -25)
C	*10*	4	3.2	(1.7- 5.2)	9.4	(1.9 -54)	4.6	(0.8 -24)	1.9	(0.3 -15)	2.5	(0.7 -25)
D	*4*	5	3.3	(1.7- 5.1)	17	(4.6 -80)	9.3	(2.2 -108)	8.1	(2.4 -25)	16.7	(7.4 -34)
E	*12*	4 or 5	3.0	(1.2- 5.2)	17	(1.9 -80)	9.6	(1.3 -108)	4.2	(0.1 -25)	5.4	(1.0 -34)
Controls												
F	*13*	4	2.5	(1.6- 3.1)	0.4	(0.3 -0.9)						
G	*13*	5	2.9	(2.0- 3.7)	96	(1.5 -582)	66	(1.5 -567)	19	(0.2 -249)	24	(2.2-495)

Table 1. Anti-Hib and anti-tetanus toxoid antibody concentrations

IgG, IgG1 and IgG2 anti-Hib antibodies and IgG1 anti-TT antibodies were determined by ELISA in Hib failure patients (A-E) and controls (F and G). Geometric mean antibody-concentrations as well as the ranges in antibody concentration are expressed in µg/ml.
A) sera at onset of Hib disease; B) reconvalescent sera; C) reconvalescent sera from fully (4 times) vaccinated patients; D) sera from patients boostered after reconvalescence; E) the 12 Hib-failure patient sera after disease or booster vaccination: from each patient the serum with maximal antibody concentration is included; F) sera of controls after four Hib conjugate vaccinations); G) sera of controls after five Hib vaccinations.

We therefore booster vaccinated the 4 patients with low reconvalescence anti-Hib antibody concentrations with Hib conjugate vaccine. The two young patients received their regular fourth Hib conjugate vaccination (booster 1) as well as an additional booster vaccination (booster 2). These 4 patients thus received five Hib vaccinations in total. We compared their antibody responses to booster vaccination with those of the controls after booster vaccination. Although IgG anti-Hib antibody concentrations in the Hib patients were still lower (geometric mean 17.4 µg/ml compared with 96 µg/ml in controls) due to the small groups this difference did not reach significance (p=0.062). The same trend was observed for IgG1 and IgG2 anti-Hib antibodies (Table 1).
Ultimately, with or without booster vaccination, all patients reached anti-Hib antibody levels above 1.0 µg/ml. However, compared to revaccinated controls, the maximal antibody concentrations were still significantly lower in the Hib failure patients ($p < 0.01$, $p< 0.01$ and $p< 0.05$ for total IgG, IgG1 and IgG2, respectively). For this reason, we conclude that in Hib vaccine failure patients a quantitative defect in Hib antibody production is demonstrable. This defect may be intrinsic, and thus correlate with susceptibility to Hib disease, but the lower anti-Hib antibody response in Hib failures may also be caused by the presence of residual Hib polysaccharide after invasive Hib disease, leading to tolerance, as has been described for other polysaccharides [19].

Anti-TT antibody concentrations also were much lower in Hib failure patients than in controls. This may result from the fact that the controls had received one additional vaccination (Hib polysaccharide conjugated to TT) containing TT. When the 4 Hib vaccination failure patients who had received five Hib vaccinations were compared with the controls having received five Hib vaccinations, no significant difference in IgG1 anti-TT antibody concentration was found (geometric mean 16.7 µg/ml and 24.3 µg/ml, respectively; see Table 1).

The Hib conjugate vaccine induces a predominant IgG1 antibody response; indeed in the control group 78% of IgG antibodies is of the IgG1 subclass. The ratio of IgG1/IgG2 anti-Hib antibodies, however, does show considerable individual variation both in controls as well as in patients, with observed ranges in IgG1-anti-Hib antibody as percentage of total antibody from 13 to 93% in patients and from 12 to 99% in controls. IgG1 anti-Hib antibodies are generally more effective than IgG2 antibodies in activating complement mediated bacteriolysis or in opsonic activity, however in some individuals the reverse can be found [20-

22]. Hib vaccination failure can therefore not be attributed to low IgG1/IgG2 anti-Hib antibody ratios in some individual patients.

Antibody avidity

Avidity of IgG1 and IgG2 antibodies against Hib and of IgG1 antibodies against TT was evaluated by two parameters, i.e. the avidity index (AI) and the Shannon's coefficient (SC). Results are summarized in Table 2. Because of low IgG, IgG1 and/or IgG2 anti-Hib antibody concentrations, Hib avidity-assays could not be performed on all serum samples nor for all isotypes.

	n	vaccina-tions	age (years)	range	Hib IgG1			Hib IgG2			TT IgG1		
Patients					*n*	AI	SC	*n*	AI	SC	*N*	AI	SC
A	*8*	3 or 4	2.4	(0.8 -4.7)	*4*	0.37	10.0	*1*	0.07	7.0	*8*	1.85	14.2
B	*12*	3 or 4	2.8	(0.9 -5.2)	*10*	0.33	10.4	*6*	0.10	7.6	*12*	1.93	14.5
C	*10*	4	3.2	(1.7 -5.2)	*10*	0.33	10.4	*6*	0.10	7.6	*10*	2.05	14.6
D	*4*	5	3.3	(1.7 -5.1)	*4*	0.35	10.8	*4*	0.12	7.2	*4*	2.71	14.9
E	*12*	4 or 5	3.0	(1.2 -5.2)	*12*	0.30	11.0	*10*	0.12	7.5	*12*	2.19	14.7
Controls													
G	*13*	5	2.9	(2.0 -3.7)	*13*	0.52	11.0	*11*	0.40	9.6	*13*	2.58	14.2

Table 2. Avidity of anti-Hib and anti-tetanus toxoid antibodies

Avidity of IgG1 and IgG2 antibodies against Hib and of IgG1 antibodies against tetanus toxoid (TT) was evaluated by the relative avidity index (AI) and heterogeneity of avidity by the Shannon's coefficient (SC). The average AI and SC are given for the individual groups. The number of vaccinations received is indicated, as well as the number of sera that could be analyzed by elution ELISA (*n*).
A) sera at onset of Hib disease; B) reconvalescent sera; C) reconvalescent sera from fully (4 times) vaccinated patients; D) sera from patients boostered after reconvalescence; E) the 12 Hib-failure patient sera after disease or booster vaccination: from each patient the serum with maximal antibody concentration is included; G) sera of controls after five Hib vaccinations.

The relative AI of IgG1 anti-Hib antibodies at onset of disease, after reconvalescence as well as after booster vaccination, was comparable with that in control sera (Table 2).
From 5 individual patients, consecutive serum samples could be analyzed by elution ELISA; an increase in avidity of IgG1 anti-Hib antibodies was observed in three of these patients, whereas in the two others no avidity maturation was observed (Figure 1). The avidity of IgG2

anti-Hib antibodies was lower in patients compared with controls (Table 2), and no maturation was observed in the 3 individual patients from whom consecutive serum samples could be analyzed (Figure 1). At the time of maximal IgG2 anti-Hib antibody concentrations, the relative avidity index was 0.1 in patients, compared with 0.4 in controls (p=0.08). Avidity heterogeneity of IgG2 anti-Hib antibody as represented by the Shannon's coefficient was also lower in patients than in controls (SC 7.5 and 9.6, respectively, p=0.05).

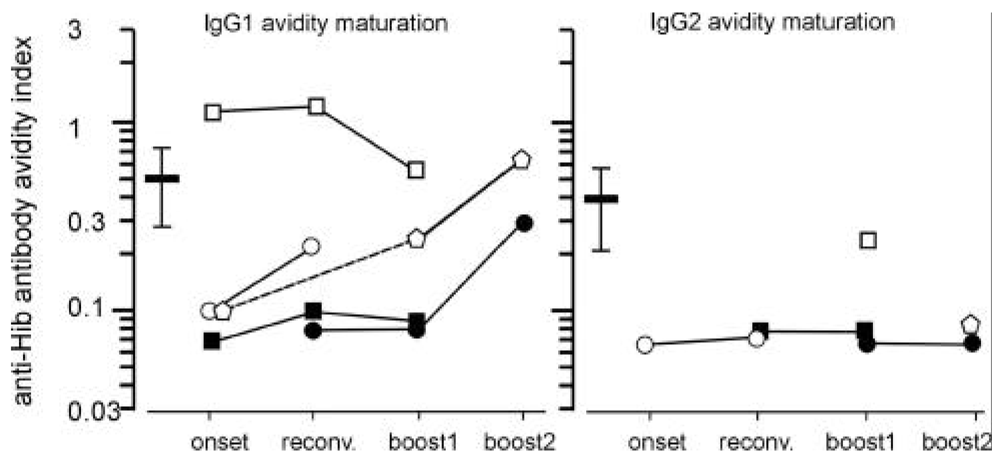

Figure 1. Avidity maturation of IgG1 and IgG2 anti-Hib antibodies in 5 individual patients during Hib disease and after booster vaccination.

In two young patients the regular fourth Hib vaccination is marked 'boost 1'; and a fifth Hib vaccination, 'boost 2', respectively. Horizontal bars depict the average avidity index (± SEM) in control sera.

In most control children, the similarity of IgG1 and IgG2 anti-Hib avidities was striking; in 5 out of the 10 analyzed Hib failure patients the IgG2 avidity was significantly lower than the IgG1 avidity (Figure 2).

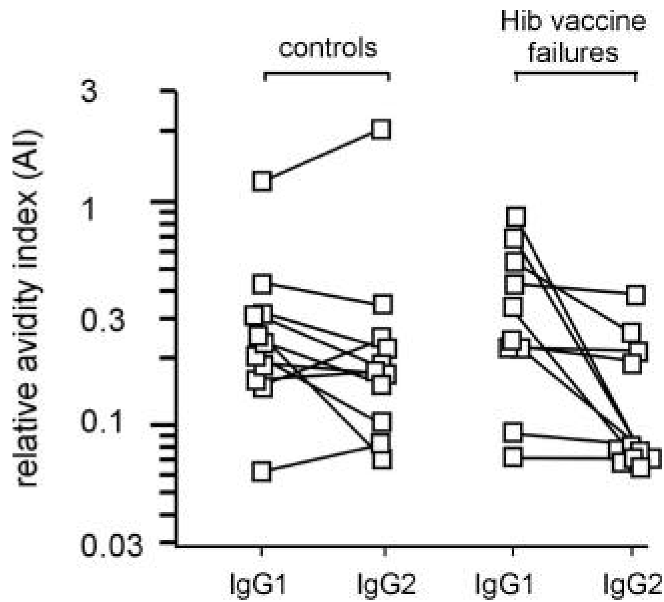

Figure 2. Correlation between IgG1 and IgG2 anti-Hib antibodies in controls after revaccination (n=11) and in Hib failure patients at maximal antibody concentration (n=10).

The relative AI of IgG1 anti-TT antibodies was significantly lower in the reconvalescence sera of 12 patients than in the sera of 13 controls ($p<0.01$). This observation may result from the fact that the controls had received one additional vaccination containing TT (Hib polysaccharide conjugated to TT) after completion of the National Vaccination Program. When the Hib vaccination failure patients that were vaccinated five times were compared with those controls, no significant difference in anti-TT antibody avidity was found. Alternatively, the low relative AI of IgG1 anti-TT antibodies in the reconvalescent sera from fully vaccinated patients compared to the controls suggested that also the maturation of the antibody response to protein antigens might be delayed in Hib failure patients.
In both patients and controls, the avidity parameters of IgG1 anti-TT antibodies are significantly higher ($p<0.01$) than those of anti-Hib antibodies, as exemplified in Figure 3. This finding is probably a reflection of the different nature of Hib and TT antigens. The limited variety of available Hib capsular polysaccharide epitopes is known to be associated with an oligoclonality of the serum anti-Hib antibody response [23] and a restricted use of V_H genes [24;25]. The restricted V_H gene usage makes avidity maturation of anti-Hib-antibodies after repeated vaccinations less likely compared to the response to a complex protein antigen as TT.

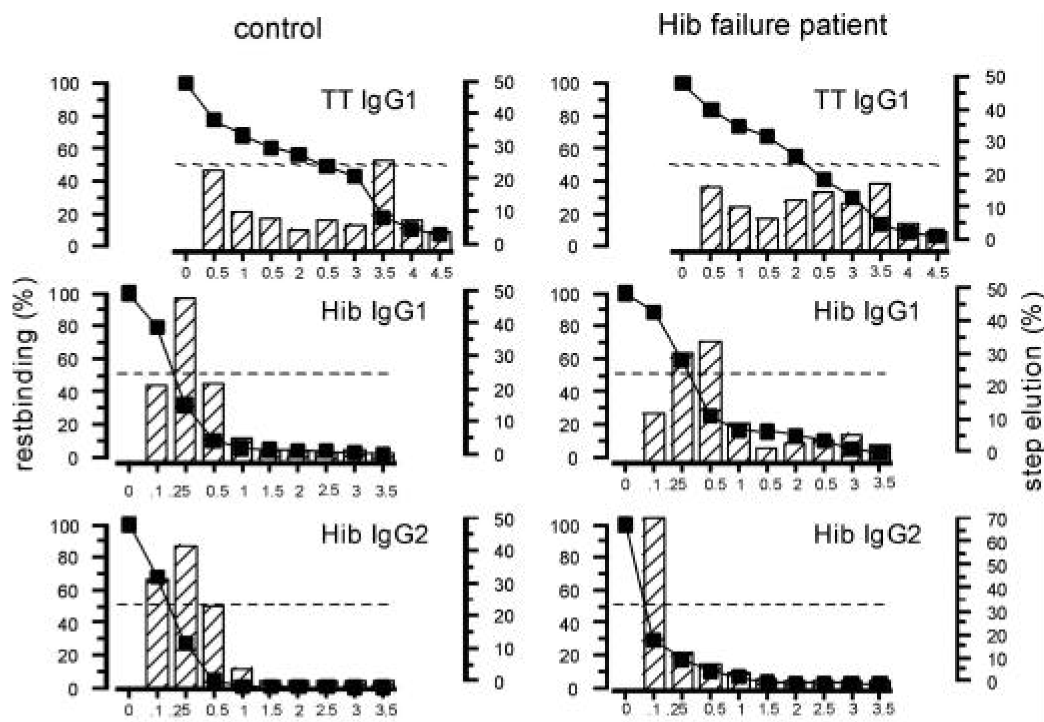

Figure 3. Thiocyanate elution profiles of IgG1 and IgG2 Hib antibodies and IgG1 anti-TT antibodies of a control and a Hib failure patient.

Each histogram bar represents the percentage (right y-axis) of total antibody eluted by the thiocyanate concentration indicated on the X-axis. The black squares (■) represent the percentage of restbinding (left y-axis) after each elution step. The relative avidity index is defined as the thiocyanate concentration resulting in 50% restbinding.

We thus conclude that Hib failure patients not only have a quantitative defect in the production of anti-Hib antibodies, but also a qualitative defect. This qualitative defect also appears to be selective for Hib, and more particular for IgG2 anti-Hib antibodies. This is illustrated by the low relative AI for IgG2 anti-Hib antibodies, and by the low SC suggesting a limited diversity of IgG2 anti-Hib producing B cell clones.

Conclusions

Protective function of anti-Hib polysaccharide antibody depends upon the density of antibodies present on the bacterial surface. This density will depend not only on antibody concentration, but also on antibody avidity. Correlations between antibody avidity and effector functions have been observed [4;26;27] and the effect of lower overall avidity should

become more pronounced in situations where antibody concentrations are limited. Hib conjugate vaccines prime for memory responses, which are characterized by rapid production of antibodies of relatively high avidity. Antibody concentration and avidity measures are therefore correlated surrogates of protection. High antibody responses following encounter with the antigen indicate successful priming, as does the presence of high avidity antibodies. In individuals with low antibody titers, antibody avidity may be able to discriminate between those subjects successfully primed in infancy -who may be considered to be protected-, and those with low-avidity antibodies, who thus are not primed sufficiently, and therefore may be at risk of invasive disease.

In patients with Hib vaccination failure without obvious clinical risk factors or obvious immunological deficiencies, we found both quantitative and qualitiative defects in the specific anti-Hib antibody production. Our findings have to be substantiated further by the investigation of a large group of vaccine failures and healthy controls. Furthermore, it would be informative to investigate the opsonic activity of sera obtained from patients and controls against *H. influenzae* type b.

The low specific anti-Hib antibody responses after invasive Hib disease and the relatively low avidity of (IgG2-) anti-Hib antibodies suggest that the patients who developed invasive Hib disease despite three or four Hib conjugate vaccinations were inadequately primed by these vaccinations, which might have contributed to their susceptibility for Hib disease.

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Chapter 8

Summary and Discussion

Antibodies to capsular polysaccharides are essential in the defense against encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae* type b. Polysaccharide vaccines induce a protective antibody response in adults and children older than 18-24 months of age, leaving the group most at risk for infections with encapsulated bacteria, infants and young children, unprotected. Polysaccharide-protein conjugate vaccines are immunogenic in infants and toddlers, and also in several other groups at risk for infections with encapsulated bacteria due to impaired anti-polysaccharide antibody production. In this thesis, the immunobiology and the application of polysaccharide- and polysaccharide conjugate vaccines were studied in an experimental rat model and in selected patient groups.

The importance of the splenic marginal zone in the anti-polysaccharide antibody response

The concept that marginal zone B cells are involved in the response to polysaccharide antigens originates from the observation that high expression of complement receptor 2 (CD21) on marginal zone B cells occurs only late in ontogeny; a time frame that coincides with the acquisition of anti-polysaccharide antibody responsiveness. The importance of splenic marginal zone B cells, complement receptors, and complement in the antibody response to TI-2 antigens has been shown in recent studies in $C3^{-/-}$, $CRI/II^{-/-}$, and $Pyk2^{-/-}$ mice [1]. In these studies, splenic marginal zone B cells expressing high levels of CD21 appeared to be an absolute requirement for the generation of an antibody response to the synthetic TI-2 antigen TNP-Ficoll. It is likely that the ability of TI-2 antigens to activate complement and to bind in particular to C3d facilitates the localization of these antigens on marginal zone B cells. In addition, the ability of complement-factor C3d to activate B cells by cross-linking of B cell receptor and CD21 may directly facilitate marginal zone B cells to induce TI-2 antibody responses [2].

We studied the initiation of the anti-polysaccharide antibody response in a rat model (**chapters 2** and **3**). The induction of anti-capsular antibodies by polysaccharide vaccine proved to be complement-dependent, most likely because polysaccharide localization on splenic marginal zone B cells depends on complement. This localization on marginal zone B cells seems crucial for the initiation of an anti-polysaccharide antibody response. This is

supported further by our results in splenectomized rats which show impaired anti-polysaccharide antibody responses following polysaccharide vaccination (**chapter 3**).

In man, functional or anatomical asplenic individuals are highly susceptible to infections with encapsulated bacteria and this is thought to reflect the critical role for the splenic marginal zone in alerting the immune system to these pathogens. The localization of marginal zone B cells is at the point were blood enters the spleen, so that marginal zone B cells are well situated for the capture of blood-borne pathogens. In addition, marginal zone B cells have a distinct phenotype, with high expression of complement receptor 2 (CD21), which greatly enhances the B cell response to complement-coated bacteria. This strategic localization and expression of CD21 makes marginal zone B cells remarkably well suited for the capture of complement-coated bacteria. *In vitro* studies have established that when antigen is recognized, marginal zone B cells differentiate with unmatched speed into antibody secreting cells [3]. A number of studies (reviewed in [4]) suggest that marginal zone B cell responses can be amplified or switched through cytokines released by non-conventional helper cells. This help might come from CD1 restricted T cells [5], which are known to secrete very promptly cytokines such as IL-4 and IFN-γ. The CD1 family of proteins are non-classical MHC molecules, evolutionarily highly conserved and prominently expressed on professional antigen presenting cells. This suggests that CD1 proteins may function in recruiting a form of innate help from cytokine-producing T cells to antigen-presenting cells. Certain CD1 family members (notably CD1d) are specialized in presenting glycolipids. This would also allow for presentation of polysaccharides because the biosynthesis of these molecules starts on lipid carriers and capsular polysaccharides do carry terminal lipid tails. Interestingly, marginal zone B cells express very high levels of CD1, and this strengthens the idea that they may be involved in polysaccharide antigen presentation.

It is not known whether the availability of complement and antigen localization on marginal zone B cells is also important for antibody responses to polysaccharides conjugated to proteins. There is circumstantial evidence that conjugates do not depend on localization on marginal zone B cells for induction of an immune response: infants with a low CD21 expression on B lymphocytes still are able to respond to polysaccharide conjugate vaccines. We showed in chapter 2 that conjugate vaccine can induce anti-polysaccharide antibodies in the absence of complement. More detailed information came from the rat model, showing polysaccharide conjugate vaccine to localize in the splenic marginal zone, but in a pattern

different from polysaccharides (in association with marginal zone macrophages) and in a less complement-dependent way. This latter splenic localization is not obligatory for the induction of an antibody response: we demonstrated that an adequate anti-polysaccharide antibody response can be induced by conjugate vaccines in the absence of a spleen (**chapter 3**). The initiation of an anti-polysaccharide response by conjugate vaccines can thus take place in lymphoid tissue other than the spleen

PPS 19 localisation 2 hours after vaccination

			Spleen	Liver	Thymus	Gut	Ln 1	Ln 2	Ln 3
1	Sham	PPS	++++	++++	+/-	-	++	++	
2			++++	++++	+/-	-	+/-	+/-	
3		PCV		+++	+/-	-		++	++
4			++	++	+/-	-	+	+/-	++
5	Sx	PPS			+/-	-	++	+++	+++
6				++++	+/-	-	++	++	++
7		PCV		++			+	++	++
8				+++	+/-	-	+/-	+	++
9	Auto Tx	PPS	++++	+++	+/-	+		+++	++
10			++++	+++	+/-	++	+++	+	++
11		PCV	++++	+++	+/-	-	+++	++	+++
12			++++	+++	+/-	-	+++	+++	++

PPS 14 localisation 2 hours after vaccination

			Spleen	Liver	Thymus	Gut	Ln 1	Ln 2	Ln 3
1	Sham	PPS	++	+++	+/-	-	+	+/-	
2			++	+++	+/-	-	+	-	
3		PCV	++	++	+/-	-	+	+/-	+/-
4			++	++	-	-	+/-	-	+/-
5	Sx	PPS			+/-	-	+/-	+/-	
6				++	-	-	+/-	-	+/-
7		PCV		++			+/-	+/-	+/-
8				++	-	-	+/-	+/-	
9	Auto Tx	PPS	+	++	+/-	-		+/-	-
10			++	++	+/-	-	-	-	+
11		PCV	+/-	++	+/-	-	+	-	-
12			+/-	++	+/-	-	+	-	+/-

Table 1. Localization pattern of pneumococcal polysaccharide vaccine (PPS, containing 25 µg polysaccharide of the serotypes 4, 6B, 9V, 14, 18C, 19F and 23F) and pneumococcal conjugate vaccine (PCV, containing the polysaccharides of the pneumococcal serotypes 4, 9V, 14, 19F and 23F and an oligosaccharide of serotype 18C (2 µg each) and polysaccharide type 6B (4 µg), conjugated to CRM_{197} carrier protein (Wyeth Lederle Vaccines and Pediatrics, Rochester, NY) two hours after intraveneous (in the tail vein) and subcutaneous (in the hind leg) injection. Rats were sham operated (Sham), splenectomized (Sx) and splenectomized followed by autotransplantantion (Auto Tx). Ln 1=abdominal lymph node; Ln 2=cervical lymph node, Ln 3=axillary lymph node. Localization of PPS-14 and PPS-19 is evaluated by immunohistochemistry using specific antibodies on 4 micron frozen tissue sections using a semiquantitative scale.

Recent additional experiments showed that in splenectomized rats, two hours after injection, conjugated polysaccharides localize most obviously in lymph nodes (Table 1). This suggests

that lymph nodes are an important site of antigen localization after polysaccharide conjugate vaccination. After splenectomy, polysaccharides are also mostly found in lymph nodes (Table 1). Because the antibody response to a polysaccharide vaccine is abrogated after splenectomy (**chapter 3**), this indicates that mere localization of polysaccharides in lymph nodes is not sufficient for the induction of an anti-polysaccharide response. For the induction of an antibody response by nonconjugated polysaccharides additional factors are required, and apparently these requirements can not be met in an environment other than the splenic marginal zone. In contrast, the induction of an antibody response to polysaccharide conjugates does not require this specialized splenic marginal zone compartment.

The role of T cells in the anti-polysaccharide antibody response

Chapter 3 also provides insight in the way T cells can influence the *in vivo* antibody response to polysaccharides. By definition, TI-2 polysaccharide antigens can induce an antibody response in the absence of T lymphocytes. Also by definition, T lymphocytes can augment the antibody response to polysaccharides. The T lymphocytes that exert this effect differ from classical MHC restricted, antigen specific $CD4^+$ T helper cells and therefore are operational defined as "amplifier T cells". As already discussed above, amplifier T cells may be CD1 restricted. In a conjugate, the polysaccharide antigen is covalently coupled to a protein. These conjugates induce an anti-polysaccharide antibody response with characteristics of a T cell dependent (TD) antibody response. The prevalent model of how the conjugate would lead to a TD B cell activation is that polysaccharide-specific B cells receive T cell help from peptide-specific T cells (see Figure 2 in the Introduction). In **chapter 3** we show in the rat model that admixture of non-conjugated polysaccharides with the conjugate does not lead to a better response to the native polysaccharide. Yet, both native polysaccharides and conjugated polysaccharides localize in the same region of the spleen (although polysaccharides localize specifically on marginal zone B lymphocytes, and the conjugate shows more diffuse presence in this area). This would indicate that the mere secretion of B cell stimulatory cytokines by peptide-specific T cells in the proximity of polysaccharide-specific B cells is not sufficient to lead to *in vivo* augmentation of the B cell response. This suggests that direct cell-cell contact between polysaccharide-specific B cells and peptide-specific T cells is necessary in the *in vivo* primary response.

Memory induction and isotype switching

Above data indicate that optimal environment for induction of a primary anti-polysaccharide response by native polysaccharides is the splenic marginal zone, where co-ligation of CD21 together with antigen-receptor crosslinking stimulates polysaccharide-specific marginal zone B cells. The requirement for CD21 mediated co-stimulation can be overcome by polysaccharide conjugates that provide co-stimulation through T-cell-dependent B cell activation. Once polysaccharide-specific memory B cells are formed by vaccination with conjugate, requirements for anti-polysaccharide antibody production may be different. This is illustrated by the fact that infants who have been primed by conjugate vaccines are capable of producing anti-polysaccharide antibodies following booster vaccination with native polysaccharides, even at an age when their marginal zone is still immature and they normally would be unresponsive to polysaccharides [6]. Polysaccharide-specific memory B cells, generated by conjugate vaccination, thus differ from their primary B cell progenitors in that they can be activated by crosslinking of membrane immunoglobulins by polysaccharides (and, potentially, co-stimulation of CD21) without the need for the specialized marginal zone environment. Also the *in vitro* experiments described in **chapter 4** do support the concept that polysaccharide specific memory B cells differ from virgin B cells in their activation requirements. We showed that polysaccharide-specific B cells that are *in vivo* activated by polysaccharide-conjugate vaccination, do not require direct contact with T cells for *in vitro* production of anti-polysaccharide antibodies.

Once polysaccharide-specific memory B cells are generated by polysaccharide-conjugate priming, isotype switching can be facilitated by boosting with a polysaccharide vaccine (**chapter 5**). In young children a primary immunization with pneumococcal conjugate vaccine induces a predominant IgG1 type anti-pneumococcal antibody response. After priming with pneumococcal conjugate vaccine, booster vaccination with 23-valent pneumococcal polysaccharide vaccine leads to IgG2- and IgA-type anti-pneumococcal antibody production in these young children. Booster vaccination with conjugate does not induce this isotype switching. The relevance and impact of this finding on vaccination strategies might be substantial, as will be discussed below.

Protection against mucosal infections

One of the main purposes of the development of pneumococcal conjugate vaccines is to reduce the incidence of mucosal infections like acute otitis media (AOM), sinusitis and bronchitis, that are often caused by *S. pneumoniae*. In healthy infants and toddlers, immunization with pneumococcal conjugate vaccines indeed leads to reduction of nasopharyngeal carriage of pneumococci, and is shown to induce immune responses at mucosal membranes [7]. For this reason, pneumococcal conjugate vaccines are candidate tools that can lead to reduction of respiratory tract infections. Data from the first two large efficacy trials in healthy infants show a reduction in the occurrence of AOM caused by vaccine-serotypes up to 57% [8;9]. In this respect the more than 90% reduction of invasive infections [8] is more convincing. This suggests that the requirements for protection at mucosal surfaces are different from those for protection against invasive infections. In infants and young children, IgG1 type antibodies dominate the antibody response against polysaccharide-conjugate vaccines, and these antibodies are apparently effective in protecting against invasive disease. However, IgG2 and IgA antibodies may be more important for protection against mucosal infections with encapsulated bacteria, which might explain the less convincing protective efficacy for otitis media of vaccination schedules using only pneumococcal conjugate vaccines in infants.
Otitis prone children as well as other patient groups at risk for mucosal infections with encapsulated bacteria are often characterized by low IgA and IgG2 type anti-pneumococcal antibodies [10;11]. We demonstrated the induction of IgG2- and IgA anti-pneumococcal antibodies by pneumococcal conjugate priming followed by booster immunization with 23-valent pneumococcal polysaccharide vaccine in children with frequent recurrent upper respiratory tract infections such as recurrent (AOM) (**chapter 5**), even in those with partial IgG2 and/or IgA deficiency (**chapter 5b**). Sequential immunization with pneumococcal conjugate vaccine followed by polysaccharide vaccine thus overcomes the apparent class switch defect in this patient group, which might be of utmost importance for protection against mucosal infections with encapsulated bacteria.
In the Netherlands, a large double-blinded randomized clinical trial measuring the efficacy of this vaccination schedule in preventing recurrent episodes of AOM in otitis prone children (OMAVAX-study) is currently ongoing.

Correlates of protection

Next to establishing the clinical efficacy of pneumococcal conjugate vaccines, the OMAVAX-study and other efficacy trials will yield a valuable set of laboratory and clinical data which will facilitate the development and validation of laboratory correlates of protection against invasive disease and mucosal infections. Since opsonophagocytosis is crucial in the defense against encapsulated bacteria, functional antibody levels measured by phagocytosis assays may be used as correlates of protection. We employed an assay that uses human polymorphonuclear leukocytes (PMN) as phagocytic cells to evaluate the opsonophagocytic activity of sera obtained after vaccination of healthy adults with pneumococcal conjugate vaccine (**chapter 6**). The level of phagocytosis correlates with IgG2 anti-pneumococcal antibody concentrations, suggesting that not only after polysaccharide vaccination but also after conjugate vaccination, the functionally most active antibodies are of the IgG2 isotype. These data correspond with findings of others [12;13] supporting an important role for IgG2 and IgA in the host defense against pneumococci. Together with clinical data on pediatric otitis-prone patients characterized by normal or even high IgG1, but low IgG2 and IgA type anti-pneumococcal antibodies [10;11], these results are consistent with the view that IgG2 and IgA represent the main opsonins in conferring clinical protection against mucosal pneumococcal infections.
Receptors for the constant region of antibodies (FcR) play a crucial role in the process of opsonophagocytosis. Two subclasses of the leukocyte IgG receptor (FcγR) family, FcγRIIa (CD32) and FcγRIIIb (CD16) are constitutively expressed on PMN. Both receptors exhibit genetically determined functional polymorphisms. Both FcγRIIa and FcγRIIIb can bind IgG1- and IgG3-containing complexes, but only FcγRIIa–H131 can effectively interact with complexed IgG2 [14]. The capacity to clear IgG2-opsonized particles thus depends on FcγRIIa genotype. We indeed found that the H131/R131 FcγRIIa polymorphism on PMN had profound influence on phagocytic efficiency (**chapter 6**).

Vaccination strategies for risk groups

The efficacy of polysaccharide conjugate vaccines in protecting infants and young children against invasive disease is high: this holds true for both the heptavalent pneumococcal conjugate vaccine [8] and the *Haemophilus influenzae* type b conjugate vaccine [15].

Introduction of latter conjugate vaccine in childhood vaccination programs has dramatically reduced the incidence of invasive *H. influenzae* type b disease; only sporadically cases of vaccine failure are reported (**chapter 7**). These successes of conjugate vaccines in infants may open up ways to apply conjugate vaccines in other groups at risk for infections with encapsulated bacteria and impaired responses to the plain polysaccharide.

The fact that in rats polysaccharide conjugate vaccines are able to induce robust anti-polysaccharide antibody levels after splenectomy (**chapter 3**) suggests that asplenic or hyposplenic patients may benefit from polysaccharide-conjugate vaccines. Another group to benefit from (pneumococcal) conjugate vaccine may be the elderly. They are, next to children under 5 years of age, the group at greatest risk for invasive pneumococcal infections and pneumococcal pneumonia. Elderly respond sub optimally to pneumococcal polysaccharide vaccines, particularly those with underlying disease. Furthermore, because polysaccharide vaccines do not induce classical immunological memory, antibody levels to clinically important serotypes decline to prevaccination values within 3-7 years. Although revaccination with polysaccharide vaccines is safe and may prolong the duration of protection, many older individuals will remain incompletely protected. The enhanced immunogenicity of pneumococcal conjugate vaccines, and more importantly, their potential for prolonged protection because of memory induction, opens new possibilities to develop pneumococcal conjugate vaccination strategies for additional risk groups like elderly and patients with a secondary immune deficiency.

A limitation of the conjugate vaccines in adults and elderly is the limited coverage of pneumococcal serotypes causing invasive disease in these age-groups. In developed countries an 11-valent pneumococcal conjugate vaccine would cover 75-85% of invasive isolates in young children (not counting possibly cross-reacting serotypes) but only 65-68% of those in adults. Yet, among these 11 vaccine-serotypes a substantial number is relatively poorly immunogenic, and the 11-valent conjugate vaccine includes virtually all of the serotypes associated with resistance to antimicrobials. Therefore, adults at risk for pneumococcal infections may benefit from the existing pneumococcal conjugate vaccines, even though serotype coverage is limited. To extend serotype coverage, both in adults and in children, pneumococcal conjugate vaccines could be part of a vaccination schedule that also includes a dose of 23-valent polysaccharide vaccine.

Impact of conjugate vaccination on carriage and antimicrobial resistance

A very important feature of polysaccharide conjugate vaccines is their effect on nasopharyngeal carriage. Nasopharyngeal carriage rates of *H. influenzae* type b are reduced in vaccinated populations, and immunization with pneumococcal conjugate vaccines also results in reduction in the prevalence of nasopharyngeal carriage of pneumococci of the vaccine serotypes. The serotype specificity of these conjugate vaccines has led to the concern that their use might result in increased carriage of and disease from serotypes not included in the vaccine. Replacement has not occurred with the use of *H. influenzae* type b conjugate vaccines, but increased nasopharyngeal carriage of non-vaccine type pneumococci has been found in trials with pneumococcal conjugate vaccines. In an early study in Gambian infants immunized with a pentavalent pneumococcal conjugate vaccine, and then reimmunized with a 23-valent pneumococcal polysaccharide vaccine, a reduction of the prevalence of nasopharyngeal carriage of pneumococci of the conjugate vaccine types was shown. However, replacement with pneumococci of non-vaccine serotype(s) occurred, so the overall pneumococcal carriage in vaccinated children changed little [16]. A second study of Gambian children 1-4 months after completion of a primary course of vaccination with a nonavalent pneumococcal conjugate vaccine at the ages of 2, 3 and 4 months showed a similar trend [17]. Although a first study in Israel did not find evidence for replacement [18], a subsequent study did so [19]. In a study in Soweto, South Africa, where 500 infants were vaccinated with a 7-valent pneumococcal conjugate vaccine at 6, 10 and 14 weeks of age, nasopharyngeal carriage rates of vaccine serotypes decreased in vaccinees at 9 months of age (from 36% to 18%), but carriage rates of non-vaccine serotypes increased from 25% to 36%. Of concern is that carriage of one of the non-vaccine serotypes most commonly isolated in invasive pneumococcal disease in South Africa, serotype 15, was significantly increased in vaccinees compared with controls [20]. Whether this replacement will lead to the increase of invasive disease or respiratory tract infections caused by non-vaccine serotypes is difficult to predict. Pneumococci of non-vaccine serotypes may be inherently poor at causing disease, and increased carriage and transmission of these isolates might remain without clinical consequences. Alternatively, pneumococci of some non-vaccine serotypes that compete poorly with vaccine serotypes for colonization of the nasopharynx might become equally important causes of disease now they are given a competitive advantage because of vaccination.

High level antibiotic resistance is almost exclusively found among pneumococci of the vaccine serotypes, presumably because selective pressure for resistance is strongest on isolates carried in the nasopharynx of children, which are predominantly vaccine serotypes. Immunization should therefore lead to reduced prevalence of resistant isolates. However, selection pressure induced by vaccination, in combination with pressure from continued exposure to antibiotics might favor the appearance of non-vaccine serotype variants of major resistant clones.
Because the full effects of conjugate vaccination on the pneumococcal serotype distribution spectrum may be observed only after a few years of vaccine implementation, when immune selective pressures are maximal, close monitoring of pneumococcal colonization in the following years is necessary. It should be possible to incorporate capsular polysaccharides of non-vaccine serotypes into the conjugate vaccine, if and when they become important causes of disease. The number of additional serotypes that can be incorporated in a single conjugate vaccine however is technically limited.

The current pneumococcal conjugate vaccines certainly are an important, but possibly not the final solution in the prevention of pneumococcal disease, particularly not concerning mucosal infections. Additonal prevention strategies therefore should also be explored.
Furthermore, with the increasing prevalence of antibiotic-resistant pathogens, judicious use of antibiotics is warranted [21]. The Dutch guidelines on AOM, the most frequent reason for prescribing antibiotics in childhood, advise physicians to manage AOM in children older than 2 years with an initial observation period of three days, and to limit prescription of antibiotics to children with severe illness, to those who have an irregular course of AOM, to infants, and to children considered high risk. The percentage of patients given antibiotics for AOM in the Netherlands is limited to only 31%, whereas in many countries the prescription rate exceeds 95% [22]. As a consequence of the limited use of antibiotics, the percentage of penicillin resistant *S. pneumoniae* in the Netherlands is currently still under 1% [23].

Alternative prevention strategies

The use of antibiotic prophylaxis for recurrent otitis media may be unnecessary if alternative strategies to decrease the rate or intensity of colonization of the respiratory tract by bacterial pathogens are effective. Xylitol has been shown to inhibit growth and adherence of *S.*

pneumoniae [24;25] and daily use of oral xylitol reduced the incidence of AOM by 40% [26]. However, the doses of xylitol required to achieve protection are high, which limits its practical use. More importantly, oral xylitol intake does not lead to a reduction in pneumococcal carriage. Oligosaccharides that can inhibit binding of several bacteria to epithelial cells may also prevent AOM [27;28], although in a first randomized placebo-controlled trial oligosaccharides had no beneficial effect on the occurrence of AOM in children or on the nasopharyngeal carriage of bacteria [29].

Although pneumococcal conjugate vaccines have a promising capability in the prevention of invasive disease, these vaccines have certain shortcomings, particularly in prevention of mucosal infections. Their efficacy in preventing AOM in healthy infants for pneumococcal serotypes included in the vaccines, is less than 60% [9], and more effective vaccines would be desirable. Stronger immunity against pneumococcal polysaccharides may be induced in the future by using pneumococcal vaccine with immunomodulating cytokines or lymphocyte stimulating antibodies [30;31]. Pneumococcal polysaccharides have also been used as mucosal vaccines, together with novel adjuvants, and both serum and mucosal immune responses have been successfully induced [32;33]. They may be an alternative approach to current strategies to protect against mucosal infections by encapsulated bacteria. Furthermore, research into vaccines that can provide protection against disease caused by pneumococci of all serotypes should be continued. Inactivated pneumolysin toxin, PspA and a 37-kDa metal-binding lipoprotein (PsaA) are commonly mentioned as potential pneumococcal protein vaccines [34]. They can be used either as a pure protein [35-37] or conjugated with pneumococcal polysaccharides [38;39].

Concluding remarks

Vaccines represent some of the most important tools available for prevention of disease. Vaccines protect the immunized individual from developing a potentially serious disease, and they also help to protect the community by reducing the spread of infectious agents. Vaccination as a deliberate attempt to protect humans against disease has a long history. Edward Jenner, in his “Inquiry to the Causes and Effects of the Variolae Vaccinae” in 1798 was the first to observe that pustular material from the lesions of cowpox when inoculated into humans, protected them from infection with smallpox [40]. Since then, vaccination has

controlled nine major disease, at least in parts of the world: smallpox, diphtheria, tetanus, yellow fever, pertussis, poliomyelitis, measles, mumps and rubella. In the case of smallpox, the dream has been fulfilled, as this disease is eradicated [41].

Prevention of these diseases, caused by viruses or bacterial toxins, is achieved by vaccination with protein antigens, such as attenuated virus strains and toxoids. Control of diseases caused by encapsulated bacteria, necessitating the use of polysaccharides for vaccine production, has proven to be more difficult. However, covalent attachment of polysaccharides to carrier proteins has been shown to enhance the immunogenicity of polysaccharides [42]. Conjugate vaccines of capsular polysaccharides from *Haemophilus influenzae* type b (Hib), *Streptococcus pneumoniae*, and several other bacteria are now available for human use.

Since the introduction of Hib conjugate vaccines in several countries, the incidence of invasive Hib disease in these countries has declined dramatically. The Hib conjugate vaccines have also been demonstrated to markedly reduce carriage of *Haemophilus influenzae* type b. It should therefore be possible to eliminate invasive Hib disease with the widespread use of Hib vaccines. Importantly, neither increases in carriage rates of other *H. influenzae* serotypes [43], nor increased incidence of meningitis caused by other encapsulated bacteria have, so far, been observed.

Recent clinical trials of pneumococcal conjugate vaccines suggest that they, too, are highly effective at preventing invasive disease caused by vaccine serotypes. However, the pneumococcal conjugate vaccines target only 7-11 of the more than 90 serotypes, and replacement of vaccine serotype pneumococci by non-vaccine type pneumococci has occurred. A major concern is now, whether the incidence of disease caused by non-vaccine serotype pneumococci will also increase. Eradication of pneumococcal carriage by vaccination is unlikely to occur; therefore complete elimination of pneumococcal disease by pneumococcal conjugate vaccination will not be accomplished. However, the development of these conjugate vaccines, with further adaptations to come, can be considered a major step in the prevention of pneumococcal disease.

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Nederlandse samenvatting

Klinische en experimentele studies met pneumococcen- en *Haemophilus influenzae* type b-conjugaatvaccins

Pneumococcen (*Streptococcus pneumoniae*) en *Haemophilus influenzae* type b (Hib) zijn twee soorten bacteriën die ernstige infecties kunnen veroorzaken. Pneumococcen zijn de verwekkers van onder andere middenoorontsteking (otitis media), longontsteking (pneumonie), hersenvliesontsteking (meningitis) en bloedvergiftiging (sepsis). *Haemophilus influenzae* type b staat bekend als de verwekker van hersenvliesontsteking en van ontsteking van het strotklepje (epiglottitis) en kan ook long- en gewrichtsontstekingen veroorzaken. Het afweersysteem speelt een belangrijke rol bij het voorkómen van infecties door deze bacteriën. Hib en *S. pneumoniae* worden bij een aanzienlijk percentage van de gezonde bevolking in de bovenste luchtwegen aangetroffen, maar dragerschap van de bacterie leidt slechts in een beperkt aantal gevallen tot ziekte. Met name jonge kinderen, ouderen en patiënten met een gestoorde afweer zijn kwetsbaar. Pneumococcen en Hib zijn gekapselde bacteriën; ze worden omhuld door een suiker- (polysaccharide) kapsel dat hen beschermt tegen het afweersysteem. Pneumococcen kunnen op grond van verschillen in kapsel-polysacchariden worden onderverdeeld in meer dan 90 verschillende typen; *Haemophilus influenzae* bacteriën met het type b-kapsel-polysaccharide zijn verantwoordelijk voor het overgrote deel van de ernstige *Haemophilus influenzae* infecties.

In de afweer tegen gekapselde bacteriën spelen antistoffen (immuunglobulines, Ig) een zeer belangrijke rol. Antistoffen worden geproduceerd door speciale witte bloedcellen, de B-cellen. Ze herkennen heel specifiek een bepaalde structuur van een bacterie (of virus). In het geval van gekapselde bacteriën zijn met name antistoffen gericht tegen het kapsel-polysaccharide (anti-polysaccharide antistoffen) van belang. Deze antistoffen binden aan het kapsel, en daarmee worden ook andere, minder specifieke componenten van het afweersysteem geactiveerd, bijvoorbeeld complement-factoren (een reeks eiwitten, C1-C9, in het bloed). Met antistoffen en complement beladen bacteriën worden herkend door andere witte bloedcellen, fagocyten, die de bacteriën vervolgens opruimen (fagocytose).

Afweer moet worden opgebouwd: pas ná contact met een bepaalde bacterie of virus worden er antistoffen geproduceerd die kunnen beschermen tegen infecties veroorzaakt door die specifieke verwekker. Door vaccinatie kan de afweer vervroegd op peil worden gebracht. Reeds op zeer jonge leeftijd kunnen antistoffen worden gevormd tegen bacterie- of virus-eiwitten, zoals de bestanddelen van het DKTP-vaccin. Vaccinaties leiden, net als natuurlijk contact met een ziekteverwekker, ook tot geheugenvorming in het afweersysteem. Als er eenmaal contact is geweest met het vaccinbestanddeel of met de ziekteverwekker zelf, dan zal het afweersysteem bij hernieuwd contact direct op de ongewenste indringer reageren en deze versneld onschadelijk maken, zodat het niet tot ziekteverschijnselen komt.

De vorming van antistoffen tegen polysacchariden treedt pas op vanaf de leeftijd van 18-24 maanden. Een tweede groot nadeel van polysaccharide-vaccins is het ontbreken van geheugenvorming; als een tijd na vaccinatie de antistofconcentraties in het bloed weer laag zijn, is de bescherming verdwenen. Het onvermogen om vroeg in de ontwikkeling anti-polysaccharide antistoffen te maken verklaart waarom juist jonge kinderen het meest kwetsbaar zijn voor oorontsteking, longonsteking, en hersenvliesonsteking met deze gekapselde bacteriën. Inenting met vaccins die kapsel-polysacchariden bevatten (polysaccharide-vaccins) is onder de leeftijd van twee jaar dus niet zinvol.

B-cellen kunnen, zoals gezegd, al wel voor de leeftijd van twee jaar antistoffen produceren tegen eiwitten; dit dankzij de hulp van andere witte bloedcellen, de helper T-cellen. De antistofvorming tegen eiwitten wordt T-cel afhankelijk genoemd. De boven beschreven vorming van antistoffen tegen polysacchariden is T-cel onafhankelijk. Dat wil zeggen dat de B-cel geen hulp van T-cellen nodig heeft om antistoffen tegen polysacchariden te maken. T-cellen kunnen ook geen polysacchariden herkennen; polysaccharide-specifieke T-cellen zijn nooit aangetoond. Een T-cel afhankelijke respons onderscheidt zich van een T-cel onafhankelijke respons door o.a. twee belangrijke kenmerken: een zuigeling kan vanaf de leeftijd van twee weken al antistoffen maken tegen eiwitten en herhaalde vaccinatie leidt tot steeds hogere antistofconcentraties dankzij het geheugen dat wordt opgebouwd.
De antistofvorming tegen polysacchariden loopt dus heel anders en is o.a. afhankelijk van de aanwezigheid van een bepaald type B-cellen in de milt, de zogenaamde marginale zone B-cellen. Vanaf de leeftijd van 18-24 maanden komt er op het oppervlak van deze cellen een eiwit-structuur, CD21, in grote hoeveelheden tot expressie. CD21 bindt complement-factor C3d. Dit C3d wordt geactiveerd en gebonden door bacteriële kapsel-polysacchariden. De

marginale zone B-cel is dus via CD21-C3d interactie in staat polysacchariden aan zijn oppervlak te binden. Vervolgens worden B-cellen geactiveerd die specifieke antistoffen tegen dit kapsel-polysaccharide kunnen maken. De slechte antistofvorming tegen polysacchariden bij kinderen onder de twee jaar is mogelijk deels te verklaren door het feit dat bij zuigelingen in de milt CD21 in slechts zeer geringe hoeveelheden tot expressie komt. Er kan dus geen CD21-C3d interactie plaatsvinden, en B-cellen worden dus niet tot antistofvorming tegen polysacchariden aangezet.

Koppeling van kapsel-polysacchariden aan eiwitten leidt ertoe dat de antistofvorming tegen deze polysacchariden T-cel afhankelijke kenmerken krijgt: al op zeer jonge leeftijd worden er antistoffen tegen kapselpolysacchariden gevormd als deze gekoppeld zijn aan eiwitten. Blijkbaar is de antistofvorming tegen aan eiwit gekoppelde polysacchariden niet afhankelijk van de aanwezigheid van rijpe marginale zone B-cellen in de milt -want die zijn er nog niet op deze jonge leeftijd-, en dus onafhankelijk van CD21-C3d-interactie.
Vaccins bestaande uit polysacchariden gekoppeld aan eiwitten worden polysaccharide-conjugaat-vaccins genoemd. In **hoofdstuk 2** en **3** tonen we in een ratmodel aan dat pneumococcen-polysaccharide-conjugaat-vaccins (PCV) zich inderdaad heel anders gedragen dan ‘kale’ polysaccharide-vaccins. In **hoofdstuk 2** wordt beschreven dat de kale pneumococcen-polysacchariden na inspuiting in de bloedbaan van de rat lokaliseren op de marginale zone B-cellen van de milt. Deze lokalisatie is afhankelijk van de aanwezigheid van complement. In ratten die zijn behandeld met een bestanddeel van slangengif (Cobra Venom Factor, CVF), dat de complement-eiwitten in het bloed ‘uitput’, vinden we deze specifieke milt-lokalisatie niet, en blijft het polysaccharide langer in de bloedbaan. Bovendien is de antistofvorming tegen kale polysacchariden in met CVF behandelde ratten veel slechter. Conjugaat-vaccins lokaliseren minder specifiek op marginale zone B-cellen, en ook in afwezigheid van complement-eiwitten vinden we polysaccharide-conjugaat terug in de milt. De productie van antistoffen tegen polysaccharide-conjugaten is zelfs beter na CVF-behandeling. De antistofvorming tegen polysacchariden gekoppeld aan eiwitten is dus minder afhankelijk van de aanwezigheid van complement dan die tegen kale polysacchariden. Dit, in combinatie met het gegeven dat antistofvorming tegen conjugaten al op zeer jonge leeftijd kan plaatsvinden, als er nog vrijwel geen CD21 op marginale zone B-cellen tot expressie komt, wijst er op dat PCV niet via CD21-complement factor C3d interactie aanzetten tot antistofproductie.

In **hoofdstuk 3** laten we zien dat de antistofproductie tegen PCV zelfs milt-onafhankelijk is: in ratten zónder milt is de antistofvorming tegen conjugaat-vaccins minstens even goed als in ratten mèt milt. De antistofvorming tegen kale polysacchariden daarentegen is bijna afwezig in ratten zonder milt. De milt is van eminent belang voor de vorming van antistoffen tegen polysacchariden, en patiënten zonder goed functionerende milt zijn bevattelijk voor ernstig verlopende pneumococcen-infecties. Als de kapsel-polysacchariden gekoppeld zijn aan eiwitten, zijn er blijkbaar gebieden buiten de milt waar de antistofproductie tegen polysacchariden in gang kan worden gezet. Conjugaat-vaccins zouden dus niet alleen preventief kunnen worden toegepast bij jonge kinderen, maar ook bij patiënten zonder milt, en andere patiënten met verhoogd risico voor infecties met gekapselde bacteriën.

Hóe conjugaat-vaccins de productie van antistoffen tegen polysacchariden stimuleren is nog grotendeels onduidelijk. Wel duidelijk is dat het polysaccharide daadwerkelijk gebonden moet zijn aan het eiwit. Menging van losse kapsel-polysacchariden met een conjugaat-vaccin leidt niet tot een betere antistofvorming tegen het losse polysaccharide (**hoofdstuk 3**). Het veronderstelde werkingsprincipe van conjugaat-vaccins is dat helper T-cellen het eiwitgedeelte van het conjugaat herkennen, en hulp verlenen aan naburige B-cellen die specifiek het polysaccharidedeel herkennen. De T-cellen zouden activerende factoren produceren waardoor de B-cel aangezet kan worden tot antistofvorming tegen polysacchariden zonder afhankelijk te zijn van activatie via CD21-C3d interactie. Het is daarbij nodig dat de T- en B-cellen in elkaars nabijheid liggen; het polysaccharide-eiwit-conjugaat-vaccin bewerkstelligt dit nauwe contact. In laboratoriumproefopstellingen (in een reageerbuis) liggen T- en B-cellen sowieso heel dicht bij elkaar, en is menging van eiwit en polysaccharide voldoende om een antistofrespons tegen het polysaccharide te krijgen (**hoofdstuk 4**). Toch blijkt er ook dan een verschil te bestaan tussen de antistofvorming tegen eiwitten en polysacchariden: de antistofproductie tegen polysacchariden kan op gang worden gebracht door B cel-activerende oplosbare factoren alleen, terwijl voor de antistofproductie tegen eiwitten contact van B- met T-cellen nodig is.

Tot nu toe is er steeds gesproken over antistoffen in het algemeen, maar antistoffen (immuunglobulines, Ig) kunnen verder onderverdeeld worden in een aantal klassen, aangeduid met een hoofdletter: IgG, IgM, IgA, IgE en IgD. IgG en IgA worden nog verder onderverdeeld in de subklassen IgG1, IgG2, IgG3 en IgG4, en IgA1 en IgA2. Voor de afweer zijn met name IgA, IgG en IgM van belang. IgA is de antistofklasse die met name op de

slijmvliezen van luchtwegen, darm en urinewegen voorkomt, maar ook in het bloed; IgG is de belangrijkste antistofklasse in het bloed. Tegen eiwitten worden voornamelijk IgM en IgG1-type antistoffen gemaakt. Vaccinatie met polysaccharide-conjugaat-vaccins geeft bij jonge kinderen IgM en meestal ook IgG1 antistoffen. Volwassenen maken na inenting met pneumococcen-vaccins IgM en met name IgG2-antistoffen.
Afwijkingen in de antistofproductie leiden tot verhoogde infectiegevoeligheid. Patiënten die veel te weinig of helemaal geen antistoffen maken (patiënten met een hypo- of a-gammaglobuliniaemie) zijn relatief zeldzaam. Veel vaker komt een verminderde antistofvorming in een van de (sub-) klassen voor. Voldoende IgG2- en IgA antistofconcentraties tegen polysacchariden lijken van belang voor adequate bescherming tegen infecties met gekapselde bacteriën. Patiënten met frequent terugkerende luchtweginfecties en ernstige middenoorontstekingen hebben vaak een verlaagde concentratie IgG2-antistoffen in het bloed. IgA-antistofdeficiëntie (het niet kunnen maken van IgA antistoffen) gaat ook vaak gepaard met een verhoogde infectiegevoeligheid. In 1985 werd voor het eerst een patiënt beschreven die normale totale concentraties van IgM, IgA, IgG en IgG subklassen had, en een goede antistofproductie tegen eiwitten, maar die geen specifieke antistoffen kon maken tegen bacteriële kapsel-polysacchariden. Deze patiënt had zeer vaak long- en oorontstekingen doorgemaakt. Dit was de eerste beschrijving van de relatie tussen het optreden van frequente, ernstige luchtweginfecties en een selectieve antistofdeficiëntie tegen polysacchariden. Sindsdien zijn er meerdere studies verschenen over patiënten met frequent recidiverende bacteriële luchtweginfecties en verlaagde antistoffen tegen polysacchariden.

Kinderen met recidiverende bacteriële luchtweginfecties produceren meestal wel IgM- en IgG1-, maar vaak verminderd IgG2- en IgA anti-pneumococcen-polysaccharide antistoffen. Dit in tegenstelling tot gezonde controlekinderen, die wel IgG2 en IgA anti-polysaccharide antistoffen kunnen maken. IgG2- en IgA-antistoffen tegen polysacchariden lijken dus een factor voor optimale bescherming tegen oorontstekingen en andere luchtweginfecties veroorzaakt door gekapselde bacteriën als de pneumococ. Omdat ook na vaccinatie met het pneumococcen-polysaccharide-vaccin deze kinderen slecht IgG2 en IgA anti-pneumococcen-antistoffen maken, werkt het vaccin onvoldoende beschermend. In **hoofdstuk 5a en 5b** laten we zien dat bij kinderen met frequent terugkerende bacteriële luchtweginfecties inenting met een pneumococcen-conjugaat-vaccin, gevolgd door een vaccinatie met een polysaccharide-vaccin, wèl leidt tot IgG2- en IgA-antistofvorming. Dit geldt voor patiënten met normale

totale concentraties aan IgG2- en IgA in het bloed (**hoofdstuk 5**), en zelfs voor patiënten met verlaagde totale concentraties aan IgG2- en/of IgA (**hoofdstuk 5b**). In Nederland is momenteel een grote, dubbelblinde studie gaande (de OMAVAX-studie), die in kinderen met recidiverende oorontstekingen, de zogeheten otitis-prone kinderen, meet of dit vaccinatie-schema, pneumococcen-conjugaat-vaccin gevolgd door pneumococcen-polysaccharide-vaccin, ook daadwerkelijk het optreden van oorontstekingen voorkómt.

Voor een goede afweer tegen gekapselde bacteriën is opname van bacteriën door witte bloedcellen noodzakelijk. Dit opname-proces wordt fagocytose genoemd. Voor efficiënte fagocytose moeten antistoffen worden gebonden aan het oppervlak van de bacterie. Deze binding heet opsonisatie, het 'lekker maken' van de bacterie, zodat de fagocyt de bacterie makkelijker opneemt. Fagocyterende witte bloedcellen zijn uitgerust met structuren op hun oppervlak die antistoffen binden, en aldus de geopsoniseerde bacterie kunnen opnemen. Deze structuren heten Fc-receptoren, waarvan er verschillende soorten zijn. Voor de herkenning van IgA is er de Fcα-receptor (FcαRI), en voor de herkenning van de verschillende IgG subklassen zijn er een groot aantal Fcγ-receptoren, onderverdeeld in 3 klassen (FγRI, FcγRII, FcγRIII) die weer in subklassen worden onderscheiden. Voorts bestaan er individuele verschillen in de structuur van FcγR (zogeheten polymorfismen), die de interactie met IgG subklassen kunnen beïnvloeden. IgG tegen pneumococcen blijkt de fagocytose van pneumococcen voornamelijk te bevorderen via FcγRIIa. Er bestaan twee polymorfismen van FcγRIIa, R131 en H131. FcγRIIa-H131 is de enige receptor die IgG2 kan binden. Fagocyten met FcγRIIa-H131 op hun oppervlak fagocyteren bacteriën beladen met IgG2 veel beter dan fagocyten zonder deze receptor. In **hoofdstuk 6** tonen we aan dat, na vaccinatie van gezonde volwassenen met pneumococcen-conjugaatvaccin, de hoeveelheid IgG2-antistoffen tegen pneumococcen-polysaccharide correleert met de efficiëntie van fagocytose door fagocyten met FcγRIIa-H131 op hun oppervlak. Er is geen relatie tussen fagocytose en IgG1-antistofconcentratie. Ook na vaccinatie met conjugaat, dat zoals gezegd voornamelijk IgG1-antistoffen induceert, lijkt IgG2 dus de meest functionele antistofklasse.

Koppeling van kapsel-polysacchariden aan eiwitten ligt aan de basis van de ontwikkeling van conjugaat-vaccins. Vaccinatie met polysaccharide-conjugaat-vaccins leidt er toe dat de afweer tegen polysacchariden kenmerken krijgt van de afweer tegen eiwitten: naast het feit dat er al op zeer jonge leeftijd antistofvorming mogelijk is, wordt er ook geheugen opgebouwd. Bij elk

volgend contact met het polysaccharide (als vaccin of als hele bacterie) reageert het afweersysteem met snelle productie van veel antistoffen. Deze antistoffen worden ook kwalitatief beter: als er vaker contact met het polysaccharide is geweest, worden er sterker bindende antistoffen (antistoffen met gemiddeld een hogere affiniteit voor het polysaccharide, zogenaamde hoog-avide antistoffen) gevormd. Bovendien blijken conjugaat-vaccins ook het dragerschap van gekapselde bacteriën te sterk verminderen. Het daadwerkelijk optreden van infectie kan daarmee worden voorkomen.

Het Hib-conjugaatvaccin, bestaande uit het kapsel-polysaccharide van Haemophilus influenzae type b (Hib) gekoppeld aan tetanus-toxoid, dat in Nederland sinds 1993 gegeven wordt aan kinderen op de leeftijd van 2, 3, 4 en 11 maanden, heeft geleid tot een enorme reductie in het aantal ernstige infecties veroorzaakt door Haemophilus influenzae type b. Vóór introductie van het Hib-vaccin werden jaarlijks bijna 700 ernstige Hib-infecties gemeld. Sinds opname van het Hib-vaccin in het Rijks Vaccinatie Programma heeft slechts een klein aantal kinderen -gemidddeld 3 per jaar- ondanks volledige vaccinatie, dat wil zeggen na tenminste 3 Hib-inentingen, nog een hersenvliesontsteking of ontsteking van het strotklepje doorgemaakt. In die sporadische gevallen spreken we van 'Hib-vaccinatie-falen'. In andere landen waar het Hib-vaccin wordt gegeven, worden soortgelijke successen behaald.
Wij waren in staat om bij het merendeel van de kinderen met Hib-vaccinatie-falen, namelijk 14 van de 21, onderzoek te doen naar de afweer. Twee van deze 14 kinderen bleken een algemene afwijking in antistofproductie te hebben: een meisje had een hypogammaglobulinaemie, en een jongen had een lage concentratie totaal IgG2 in het bloed. Een stoornis in de specifieke anti-Hib antistofproductie bleek vaker voor te komen: een deel van de kinderen had onvoldoende antistoffen tegen Hib gemaakt na vaccinatie en/of na infectie, zoals blijkt uit lage anti-Hib antistofconcentraties die werden gevonden in bloed afgenomen tijdens of na het doormaken van de infectie (**hoofdstuk 7a en 7b**). Lage concentraties anti-Hib antistoffen worden vaker gezien bij kinderen, zeker als de vaccinatie een tijd geleden is gegeven. Maar na Hib-conjugaat-vaccinatie zou er geheugenvorming moeten zijn opgetreden, en bij hernieuwd contact met Hib zou er dus snel een hoge antistofrespons moeten optreden die infectie zou moeten voorkomen. Het feit dat er ook na doormaken van een Hib-infectie slechts lage anti-Hib antistofconcentraties worden gevonden wijst er dus op dat er bij de patiënten met Hib-vaccinatie falen geen goede geheugenvorming is opgetreden. En als er bij deze patiënten al antistoffen tegen Hib geproduceerd werden, na ziekte of na opnieuw vaccineren, dan bleken deze antistoffen bovendien van minder goede

kwaliteit te zijn: de bindingssterkte (aviditeit) van de anti-Hib antistoffen van patiënten met Hib-falen was geringer dan de aviditeit van anti-Hib-antistoffen van controles. Dit is ook een aanwijzing voor onvoldoende antistofrespons tegen Hib. Deze zowel kwantitatief als kwalitatief slechtere anti-Hib antistofrespons kan de verhoogde vatbaarheid voor Hib-infectie verklaren.

In **hoofdstuk 8** worden nog enkele algemene opmerkingen betreffende pneumococcen- en *Haemophilus influenzae* type b-conjugaat-vaccins geplaatst. Het succes van het Hib-conjugaat-vaccin, dat het dragerschap van en infecties veroorzaakt door Hib sterk heeft verminderd, heeft de ontwikkeling van pneumococcen-polysaccharide-conjugaat-vaccins (PCV) een extra impuls gegeven. De productie van PCV is echter ingewikkelder, omdat in plaats van slechts één type (type b in het geval van Hib), nu meerdere typen polysacchariden in het vaccin moeten zitten. Het is technisch nog niet mogelijk om meer dan 7 tot 11 pneumococcen-typen in het vaccin op te nemen. Gekozen is om díe kapsel-polysacchariden te includeren, die bij kinderen (in de Westerse wereld) het vaakst verantwoordelijk zijn voor infecties. Inmiddels is uit eerste grote studies bij jonge kinderen gebleken dat deze pneumococcen-conjugaat-vaccins erg effectief zijn in het voorkómen van longontsteking, hersenvliesontsteking en bloedvergiftiging (zogenaamde invasieve infecties) veroorzaakt door pneumococcen die in het conjugaat-vaccin vertegenwoordigd zijn. PCV zijn helaas minder effectief in het voorkómen van ‘oppervlakkige’ infecties van de slijmvliezen, zoals acute middenoorontsteking. Blijkbaar zijn de eisen die worden gesteld aan het afweersysteem om te kunnen beschermen tegen infecties van de slijmvliezen anders dan die voor bescherming tegen invasieve infecties. Na inenting met PCV worden voornamelijk IgG1-antistoffen gevormd, en deze antistoffen zijn dus effectief in het voorkómen van invasieve pneumococceninfecties. Voor afdoende bescherming tegen ‘oppervlakkige’ luchtweginfecties zijn misschien IgG2- en IgA-antistoffen tegen pneumococcen-polysacchariden nodig, die niet of nauwelijks worden geproduceerd na vaccinatie met PCV. Mogelijk leert de eerder beschreven OMAVAX-studie die momenteel in Nederland gaande is welke antistoffen nodig zijn voor bescherming tegen oorontsteking.

Vaccinatie met PCV leidt niet alleen tot reductie van het aantal infecties met pneumococcen die in het vaccin zitten, maar ook tot vermindering van dragerschap van deze types. In de bestaande PCV zijn de meeste pneumococcen-types opgenomen die resistent zijn tegen veel gebruikte antibiotica. Een voordeel van uitgebreide vaccinatie met PCV kan dus zijn dat

resistente pneumococcen worden teruggedrongen. Maar uit de studies die tot nu toe gedaan zijn blijkt ook dat vaccin-type pneumococcen worden vervangen door pneumococcen die niet in het vaccin zitten. Het is denkbaar dat vervolgens ook het aantal infecties veroorzaakt door niet-vaccin typen zal oplopen, en dus de vaccin-samenstelling zal moeten worden veranderd.

Omdat vaccinatie met Hib-vaccins ook het Hib-dragerschap vermindert, zonder dat er toename lijkt op te treden in dragerschap van andere *Haemophilus influenzae* types, zou het mogelijk moeten zijn om door wereldwijde vaccinatie met Hib-conjugaat-vaccins ernstige *Haemophilus influenzae* infecties te elimineren. Bij pneumococcen is er echter het risico van verschuiving van vaccin-type-dragerschap naar dragerschap van niet-vaccin type pneumococcen. Alternatieve preventie-strategieën zullen moeten worden ontwikkeld. De bestaande PCV zijn dus zeker niet de definitieve oplossing, maar wel een belangrijk middel om ernstige pneumococceninfecties te helpen voorkómen.

Dankwoord

Zomer 1991. De wachttijd tussen het doctoraalexamen Geneeskunde en de aanvang van de co-schappen diende natuurlijk nuttig te worden besteed. Op het laboratorium Immunologie van het Wilhelmina Kinderziekenhuis deed een enthousiaste Engelsman, Robert Feldman, leuk onderzoek. Hij kon wel een student gebruiken. Robert, jij legde de basis. Je bracht me zowel praktisch als theoretisch de beginselen van onderzoek doen bij. In Utrecht en later ook in Londen heb ik steeds met erg veel plezier met je gewerkt.

Zo kwam ik dus bijna 10 jaar geleden binnen op het laboratorium van Prof. dr. B.J.M. Zegers. Beste Ben, vanaf dat moment heb je je steeds voor me ingezet. Dankzij jou kreeg ik een duidelijk beeld van wat ik nu eigenlijk wilde met mijn opleiding. Bovendien zorgde je ervoor dat ik dat ideaalbeeld ook kon realiseren. De AGIKO-plek bezet ik nog steeds met veel plezier, al ben ik erg blij dat dit proefschrift, mede dankzij jou, nu af is.

Dr. G.T. Rijkers, beste Ger; jouw deur staat altijd open. Als student kwam ik bij je voor scriptie-begeleiding en nog steeds loop ik zeer frequent je kamer binnen. Jij bent grotendeels verantwoordelijk voor de directe begeleiding van het onderzoek dat beschreven staat in dit proefschrift. Altijd kon ik bij je terecht, of het nu ging om het uitdenken van experimenten, het bespreken van resultaten of het opschrijven van conclusies. En al kan ik nu inmiddels zelf iets beter omgaan met computers, jouw hulp was tot het eind toe onmisbaar.

Op het onderzoek van Dr. E.A.M. Sanders kon ik voortbouwen. Beste Lieke, jij staat steeds garant voor de link naar de kliniek. Je bent kundig en praktisch, je weet feilloos hoofd- en bijzaken van elkaar te onderscheiden en je boodschap is altijd helder. Van jouw klinische en onderzoekservaring kan ik nog veel leren.

Marleen Voorhorst en Adrie van den Muijsenberg, bedankt voor jullie hulp op het lab in respectievelijk de eerste jaren en het laatste jaar van het onderzoek. En natuurlijk voor de bereidheid me op het eind bij te staan, terwijl jullie eigenlijk al van me af dachten te zijn. Jelle Adelmeijer en de mensen van de serumhoek dank ik voor de vele hand- en spandiensten en de talloze bepalingen die altijd tussendoor gedaan konden worden.

De combinatie van onderzoek en opleiding bracht de afgelopen 6 jaar heel wat afwisseling met zich mee. Eerst een paar maanden onderzoek, dan wat maanden kliniek, daarna weer een labperiode gevolgd door een jaar klinisch werk, een jaar lab, een jaar opleiding in

Nieuwegein, een jaar lab en nu weer de kliniek. En dan ook nog twee keer zwangerschapsverlof tussendoor. Ik wil alle mensen op het laboratorium en in de kliniek bedanken voor hun flexibiliteit: jullie pakten steeds de draad weer mee op. Ondanks het feit dat ik deze 6 jaar overal meer af- dan aanwezig was, voelde ik me toch steeds weer snel op mijn plek.

Eigen haard is goud waard, maar het is goed om je licht ook eens ergens anders op te steken. Zo werd een belangrijk deel van het onderzoek beschreven in dit proefschrift verricht in samenwerking met de Afdeling Pathologie en Laboratoriumgeneeskunde van het Academisch Ziekenhuis Groningen, onder begeleiding van Prof. dr. W. Timens. Beste Wim, ik voelde me in Groningen dankzij jullie gastvrijheid meteen thuis. Het gezamenlijke onderzoek naar de rol van de milt in de anti-polysaccharide-respons is erg leuk en verdient te worden voortgezet. André Zandvoort, Monique Lodewijk, Pieter Klok en Geert Harms, bedankt voor al het werk dat jullie verricht hebben aan de ratten.
Natuurlijk wil ik ook iedereen die verder direct of indirect heeft bijgedragen aan de totstandkoming van dit proefschrift op deze plaats bedanken. Regelmatig overleg en gezamenlijk onderzoek vond plaats met mensen van binnen Utrecht (het Eijkman-Winkler Instituut) en daar buiten (het RIVM, Alkmaar, Nieuwegein). De immunologische evaluatie van patiënten met Hib-vaccinatie-falen is mogelijk dankzij de samenwerking met het Nederlands Referentie Laboratorium voor Bacteriële Meningitis. Verfijning van de immunologische bevindingen vond plaats in het Laboratorium Kindergeneeskunde van het Leiden Universitair Medisch Centrum.

Familie en vrienden, dankzij jullie is er een leven naast mijn opleiding.
Lieve mama, in opleiding tot kinderarts, daar kun je je nog wel wat bij voorstellen; wat de lol is van laboratoriumonderzoek vind je minder makkelijk te begrijpen, al ben je natuurlijk wel trots op een gepromoveerde dochter. Ik hoop dat (de Nederlandse samenvatting in) dit boekje aan jou en verdere familie en vrienden duidelijk heeft kunnen maken waar ik de afgelopen jaren op dat lab mee bezig ben geweest.
Fem, dit proefschrift is natuurlijk niet een "letteren-levenswerk", maar dankzij jouw inspanningen is het wel net iets meer geworden dan een bundel artikelen met een kaft eromheen.

Lieve Pien en Niels, twee keer heb ik een deel van mijn onderzoekstijd thuis doorgebracht als zwangerschapsverlof. Dit proefschrift is door jullie komst wellicht wat ‘minder’, maar mijn leven is er zeker ‘meer’ door geworden.
Lieve Kees, de korte termijn-planning is mij wel toevertrouwd; jij houdt steeds de lange termijn voor ogen en zo nodig stuur je bij. Dankwoorden eindigen meestal met de belofte dat er na het voltooien van het proefschrift wat meer tijd over zal zijn. Ik ben bang dat ik een dergelijke uitspraak niet waar kan maken de komende tijd. Het avondlange gerammel op de computer is wel afgelopen, maar rustiger is het er niet op geworden. Je steun blijft nodig.

Curriculum vitae

Mijke Breukels werd geboren op 11 september 1968 te Eindhoven. Na het behalen van het eindexamen Gymnasium ß aan het Van Maerlantlyceum te Eindhoven, begon zij in 1986 aan de studie Biologie en in 1987 aan de studie Geneeskunde, beide aan de Universiteit Utrecht. In 1991 behaalde zij haar doctoraalexamen Geneeskunde, en na een onderzoeksperiode van 6 maanden op het laboratorium voor Pediatrische Immunologie van het Wilhelmina Kinderziekenhuis (hoofd Prof. dr. B.J.M. Zegers, supervisor Dr. R.G. Feldman) doorliep ze van april 1992 tot augustus 1994 haar co-schappen. In deze periode werd 4 maanden onderzoek verricht op het laboratorium voor Neuromusculaire Ziekten van het Academisch Ziekenhuis Utrecht (hoofd Prof. dr. F.G.I. Jennekens, supervisor Dr. H.J.L.M. Ulenkate). Het arts-examen behaalde zij in september 1994. Daarna deed zij een half jaar onderzoek op het laboratorium van Dr. R.G. Feldman, Department of Infectious Diseases and Bacteriology, Royal Postgraduate Medical School, Hammersmith Hospital, Londen. Dit onderzoek werd gefinancierd door een VSB-beurs en werd gedaan onder verantwoordelijkheid van Prof. dr. W.P.M. Hoekstra, vakgroep Moleculaire Celbiologie, Utrecht. Hiermee rondde ze de studie Biologie in april 1995 af.
In mei 1995 werd ze op basis van een subsidie van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) aangesteld als assistent-geneeskundige in opleiding tot klinisch onderzoeker (AGIKO) Kindergeneeskunde in het Wilhelmina Kinderziekenhuis, Universitair Medisch Centrum Utrecht. In het kader van deze aanstelling heeft ze onderzoek verricht op het laboratorium voor Pediatrische Immunologie (Prof. dr. B.J.M. Zegers, Dr. Ir. G.T. Rijkers), hetgeen heeft geresulteerd in dit proefschrift. De opleiding tot kinderarts (huidige opleider Prof. dr. J.L.L. Kimpen) zal in 2002 worden afgerond.

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