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MUCOSAL ANTIBODIES TO PROTEIN AND CAPSULAR POLYSACCHARIDE ANTIGENS OF *STREPTOCOCCUS PNEUMONIAE* IN CHILDREN

Relation to pneumococcal carriage and acute otitis media

Department of Vaccines
Laboratory of Vaccine Immunology
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Relation to pneumococcal carriage and acute otitis media

by

Birgit Simell

ACADEMIC DISSERTATION

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To my family

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred in the text by their Roman numerals:

I Simell B, Korkeila M, Pursiainen H, Kilpi TM, Käyhty H. Pneumococcal carriage and otitis media induce salivary antibodies to pneumococcal surface adhesin A, pneumolysin, and pneumococcal surface protein A in children. J Infect Dis 2001;183: 887-96.

II Simell B, Kilpi TM, Käyhty H. Pneumococcal carriage and otitis media induce salivary antibodies to pneumococcal capsular polysaccharides in children. J Infect Dis 2002;186:1106-14.

III Simell B, Kilpi TM, Käyhty H. Subclass ditribution of natural salivary antibodies against pneumococcal capsular polysaccharide of type 14 and pneumococcal surface adhesin A in children. Submitted for publication.

IV Simell B, Kilpi TM, Rapola S, Lahdenkari M, Jäntti V, Käyhty H. Salivary antibodies to pneumococcal surface protein A and pneumococcal surface adhesin A and the risk of pneumococcal AOM and carriage. Manuscript.

Some additional unpublished data are also presented.

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ABBREVIATIONS

AOM	acute otitis media
C3	third component of complement
CBP	cholin-binding protein
CbpA	cholin-binding protein A
CI	confidence interval
CMIS	common mucosal immune system
CPS	cell wall polysaccharide
CSF	cerebrospinal fluid
CV	coefficient of variation
EIA	enzyme immunoassay
FBS	fetal bovine serum
FinOM	Finnish Otitis Media
GM	geometric mean
GMC	geometric mean concentration
Hic	factor H-binding inhibitor of complement
HIV	human immunodeficiency virus
Ig	immunoglobulin
IgA	immunoglobulin class A
IgG	immunoglobulin class G
IgM	immunoglobulin class M
IL	interleukin
kDa	kilodalton
KTL	Kansanterveyslaitos (National Public Health Institute)
LytA	major pneumococcal autolysin
MALT	mucosa-associated lymphoid tissue
MEF	middle ear fluid
NP	nasopharyngeal swab
NPA	nasopharyngeal aspirate
OD	optical density
OMPC	outer membrane protein complex
OR	odds ratio
PAF	platelet-activating factor
PbcA	C3-binding protein A
PBP	penicillin-binding protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Ply	pneumolysin
Pnc	pneumococcal
PncCRM7	pneumococcal capsular polysaccharide-CRM_{197} conjugate vaccine
PncOMPC	pneumococcal capsular polysaccharide-meningococcal outer membrane protein complex conjugate vaccine
PS	polysaccharide
PS14	capsular polysaccharide of pneumococcal serotype 14
PsaA	pneumococcal surface adhesin A
PspA	pneumococcal surface protein A
PspC	pneumococcal surface protein C
RR	relative risk
SC	secretory component

SD	standard deviation
sIg	secretory immunoglobulin
SpsA	*Streptococcus pneumoniae* secretory IgA binding protein
ST	serotype
TD	T cell dependent, thymus-dependent
TI	T cell independent, thymus-independent
U/ml	units per milliliter

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) continues to be a leading cause of morbidity and mortality worldwide. Along with a number of invasive infections such as pneumonia, meningitis and sepsis, pneumococcus causes local infections such as sinusitis and acute otitis media (AOM). Although usually a self-healing infection, AOM is a marked health problem among infants and young children resulting in a burden on health services and respectable inconvenience for the young patients and their families.

Mucosal surfaces of the human upper respiratory tract are the primary site of a pneumococcal (Pnc) infection. Local mucosal immunity is likely to act as an important first line defense against Pnc carriage and subsequent disease. The recurrent nature of Pnc AOM, however, indicates that the natural immune responses in early childhood are not strong enough to offer effective protection against Pnc infection. Mucosal immune responses induced by systemic immunization are modest, but stronger local antibody responses could be accomplished by mucosal (e.g., intranasal) immunization. The results gathered from animal models concerning mucosal immunization against pneumococcus have been encouraging. However, more basic research will be required before considering such applications in humans. This research includes the characterization of the development of natural mucosal immunity to various Pnc antigens.

The vaccine development against pneumococcus begun a long time ago and it still continues. The Pnc 23-valent capsular polysaccharide (PS) vaccine is efficacious among healthy adults, but it induces poor antibody responses in young children. Covalent conjugation of Pnc PS antigens to a protein carrier has improved their immunogenicity in children. However, the serotype selection of Pnc conjugate vaccines is restricted and the protection against overall Pnc AOM incidence is therefore modest. These realities have stimulated an interest in the development of vaccines based on common Pnc protein antigens. The crucial information still lacking, is whether human mucosal antibodies to Pnc protein vaccine candidates would have a role in the prevention of Pnc diseases.

The Finnish Otitis Media (FinOM) Cohort Study was originally conducted to examine the risk factors and epidemiology of Pnc carriage and AOM. During the study, 329 Finnish children were followed from 2 to 24 months of age. As a secondary endpoint, this allowed the investigation of the natural development of mucosal immunity to pneumococcus. To this end, the development of salivary antibodies to the selected Pnc protein and PS antigens has been examined in this thesis. In addition, the association between salivary antibodies to Pnc proteins and the risk of subsequent Pnc carriage and AOM has been evaluated.

REVIEW OF THE LITERATURE

1. Streptococcus pneumoniae

1.1. General description

Streptococcus pneumoniae is an exclusively human pathogen, which was isolated for the first time over 120 years ago, in 1881. Before the era of antibiotics, Pnc diseases, particularly Pnc pneumonia, were common causes of death. Nowadays, pneumococcus continues to be an important cause of both mucosal and systemic diseases worldwide. Pneumococcus has been the subject of intensive investigation for many decades. This has generated many scientific discoveries, including the comprehension of DNA being a carrier of genetic information (Avery *et al.* 1944), the therapeutic efficacy of penicillin (Abraham *et al.* 1941; Keefer *et al.* 1943), the role of the bacterial capsule in resistance to phagocytosis (Issaef 1893), and the ability of bacterial polysaccharides (PS) to induce protective antibodies (Felton *et al.* 1955; Baker 1990). Despite all intensive investigation, many questions concerning the mechanisms of the Pnc pathogenesis and the immunology against Pnc infections still remain to be answered. Furthermore, a new challenge is now being faced by the appearance and spread of Pnc strains, which are resistant to one or more antibiotic drugs.

Pneumococcus is a gram-positive, encapsulated, facultative anaerobic, lancet-shaped coccus, which usually grows in pairs or in short chains. The three major surface layers that can be distinguished on the surface of pneumococci are plasma membrane, cell wall, and PS capsule. On the basis of the differences in the structure of the PS capsule, pneumococci can be divided into more than 90 different serogroups or serotypes. Two nomenclature systems have been used to classify these types. In the American nomenclature, the serogroups or serotypes are designated in the order of their discovery. In contrast, in the Danish nomenclature, the serogroups or serotypes are designated according to the structural and antigenic characteristics of the capsule, e.g., the serologically cross-reactive serotypes 6A and 6B are placed in the same serogroup 6 (Lund *et al.* 1978). Nowadays, the Danish nomenclature is more widely adopted. The Danish nomenclature was used in the present study.

The complete DNA sequence of *S. pneumoniae* (serotype 4 from a child with meningitis) was published for the first time in November 1997. The opportunity to exploit the whole genome sequence of pneumococcus should allow the rapid discovery of regulatory networks and therapeutic targets for this pathogen, as well as new candidates for vaccine development (Baltz *et al.* 1998; Hoskins *et al* 2001; Tettelin *et al.* 2001; Wizemann *et al*. 2001).

1.2. Identification of pneumococcus

The laboratory culture of pneumococci requires multiple nutritional factors, carbon dioxide (CO_2) and an ideal pH of 7.2 to 7.4. On solid media pneumococci grow characteristically as flat round colonies with depressed centers. When grown on media containing blood, partial α-hemolysis of surrounding erythrocytes is detected. The identification of isolates with appropriate colonial morphology can be performed with a number of conventional biochemical and/or immunochemical tests, and in most cases the identification of pneumococci is rather straightforward. At the moment, however, no "gold standard" method is available for the identification of pneumococci.

The conventional methods for the identification of pneumococcus are based on optochin sensitivity and bile solubility of the organism. Sensitivity to optochin (ethylhydrocupreine; a derivative of quinine) is the most important identification criterion for pneumococcus (Lund & Henrichsen 1978; Ruoff *et al.* 1999). On the plate inoculated with pneumococci, an inhibition zone appears around the optochin disk. This test is the most frequently used method to identify pneumococci in clinical laboratories (Kaijalainen *et al.* 2002). As an additional test, the bile solubility test can be used to confirm the result. Addition of bile salts on a broth culture of pneumococci results in prompt dissolution of the bacteria due to the activation of peptidoglycan degrading autolysin enzyme. The other α-hemolytic streptococci are generally resistant to optochin and bile insoluble.

The presence and type of the Pnc PS capsule can be determined by using several immunochemical tests. The capsular swelling reaction (Neufeld's quellung reaction) is an old-time, but still useful method to identify Pnc isolates rapidly and with high certainty. Equal volumes of a bacterial suspension, methylen blue and antiserum are mixed on a glass slide, and the bacteria are examined under a light microscope.

Appearance of capsular swelling identifies genus, species and serotype. The presence of capsular PS can also be established by immunochemical methods, e.g. latex agglutination and counterimmunoelectrophoresis (CIEP). Furthermore, several rapid tests are also available for the identification of Pnc antigens directly in clinical samples, e.g., in blood, sputum, urine and cerebrospinal fluid (CSF).

In the 1990s, new possibilities for the identification of pneumococcus were introduced by the development of novel gene amplification methods for detection of pneumococci directly from the clinical samples (Virolainen *et al.* 1994; Salo *et al.* 1999; Toikka *et al.* 1999; Lawrence *et al.* 2003; Murdoch *et al.* 2003). For example, demonstration of the gene for the Pnc virulence protein pneumolysin (Ply) by polymerase chain reaction (PCR) method has been used to detect and identify pneumococci in reference laboratories (Salo *et al.* 1995; Kearns *et al.* 2000). The PCR analysis for the gene of Pnc surface protein A (*psaA*) has shown to be a sensitive tool for diagnosis of Pnc pneumonia (Scott *et al.* 2003). Hybridization methods have also been used for the identification of pneumococcus (Pozzi *et al.* 1989; Fenoll *et al.* 1990). Furthermore, the molecular characterization of Pnc isolates by multilocus sequence typing (MLST) has now become available (Enright *et al.* 1998; Meats *et al.* 2003). The above-mentioned methods, however, have not yet been introduced in routine use for the identification of Pnc isolates.

2. Pneumococcal infections

2.1. Nasopharyngeal carriage

S. pneumoniae is frequently present in the upper respiratory tract of healthy children and adults as a member of the normal nasopharyngeal bacterial flora. The bacteria spread from person to person in droplets of respiratory secretions (Figure 1). Pneumococci adhere to their receptors on the surface of the mucosal cells of the recipient and subsequently colonize the nasopharyngeal epithelium. The balance between the virulence factors of the colonizing pathogen and the defense mechanisms of the host leads to an asymptomatic carriage state, which is the prevailing outcome of Pnc encounters. This is presumably how *S. pneumoniae* manages to persist as a human

parasite. Pnc disease is rarely associated with prolonged nasopharyngeal carriage of a particular Pnc type. Instead, Pnc disease is usually caused by recently acquired strains (Gray *et al.* 1980). It has been suggested that a prolonged carriage of one Pnc type may even be beneficial to the individual in preventing colonization by other types (Gwaltney *et al.* 1975). Multiple Pnc serotypes may be carried concomitantly (Loda *et al.* 1975). In this thesis, the terms "carriage" and "colonization" are used synonymously.

2.1.1. Epidemiology of pneumococcal carriage

The human nasopharyngeral flora is established gradually during the first year of life (Aniansson *et al.* 1992). Almost half of the children in industrialized countries, such as Sweden and the U.S., are colonized with pneumococci at least once by the age of one year (Aniansson *et al.* 1992; Faden *et al.* 1997). In a study conducted in Birmingham, Alabama, the first Pnc type was acquired by a mean age of six months (Gray *et al.* 1980). Pnc carriage involves relatively few of the more than 90 different Pnc serogroups/types circulating. In industrialized countries, the most frequent serogroups isolated from the nasopharynx in young children are 6, 14, 19 and 23 (Gray *et al.* 1980; Prellner *et al.* 1984a; Syrjänen *et al.* 2001). These pediatric types are also related to most cases of Pnc AOM and invasive disease in children in these areas (Austrian *et al.* 1977; Gray *et al.* 1979; Eskola *et al.* 1992; Sniadack *et al.* 1995; Kilpi *et al.* 2001).

Nasopharyngeal carriage rates of *S. pneumoniae* vary by geographic location and population. The average carriage rates in children are 40 to 50% and in adults 20 to 30% (Ghaffar *et al.* 1999). In children, Pnc carriage rates are highest during the first two years of life and start to decrease gradually after the age of 3 to 5 years (Hendley *et al.* 1975; Loda *et al.* 1975; Ingvarsson *et al.* 1982; Syrjänen *et al.* 2001). The nasopharyngeal carriage rate in children in developing countries is generally 2 to 3 times higher than that found in industrialized countries (Greenwood 1999). High Pnc carriage rates in children have been recorded in several developing countries including Zambia (Frederiksen *et al.* 1988), Pakistan (Mastro *et al.* 1993), The Philippines (Lankinen *et al.* 1994) and The Gambia (Lloyd-Evans *et al.* 1996). In Papua New Guinea all children are intensively and persistently colonized with pneumococcus already within the first 3 months of life (Gratten *et al.* 1986; Montgomery *et al.* 1990). Also, Australian aboriginal infants are at a high risk for Pnc carriage (Leach *et al.* 1994). In industrialized countries, such as Sweden, the U.S. and Finland, slower rates of

acquisition and carriage have been reported (Aniansson *et al.* 1992; Faden *et al.* 1997; Syrjänen *et al.* 2001). The reasons for the differences between various populations are not fully understood.

The duration of Pnc carriage is dependent on age and Pnc serotype. The duration of carriage in children is generally longer than in adults (Ekdahl *et al.* 1997). The pediatric types are carried significantly longer compared to the other types (Gray *et al.* 1980), which may be linked to the poor immunogenicity of these types (Klein 1981; Douglas *et al.* 1983). In a study of infants conducted by Gray *et al.* (1980), the duration of carriage was serotype dependent being usually between 2.5 and 4.5 months (range: 1 to 17 months) during the first two years of life. In Swedish children, the duration of carriage has been reported shorter, the median duration being 19 days (Ekdahl *et al.* 1997). In adults, the average duration of Pnc carriage is 6 weeks (Musher 1992).

Several factors affect carriage of *S. pneumoniae*. Young age (<2 years) is associated with an increased risk of Pnc carriage. This is probably best explained by the close contacts between young children and their poorly developed immunity to this organism. The importance of family composition in acquiring nasopharyngeal carriage is clearly established: infants having older siblings have their first acquisition earlier and acquire slightly more serotypes compared to infants who do not have any siblings (Gray *et al.* 1980; Leino *et al.* 2001). In several studies, significantly increased nasopharyngeal carriage has been reported in children with a day-care contact - either via their siblings or theirselves (Rosen *et al.* 1984; Aniansson *et al.* 1992; Dagan *et al.* 1996b). In a Finnish study, however, day care was not associated with an increased risk of Pnc carriage during the first two years of life (Leino *et al.* 2001). Pnc carriage increases in winter (Gray *et al.* 1980, 1982). This is most likely related to closer interpersonal contacts and the viral upper respiratory tract infections that are more frequent during winter months. Higher carriage rates have been reported during episodes of respiratory tract illnesses and AOM than during health (Faden *et al.* 1991; Syrjänen *et al.* 2001).

2.1.2. The composition of nasopharyngeal flora

The colonization of the nasopharynx is a dynamic process and the nasopharyngeal bacterial flora is in a constant state of flux. Factors controlling the trafficking of bacteria into and out of the nasopharynx are poorly understood. While the nasopharynx is

predominantly colonized with avirulent bacteria (e.g., viridans streptococci, nonhemolytic streptococci, diphtheroids, *Neisseria* species), potential middle ear pathogens may also be carried asymptomatically. The most common bacterial species causing AOM such as *S. pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* seem to belong to the normal flora of the nasopharynx both in healthy and otitis prone children (Ingvarsson *et al.* 1982; Freijd *et al.* 1984; Prellner *et al.* 1984a; Stenfors *et al.* 1990; Syrjänen *et al.* 2001). A significant relationship between Pnc colonization and AOM has been documented, emphasizing the importance of the nasopharynx as a reservoir for potential middle ear pathogens (Zenni *et al.* 1995; Syrjänen *et al.* 2001, 2002). In the nasopharynx of the youngest children the most common pathogens are *S. pneumoniae* and *M. catarrhalis*, while the frequency of *H. influenzae* increases after the first year of life (Aniansson *et al.* 1992). The frequency of pathogens decreases with increasing age (Ingvarsson *et al.* 1982; Stenfors & Räisänen 1990), which may be due to an increased immunity against these bacteria. The composition of microflora in the nasopharynx may influence the outcome of Pnc carriage facilitating or impeding Pnc colonization and invasion by symbiosis or competition.

2.1.3. Antibodies and pneumococcal carriage

Colonizing pneumococci may stimulate an immune response that eventually eliminates them. The importance of this immune response is suggested by the observation that the duration of carriage is longer in children than in adults. The duration of Pnc carriage with the poorly immunogenic serogroups, such as 6 and 23, tends to persist longer in children than carriage with the immunogenic groups. Thus, the high incidence of Pnc carriage and AOM in infants and young children partly may be explained by their immature immunity to *S. pneumoniae* compared with adults (Lindberg *et al.* 1993).

The factors of both mucosal and systemic immunity probably play important roles in the defense against Pnc carriage, and the immune status of the host is an important determinant for the prevalence and duration of Pnc carriage. An important specific host factor interrupting nasopharyngeal colonization is suggested to be the production of secretory IgA (sIgA). Nasopharyngeal secretions with sIgA antibody activity against *S. pneumoniae* have been shown to interfere with Pnc adherence to mucosal epithelial cells *in vitro* (Kurono *et al.* 1991). The pre-existing type-specific serum antibodies do

not prevent acquisition of homotypic *S. pneumoniae*, but they have been suggested to shorten the duration of Pnc carriage (Gwaltney *et al.* 1975). Carriage of a particular Pnc serotype for a prolonged period does not seem to induce sufficient local or systemic immunity to prevent a subsequent reacquisition of that serotype (Loda *et al.* 1975).

2.1.4. Interventions to decrease the carriage rates

Nasopharyngeal carriage of *S. pneumoniae* is the first step in the development of Pnc disease. Thus, different interventions have been tried or proposed to decrease Pnc carriage rates. Immunization with Pnc conjugate vaccines has resulted in the reduction of carriage of the vaccine-type pneumococci in infants (Douglas *et al.* 1986; Obaro *et al.* 1996; Dagan *et al.* 1996a, 1997; Mbelle *et al.* 1999). However, serotype replacement by non-vaccine type pneumococci in carriage has raised concern (Mbelle *et al.* 1999; Lipsitch *et al.* 2000).

Many viral and bacterial respiratory pathogens bind to carbohydrate receptors on the respiratory mucosa. In mucosal secretions, such as saliva, tears, urine, sweat and breast milk, occur natural oligosaccharides that bind to the carbohydrate-binding proteins of the microbial pathogens preventing their attachment. Andersson *et al.* (1986) demonstrated that a human milk oligosaccharide could inhibit the binding of *S. pneumoniae* to human nasopharyngeal and oropharyngreal cells. A novel approach to decrease Pnc carriage has been the oligosaccharides that are structural analogues of the epithelial receptor for Pnc attachment. These sugars have been shown to be able to inhibit bacterial adhesion and even detach bacteria that have already attached to pharyngeal cells (Cundell *et al.* 1995a). Zopf and Roth (1996) described the potential of large-scale manufacture of human oligosaccharides for use in studies of prevention of various infectious diseases including AOM. These oligosaccharides have been demonstrated promising in protection against Pnc infections in animal models (Idänpään-Heikkilä *et al.* 1997). However, the intranasal administration of an oligosaccharide 3'-sialyllacto-N-neotetraose in Finnish children did not have a beneficial effect on the nasopharyngeal carriage of bacteria or on the occurrence of AOM (Ukkonen *et al.* 2000).

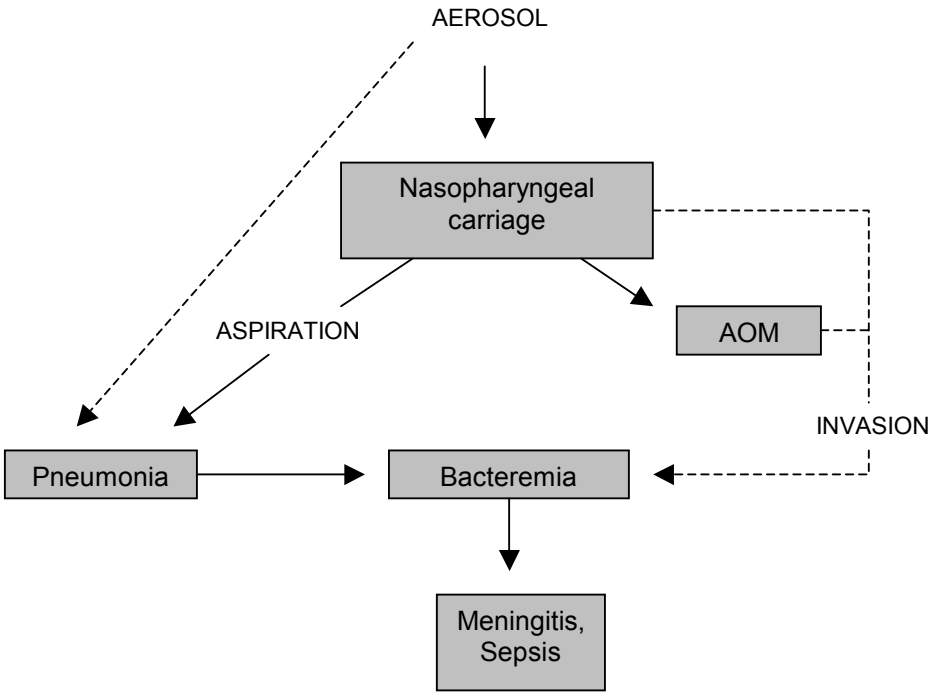

Figure 1. Natural history of diseases caused by *Streptococcus pneumoniae.*

2.2. Pneumococcal diseases

Pneumococci cause a wide variety of diseases, ranging from mild mucosal to life-threatening invasive diseases. Pneumococci cause diseases primarily near by their normal residence that is the upper respiratory tract. Clinical illness follows the spread of pneumococci to surrounding tissues from the nasopharynx. Pneumococci are a primary cause of pneumonia, meningitis and bacteremia in children and pneumonia in adults. The symptoms of all Pnc diseases are primarily due to the ability of the bacteria to evoke an intense inflammatory response, either locally or systemically. Pneumococci reach their target within the body either by direct extension from colonized mucosal surfaces causing sinusitis, AOM (Tuomanen 2000), and pneumonia (Tuomanen *et al.* 1995; Novak *et al.* 1998a), or by hematogenous spread causing sepsis and meningitis (Cundell *et al.* 1995a; Sande *et al.* 1999) (Figure 1). Despite the availability of effective antimicrobial drugs, the mortality in serious Pnc diseases remains high.

2.2.1. Epidemiology of pneumococcal diseases

2.2.1.1. The incidence of pneumococcal diseases

The most important factor determining the risk for Pnc disease is probably age and persons in the extremes of age are the high-risk groups for Pnc diseases. The incidence

of Pnc diseases is highest in infants under two years of age and in persons over 60 years of age. The risk of Pnc disease is increased in patients with predisposing conditions, including asplenia (Wara 1981), chronic medical conditions (such as underlying chronic cardiovascular disease, chronic obstructive lung disease and diabetes mellitus) (Lipsky *et al.* 1986; Taylor *et al.* 1999), alcoholism (Lipsky *et al.* 1986), or immunosuppressive illnesses, particularly human immunodeficiency virus (HIV) infection (Janoff *et al.* 1997). Cigarette smoking is a strong independent risk factor for Pnc disease among immunocompetent, nonelderly adults (Nuorti *et al.* 2000). A highly increased risk of Pnc disease has been reported in certain populations. Exceptionally high rates of disease are seen among the Alaskan native population (Davidson *et al.* 1989), North American Indians (Cortese *et al.* 1992) and Australian aboriginals (Torzillo *et al.* 1995).

The impact of Pnc disease is profound especially in developing countries, where the incidence of Pnc pneumonia and invasive diseases in children is several times higher than in industrialized countries (Hausdorff *et al.* 2000b). Not much data is available on the burden of Pnc AOM in children or that of Pnc pneumonia in adults in the developing world (Hausdorff *et al.* 2000a). In industrialized countries, Pnc AOM is a very prevalent disease in infants and young children, and Pnc pneumonia is an important cause of morbidity and mortality among the elderly population (Feldman 2001). The discrepancy between the developing and industrialized countries originates probably from socio-economic differences, but genetic risk factors associated with racial group (e.g., sickle cell disease) may also play a role. The incidence of Pnc disease is higher in black than in white populations. In certain countries the HIV epidemic has substantially increased the burden of Pnc disease (Gilks 1993; Madhi *et al.* 2000).

2.2.1.2. Distribution of pneumococcal serotypes

All Pnc capsular types are potentially able to cause disease, but the frequency with which different types are isolated in disease manifestations is remarkably different. Certain types are responsible for the majority of diseases. For instance, in young children, a relatively small number of Pnc serogroups account for most of Pnc disease. The Pnc types/groups 6, 14, 19 and 23 have been shown to be responsible for a large proportion of both invasive and local Pnc diseases in children. (Gray *et al.* 1979, 1980; Eskola *et al.* 1992; Sniadack *et al.* 1995; Lloyd-Evans *et al.* 1996; Kilpi *et al.* 2001). These pediatric serogroups are also poor immunogens in children, and the antibody

response comparable to adults against these types has been recorded only at the age of 5 years or even later (Douglas *et al.* 1983; Robbins *et al.* 1983; Koskela *et al.* 1986; Leinonen *et al.* 1986). In older children and adults, a larger number of serogroups are responsible for most cases of Pnc disease (Hausdorff *et al.* 2000a). In Europe and the U.S., the Pnc serogroups most often associated with invasive Pnc disease in older children and adults include 14, 4, 3, 9, 6, 12, 18 and 19 (Hausdorff *et al.* 2000b). Some Pnc groups/types are more likely to be carried for prolonged periods without causing an overt disease, e.g., types 6, 15, 16 and 17 among older children and adults (Nemir *et al.* 1936; Riley *et al.* 1981; Smart *et al.* 1987). Conversely, some serotypes (e.g., types 1, 3, 14, 18 and 46) have been rarely carried but frequently isolated from cases of Pnc disease (Gray *et al.* 1979, 1980; Riley & Douglas 1981; Boulnois 1992). In young children, however, the types causing disease are generally the same as those carried in the population (Gray *et al.* 1982).

There are differences in the geographical distribution and prevalence of Pnc serogroups and serotypes. In Europe and the U.S., approximately 20 serotypes are responsible for ~90% of all reported Pnc diseases, while these types account for <70% of Pnc diseases in e.g. Asian countries (Lee 1987). The seven most common serogroups in the U.S. during the last decade (14, 6, 19, 18, 23, 4 and 9) accounted for 84% of isolates from Finland and 78% from Australia, but only 33% of isolates from Rwanda and 31% from Papua New Guinea (Sniadack *et al.* 1995).

Changes in the type distribution and prevalence among more frequent Pnc types have been described over longer time periods (Lund 1970; Finland & Barnes 1977; Bruyn *et al.* 1992). In the U.S, the Pnc types 1-3 declined from most common to infrequent between 1935 and 1974 (Finland & Barnes 1977). At the same time, the proportion of Pnc infections caused by the seven serogroups now included in the lisenced conjugate vaccine (types 4, 6B, 9V, 14, 18C, 19F, and 23F) increased significantly (Feikin & Klugman 2002). The probable explanations for these shifts have been suggested to be the improvement of socioeconomic conditions, changes in antibiotic use and changes in the immunocompromised status of the populations (Feikin & Klugman 2002).

Certain Pnc serogroups have been shown to be preferentially associated with specific disease manifestations. For instance, serogroups 1 and 14 are most commonly isolated

from blood in both children and adults. Serogroups 6, 10, and 23 are most commonly isolated from CSF and serogroups 3, 19, and 23 from middle ear fluid (MEF). The pneumococci causing disease may need different strategies to remain at the sites of infection and overcome host defense mechanisms. Andersson *et al.* (1981) have shown that strains of serotypes 6A, 14, 19F and 23F adhere strongly when isolated from the nasopharynx of patients with AOM, but less strongly when isolated from the blood or CSF. Thus, strongly and poorly adhering strains may be found within the same capsule type, and the avidity of adhesion of a Pnc strain may correlate with the type of infection that results.

2.2.2. Acute otitis media (AOM)

AOM is a mild, but extremely common disease during childhood. It is the most common reason for the prescription of antibiotics to children. In Finland, 0.5 million attacks of AOM have been calculated to occur each year (Eskola *et al.* 2000). The incidence of AOM among children is highest before 2 years of age showing a peak between 6 and 18 months (Teele *et al.* 1989; Paradise *et al.* 1997; Kilpi *et al.* 2001). The annual incidence rate of AOM in Finnish children during the first two years of life is around 50% (Pukander *et al.* 1982).

AOM may be of bacterial, viral, or both bacterial and viral origin. *S. pneumoniae* causes approximately 35% of the AOM episodes, nontypeable *H. influenzae* 25% and *M. catarrhalis* 15% (Luotonen *et al.* 1981; Karma *et al.* 1985; Pelton 1998). In the FinOM Cohort Study, *S. pneumoniae, H. influenzae* and *M. catarrhalis* were isolated in 26%, 23% and 23% of AOM events, respectively (Kilpi *et al.* 2001). In a number of studies, Pnc groups 6, 14, 19 and 23 have been reported to cause the most cases of Pnc AOM (Gray *et al.* 1980; Klein 1980; Prellner *et al.* 1984b; Kilpi *et al.* 2001; Hausdorff *et al.* 2002).

Nasopharyngeal carriage of middle ear pathogens is considered to be the prequisite for AOM. The carriage of these pathogens is common particularly in young children, which may be one explanation for the high incidence of AOM among young individuals (Stenfors & Räisänen 1990). The middle ear pathogens disappear from the nasopharynx when children grow older. In a study by Stenfors and Räisänen (1990), all children under two years harboured middle ear pathogens in their nasopharynx, the predominant

pathogen being *S. pneumoniae* (70%) followed by *H. influenzae* (65%) and *M. catarrhalis* (48%). Among the older children (2-5 years), 72% were colonized by middle ear pathogens. The predominant pathogen in this age group was *H. influenzae* (44%), followed by *M. catarrhalis* (36%) and *S. pneumoniae* (16%). Several studies have reported that the prevalence of Pnc carriage starts to decrease after the age of 3-5 years (Masters *et al.* 1958; Hendley *et al.* 1975; Loda *et al.* 1975; Ingvarsson *et al.* 1982). However, the incidence rates of Pnc AOM start to decrease already at the age of 18 months when the incidence of AOM caused by *H. influenzae* starts to increase (Kilpi *et al.* 2001).

The transition of pneumococcus from a commensal to a pathogen is so far poorly understood. One important aspect of the Pnc interaction with host cells seems to be that it is benign and cleared by a capsule-specific immune response unless there is a coincident activation of host cells. Activation of nasopharyngeal cells by inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), qualitatively and quantitatively increases the presentation of receptors for pneumococci (Cundell *et al.* 1995c). Thus, the ability of pneumococcus to invade human cells may be more a result of changes in the human cell surface than in the bacteria itself (Tuomanen 2000). To gain access to the middle ear, pneumococci must ascend through the Eustachian tube from the nasopharynx. Very little is known about this process. Once in the middle ear, a major result of bacterial replication is influx of neutrophils and a profound inflammation (Tuomanen 2000).

A number of risk factors have been associated with the development of AOM. Several of them are related to the home environment. For example, the presence of siblings increases the risk of AOM through exposure to potential middle ear pathogens and respiratory viruses (Teele *et al.* 1989; Paradise *et al.* 1997). Smoking parents and exposure to cigarette smoke in the household causes irritation of the respiratory tract and is another risk factor for AOM (Teele *et al.* 1989; Owen *et al.* 1993; Ey *et al.* 1995; Uhari *et al.* 1996b). The form of infant nutrition has also been shown to affect the risk of AOM, which is decreased by breast milk and increased by infant formula (Sipilä *et al.* 1988; Teele *et al.* 1989; Owen *et al.* 1993; Uhari *et al.* 1996b; Duffy *et al.* 1997). Outside the home, the most important risk factor for AOM has been shown to be the

enrollment in day care, which increases the exposure of a child to respiratory and middle ear pathogens (Henderson *et al.* 1986; Sipilä *et al.* 1988; Uhari *et al.* 1996b).

Most cases of AOM are preceded by a viral upper respiratory tract infection. The viral infection predisposes the child to the development of AOM by causing Eustachian tube dysfunction and enhancing nasopharyngeal carriage of the middle ear pathogens (Sanyal *et al.* 1980; Faden *et al.* 1991; Bluestone 1996; Syrjänen *et al.* 2002). Rhinovirus, respiratory syncytial virus (RSV), adenovirus, influenza and parainfluenza viruses are detected in middle ear effusions of 17 to 24% of children with AOM, either alone or in combination with bacteria (Chonmaitree *et al.* 1992; Okamoto *et al.* 1993; Sung *et al.* 1993; Chonmaitree *et al.* 2000). A clear association between RSV-epidemics and AOM has been demonstrated (Ruuskanen *et al.* 1989). Furthermore, AOM associated with a viral respiratory tract infection is prolonged in comparison to AOM in the absence of a viral infection (Heikkinen 2000). AOM is predominantly a winter disease and the pattern seen with AOM is consistent with the appearance of winter respiratory viruses (Faden *et al.* 1998).

The most evident way to prevent viral or bacterial AOM is immunization. The only respiratory viral vaccine currently available is the influenza virus vaccine. Its use has been associated with a reduction of AOM episodes. Children in day-care centers who received influenza vaccine had a 33-36% reduction in the number of diagnosed AOM episodes as compared to placebo recipients (Heikkinen *et al.* 1991; Clements *et al.* 1995). The ability of the bacterial vaccines to prevent AOM has not been as promising and more effective bacterial vaccines against AOM are needed. For example, immunization of infants with a 7-valent Pnc conjugate vaccine has been shown to be only modestly beneficial against AOM, the efficacy against culture-confirmed Pnc AOM being approximately 30% and the overall efficacy rate against all AOM episodes 6-7% (Black *et al.* 2000; Eskola *et al.* 2001; Kilpi *et al.* 2003). An increase in AOM caused by non-vaccine serotypes and other bacterial pathogens of the upper respiratory tract has been detected.

Nonimmune strategies for prevention of AOM include the use of oligosaccharides and xylitol. As mentioned above, the intranasal administration of an antiadhesive oligosaccharide in Finnish children had no beneficial effect either on the

nasopharyngeal carriage or the occurrence of AOM (Ukkonen *et al.* 2000). Kontiokari *et al.* (1995, 1998) have demonstrated that xylitol inhibits the growth and adhesion of *S. pneumoniae in vitro*. This has stimulated an interest to develop xylitol-based protocols for the prevention of AOM. Two studies have demonstrated the efficacy of xylitol in preventing AOM (Uhari *et al.* 1996a, 1998). Xylitol administration during an acute respiratory infection was however found to be ineffective in preventing AOM (Tapiainen *et al.* 2002a). These results are encouraging but additional studies are needed to understand the pharmacokinetics of xylitol and to define the optimal administration doses and schedule (Tapiainen *et al.* 2002b).

2.2.3. Pneumococcal pneumonia and invasive diseases

2.2.3.1. Pneumococcal pneumonia

S. pneumoniae is the most frequent cause of community-acquired pneumonia among patients requiring hospitalization in the various geographical areas. The attack and case fatality rates of Pnc pneumonia are highest in elderly patients (Sullivan *et al.* 1972, Feldman 2001). Pnc pneumonia causes about three million deaths of children less than 5 years of age each year, nearly all of which are in developing countries (Greenwood 1999). The distinctive symptoms of Pnc pneumonia are cough and sputum production, which reflect the proliferation of bacteria and the inflammatory response in the alveoli, and fever, which results from the release of cytokines and other pyrogenic substances both locally and systemically (Musher 1992).

In most cases Pnc pneumonia results from the aspiration of pneumococci resident in the upper respiratory tract (Boulnois 1992) (Figure 1). The lower respiratory tract is protected by several specific and nonspecific defense mechanisms (Busse 1991). Failure of these defenses may facilitate access of pneumococci to the bronchi and the lungs (Boulnois 1992; Musher 1992). However, progression to pneumonia requires more than the simple association of pneumococci with alveolar cells (Cundell *et al.* 1995a).

Persons with underlying conditions or altered clearance mechanisms (such as cigarette smokers and persons having chronic bronchitis, asthma, chronic obstructive pulmonary disease, or lung cancer) are at a high risk of getting pneumonia (Musher 1992). Viral upper respiratory tract infections may play a particularly important role in facilitating Pnc invasion by compromising the nonspecific defense mechanisms of the lung and

causing epithelial cell damages. Sequelae of the common viral upper respiratory tract infection (such as excessive mucus production, disruption of the normal epithelium and dampening of ciliary function) have been shown to predispose to Pnc pneumonia (Douglas *et al.* 1979; Gray *et al.* 1989). The association between influenza and Pnc pneumonia is well documented. Prior influenza virus infection enhances the adherence of pneumococci to tracheal epithelial cells (Plotkowski *et al.* 1986). This enhancement is thought to be mediated by viral neuraminidase, which cleaves sialic acid from glycolipids in human lung tissue. This way, viral neuraminidase may expose other structures that can function as receptors for Pnc adherence.

Bacteria that colonize the lungs may gain access to the bloodstream. Bloodstream infections are a common complication of bacterial pneumonia; bacteria invade the alveolar spaces and cause enough tissue damage to disrupt the barriers between alveoli and blood vessels. Blood cultures are positive in 15-30% of cases of Pnc pneumonia, depending upon the population under study and, to a lesser extent, the Pnc serotype (Musher 1992).

2.2.3.2. Pneumococcal septicemia

Septicemia is a systemic disease in which micro-organisms multiply in the blood or are continuously seeded into the bloodstream. Pnc septicemia occurs frequently as a complication of Pnc pneumonia. Septicemia may occur also as a primary bacteremia (bacteria present in bloodstream) in the absence of a clinically evident focus of infection. This phenomenon has been recorded in 15% of bacteremias, most commonly in children (Balakrishnan *et al.* 2000). Epithelial damage caused by previous viral upper respiratory tract infections can increase the opportunity of pneumococci to reach the bloodstream.

A highest incidence of Pnc bacteremia has been repeatedly documented in infants up to two years of age (Jacobs *et al.* 1979; Kaplan *et al.* 1998). The incidence is low among teenagers and young adults, increases in patients of middle age, and reaches a high level among population over 65 years (Breiman *et al.* 1990). Invasive Pnc diseases are more common among men than women in all age groups (Burman *et al.* 1985; Eskola *et al.* 1992; Kuikka *et al.* 1992; Sankilampi *et al.* 1997). In a Finnish study, the incidence of invasive Pnc disease was 45.3 per 100 000 among children less than 2 years of age, and

24.2 per 100 000 among children less than 5 years of age (Eskola *et al.* 1992). The overall incidence of invasive Pnc diseases for all Finnish adults is 9.1 per 100 000, but 27.1 or more per 100 000 in those aged 65 or over (Sankilampi *et al.* 1997). Various studies performed between 1974 and 1987 in the U.S. show an overall incidence of Pnc invasive diseases between 16 and 82 per 100 000 children in the first 5 years of life (Mufson *et al.* 1982; Filice *et al.* 1986; Istre *et al.* 1987; Breiman *et al.* 1990).
In developing countries the incidence of invasive Pnc diseases is several times higher than in the industrialized world. Among Gambian infants a minimum incidence of 185 per 100 000 per year has been reported (Usen *et al.* 1998). An extremely high incidence, 297 per 100 000 persons, has been observed among the Australian aboriginals (Trotman *et al.* 1995). High incidence figures have also been reported from the native populations in Alaska (Davidson *et al.* 1994). Several factors may contribute to these differences in incidence rates between regions, including country-specific epidemiologic surveillance systems, differing blood culturing practices, living conditions, genetic factors, climate and age (Hausdorff 2001).

The incidence of different Pnc serotypes in invasive Pnc disease varies by age and region. In children of industrialized countries, the most frequent Pnc groups/types to cause Pnc bacteremic diseases are 6, 14, 18, 19, and 23 (Burke *et al.* 1971; Hansman 1977; Gray *et al.* 1986; Riley *et al.* 1991). The distribution is different in developing countries, where groups/types 1, 2, 3, 5, 7, 12, and 46 are found more frequently in children (Onyemelukwe *et al.* 1982; Guirguis *et al.* 1983; Barker *et al.* 1989). In adults, the most frequent groups associated with Pnc bacteremia are 3, 1, 14, 7, 4 and 8 (Nielsen *et al.* 1992).

Pnc septicemia in children seems to be associated with low risk of death, while increasing age, an extrapulmonary site of infection, the presence of chronic disease, or infection with certain serotypes (particularly type 3) contribute to a higher risk of death (Mufson *et al.* 1974). The mortality of Pnc septicemia over decades has remained stable between 25 and 29% (Gillespie 1989). The overall case fatality rate in Finland has ranged from 21 to 34% during the last decades (Kuikka *et al.* 1992; Lääveri *et al.* 1996). The case fatality rate in children is lower than in adults ranging from 2 to 15% (Douglas *et al.* 1983; Filice *et al.* 1986; Istre *et al.* 1987; Riley *et al.* 1991; Dagan *et al.* 1992; Takala *et al.* 1992).

2.2.3.3. Pneumococcal meningitis

The most severe form of Pnc disease is meningitis. Pnc meningitis is of exceptional severity and it is associated with a higher mortality than meningitis caused by other common meningeal pathogens (Baraff *et al.* 1993; Goetghebuer *et al.* 2000). The Pnc serogroups most often isolated from CSF include groups 6, 10 and 23 at all ages (Hausdorff *et al.* 2000a).

Meninges are a set of membranes that cover the brain and spinal column, protecting them from harmful substances in blood. Pnc meningitis usually occurs in relatively few individuals as a result of seeding of the meninges during high-grade bacteremia or a head trauma (Musher 1992). The actual mechanisms and route used by pneumococci to migrate to the meninges are not clear. It has been suggested that local inflammation caused by pneumococci breaches the blood-brain barrier and admits entry of bacteria and phagocytes to this fragile area. The inflammatory reaction, rather than the pathogen itself, is largely responsible for the damage that results from bacterial meningitis (Pfister *et al.* 1997). Neurological sequelae such as hearing loss, neurological deficits and neuropsychological impairment are common among survivors (Arditi *et al.* 1998; Woolley *et al.* 1999; van de Beek *et al.* 2002).

The rates of morbidity and mortality of Pnc meningitis in children of industrialized countries are approximately 30% and 10%, respectively (Kornelisse *et al.* 1995; Arditi *et al.* 1998). In developing countries the figures are dramatically higher: about 50% of children with Pnc meningitis die while in hospital (Muhe *et al.* 1999; Goetghebuer *et al.* 2000).

2.2.4. Treatment of pneumococcal diseases

Penicillin, a cheap and safe antimicrobial drug, has been the standard choice for treatment of Pnc diseases for decades. However, due to the emergence of antibiotic-resistant strains, Pnc diseases have become more difficult to treat. The first Pnc strain resistant to penicillin was isolated in 1965 and ten years later the first multi-resistant pneumococci were reported (reviewed in: Appelbaum 1992). Penicillin acts by binding to and thereby blocking the action of cell membrane transcarboxypeptidase-enzymes (also called penicillin-binding proteins, PBPs), which are responsible for the bacterial cell wall synthesis (Musher 1992). Resistance to penicillin results from changes in PBPs

that decrease their affinity for penicillin without altering their functions in cell wall synthesis. At the moment, the proportion of Pnc strains resistant to penicillin is steadily increasing in all parts of the world. The foci of high levels of resistance have been detected particularly in parts of Southern and Eastern Europe. In some areas up to 35% of Pnc isolates are resistant to penicillin (Dagan *et al.* 1994; Hofmann *et al.* 1995; Arnold *et al.* 1996; Whitney *et al.* 2000).

Epidemiologic studies have shown that frequent antibiotic use and the use of prophylactic antibiotics are the risk factors for spread of drug-resistant Pnc strains. This is true particularly in institutional settings, such as child care centers and hospitals, where person to person transmission of respiratory pathogens may be facilitated (Radetsky *et al.* 1981; Reichler *et al.* 1992). Young children are often treated with antibiotics due to the high frequency of upper respiratory tract infections. Thus, this is probably the reason why antimicrobial resistance has developed primarily in Pnc serogroups prevalent in young children, namely 6, 14, 19 and 23 (Dagan *et al.* 1994).

The rapid spread of penicillin- and multiresistant strains has required switching to more costly antibiotics. A frightening characteristic of penicillin-resistant strains of *S. pneumoniae* is that their rate of resistance also to other commonly used antibiotics such as erythromycin, tetracycline and trimethoprim-sulfamethoxazole is much higher than in penicillin-susceptible strains (Musher 1992). Acquisition of high-grade resistance is thought to originate via horizontal transfer of genetic material, probably from another bacterial species (Musher 1992). Some multiresistant Pnc isolates are susceptible only to vancomycin. However, the appearance of vancomycin tolerance (i.e., the ability of bacteria to survive but not grow in the presence of an antimicrobial drug) in pneumococci has now also emerged. To date, five penicillin-resistant and vancomycin-tolerant clinical strains of pneumococcus have been reported: three of these strains have been invasive isolates and two strains have been isolated from the nasopharynx (Novak *et al.* 1998b; McCullers *et al.* 2000; Henriques Normark *et al.* 2001; Hidalgo *et al.* 2003).

3. Pneumococcal pathogenesis and virulence factors

The pathogenesis of Pnc infection is a complex interplay between Pnc virulence factors and the defense mechanisms of the host. Because of the importance of pneumococci as a human pathogen, Pnc virulence factors have been intensively studied over many decades. Despite all this attention, many facts about the actual mechanisms of Pnc virulence are still unknown. Several approaches have been and are used for the investigation. The mouse model system is widely used to assess virulence factors and to test effectiveness of different types of Pnc vaccines. Although *S. pneumoniae* is normally a human pathogen, it can kill mice when injected peritoneally or administered intranasally in large enough doses. Primary human polymorphonuclear leucocytes and macrophages or macrophage-like cell lines (e.g., HL-60) are used to test the ability of *S. pneumoniae* to resist phagocytosis. Human umbilical vein endothelial (HUVE) cells and rat alveolar cells are used to study effects of the bacteria on endothelium and lung, respectively. In addition, the nasopharyngeal human epithelial cell line (Detroit 562) has been used to study Pnc adhesion (Romero-Steiner *et al.* 2003, Weiser *et al.* 2003).

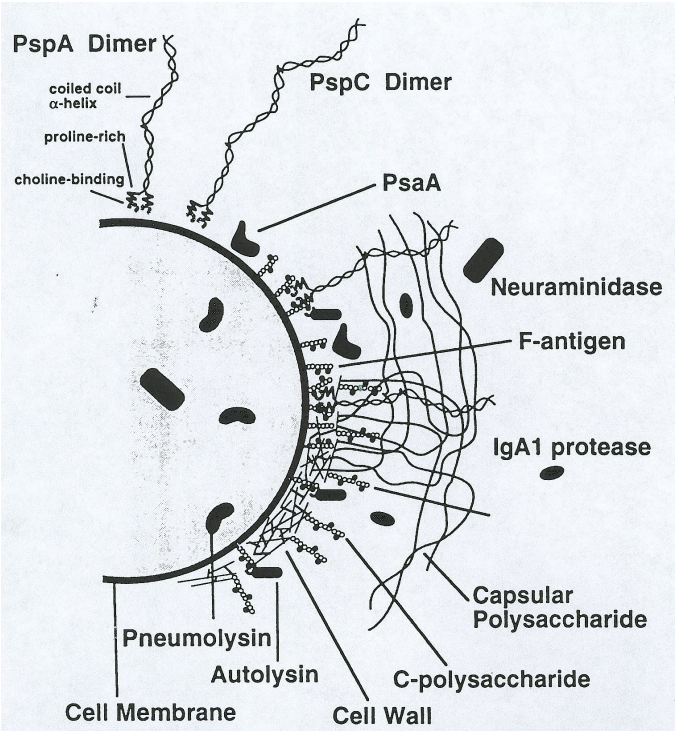

Figure 2. Cell surface of *Streptococcus pneumoniae*. The molecules that have roles in Pnc virulence and or elicitation of protection against *S. pneumoniae* are illustrated (Briles *et al.*. 2000e, with permission).

The symptoms of all Pnc diseases are primarily due to the ability of the bacteria to evoke intense inflammatory response. Four key effects of Pnc pathogenesis produced by the Pnc virulence determinants and the corresponding immune responses elicited to them are adhesion, invasion, inflammation and shock. Several Pnc surface components for which roles in Pnc virulence and/or elicitation of protection against pneumococci have been established are shown in Figure 2.

3.1 Adhesion

Adherence of pneumococci to human cells is achieved by ligand-receptor pairing between bacterial surface proteins and eukaryotic surface carbohydrates (Cundell *et al.* 1995a). Cells in the respiratory tract display a specific array of carbohydrates, which dictates the tissue tropism of the respiratory pathogens. Targets for Pnc attachment on human cells are glycoconjugates that bear a specific carbohydrate within their structure, such as sialic acid, *N*-acetyl glucosamine β1-3-galactose (GlcNAc*β*1-3 Gal), *N*-acetyl galactosamine β1-4-galactose (GalNAc*β*1-4 Gal), *N*-acetyl galactosamine β1-3-galactose (GalNAc*β*1-3 Gal) or lacto-*N*-neotetraose (Andersson *et al.* 1981, 1983, 1988; Krivan *et al.* 1988; Idänpään-Heikkilä *et al.* 1997). Cytokine activation (e.g. TNF and IL-1) of the eukaryotic cells has been shown to change the expression of receptors dramatically, allowing increased Pnc adherence (Cundell *et al.* 1995b). Sialic acid is a prominent ligand in the upper respiratory tract, being operative for the conjunctiva, Eustachian tube and nasopharynx (Barthelson *et al.* 1998). The disaccharide GalNac*β*1-4 Gal is prominent in the lower respiratory tract and is recognized by a wide variety of pulmonary bacterial pathogens (Krivan *et al.* 1988). All strains of pneumococci do not exhibit an equal ability to recognize these sugars (Andersson *et al.* 1981; Talbot *et al.* 1996). The serotype has not been shown to determine the adhesive capacity of the Pnc strains (Andersson *et al.* 1981).

The efficiency of adherence and virulence of a given Pnc strain is greatly affected by a spontaneous, high frequency phenomenon known as phase variation (Weiser *et al.* 1994). The rate of spontaneous variation is strain-specific. Pnc phase variation can be recognized by changes in colony morphology viewed by oblique light: opaque, semi-transparent and transparent phenotypes can be separated (Weiser *et al.* 1994). The transparent colony variants have a selective advantage over opaque variants for adherence to human epithelial cells and establishing nasopharyngeal colonization in the

infant rat model (Weiser *et al.* 1994; Tuomanen 1997). The opaque variants, in turn, survive better in the bloodstream than the transparent ones (Kim *et al.* 1998). The mechanism for the different adhesive and invasive capabilities between opaque and transparent forms involves modification of several surface proteins and cell wall structure. The transparent bacteria display less capsule, more cell wall phosphorylcholine and bear different proteins on their surfaces than the opaque variants, e.g., significantly greater amounts of Pnc adhesin Pnc surface protein C (PspC)/choline binding protein A (CbpA) (Weiser *et al.* 1994, 1996; Rosenow *et al.* 1997; Kim & Weiser 1998). The opaque strains, in turn, bear more capsule, less choline and more of the protective surface antigen Pnc surface protein A (PspA) (Kim & Weiser 1998). It is becoming apparent that the downregulation of capsule production enhances host cell invasion (Talbot *et al.* 1996; Weiser *et al.* 1996, 1999). It has been calculated that the PS capsule inhibits adherence and invasion of eukaryotic cells by up to 200-fold *in vitro* (Ring *et al.* 1998, 2000). After invasion, it is important to regain the capsular phase for resistance against phagocytosis.

In general, the mechanism of bacterial phase variation usually involves a control of gene expression at the level of transcription. Alternatively, an increasing number of bacterial genes are found, in which expression is switched on and off by some form of DNA arrangement, such as inversion, recombination or slipped strand mispairing (Saluja *et al.* 1995; Henderson *et al.* 1999). In the case of pneumococcus the precise mechanism of phase variation has not yet been understood at the genetic level. Recently, Waite *et al.* (2001, 2003) have shown that high-frequency capsule phase variation in Pnc serotypes 3, 8 and 37 may be controlled by spontaneous sequence duplications within the capsule genes: spontaneous sequence duplication switches the capsule production off and excision of the duplication re-enables capsule production.

3.1.1. Pneumococcal surface adhesin A (PsaA)

Pnc surface adhesin A (PsaA) is a 37-kDa surface lipoprotein essential for Pnc virulence. Initially, PsaA was thought to be a Pnc adhesin, due to two observations. First, the sequence analyses of *psaA* gene revealed a significant degree of homology with the streptococcal putative lipoprotein adhesins ScaA from *S. gordonii,* SsaB from *S. sanguis* and FimA from *S. parasanguis* (Sampson *et al.* 1994). Second, the PsaA$^-$ mutants displayed markedly reduced *in vitro* adherence to a human type II pneumocyte

cell line (Berry *et al.* 1996b). However, genomic sequence comparison (Berry *et al.* 1996b) and studies on the crystal structure of PsaA (Lawrence *et al.* 1998) revealed that it is a component of an ATP-binding cassette-type (ABC-type) permease membrane transport system. ABC-type transport systems are found in both prokaryotic and eukaryotic organisms (Higgins 1992, Tam & Saier 1993). They consist of up to three protein components: an extra-cytoplasmic protein responsible for solute binding, an integral membrane protein responsible for transport of the solute through the cell membrane, and a cytoplasmic protein that couples ATP-hydrolysis to the transport process. It is believed that PsaA is responsible for the uptake of Mn^{2+} (and possibly Zn^{2+}) into the bacterium (Dintilhac *et al.* 1997). Many genes for such metal transporters exist in the Pnc genome. Some of these proteins, such as Adc and PsaA, are particularly important for Pnc virulence (Dintilhac *et al.* 1997).

The loss of host adhesion observed in $PsaA^-$ mutants is probably indirect, with a secondary protein being rendered absent or nonfunctional via Mn^{2+} (or Zn^{2+}) deprivation. PsaA appears to have a regulatory role in adhesion by affecting the expression of choline-binding proteins on the surface of pneumococcus: mutants lacking the *psa* operon demonstrate a complete absence of PspC/CbpA (Novak *et al.* 1998a). The mutants negative for *psaA* gene have also been shown to be highly sensitive to oxidative stress (i.e., to superoxide and hydrogen peroxide), which could in part explain the reduction in virulence (Tseng *et al.* 2002). It has been suggested that PsaA might play an important role in the regulation of expression of oxidative-stress response enzymes and intracellular redox homeostasis (Tseng *et al.* 2002).

PsaA is considered as a Pnc protein vaccine candidate. In animal models, PsaA has been shown to be immunogenic and protective against invasive Pnc disease (Talkington *et al.* 1996; De *et al.* 1999; Ogunniyi *et al.* 2000). It has been found to be highly effective against nasopharyngeal carriage in mice when administered intranasally, combinations of PsaA with PspA being more effective than PsaA alone (Briles *et al.* 2000a).

3.1.2. Pneumococcal surface protein C (PspC)

Pnc surface protein C (PspC) plays an important role in Pnc pathogenesis by functioning as an adhesin (Rosenow *et al.* 1997). It also binds soluble host factors such as the secretory component (SC), the third component of complement (C3) and

complement factor H (Hammerschmidt *et al.* 1997; Cheng *et al.* 2000; Janulczyk *et al.* 2000; Dave *et al.* 2001). The gene for this protein is present in approximately 75% of *S. pneumoniae* strains.

Typical surface proteins of gram-positive bacteria are covalently anchored to the cell wall via a highly conserved hexapeptide sequence motif LPXTGE (= leucine-proline-X-threonine-glycine-glutamic acid) at their carboxy-terminal end (Navarre *et al.* 1999). Pneumococci display some of these LPXTGE motif-containing proteins. In addition, pneumococci harbor a dozen proteins that are attached to the Pnc surface by docking non-covalently with the phosphorylcholine of the Pnc cell wall. These Pnc choline-binding proteins (CBPs) have a common choline-binding carboxy-terminal (Garcia *et al.* 1998) and the amino-terminal that produces their functional diversity. The choline-binding domain is composed of multiple carboxy-terminal tandem amino acid repeats (usually approximately 10 repeat regions of approximately 20 amino acids). The Pnc CBPs include several important surface molecules, such as PspA, PspC, CbpA, SpsA (= *S. pneumoniae s*ecretory Ig*A* binding *p*rotein), Hic (= factor *H*-binding *i*nhibitor of *c*omplement), and LytA (= major Pnc autolysin). The expression of CBPs is subject to phase variation that results in the display of different combinations of proteins. This adapts the bacteria to survive on the mucosa versus the blood stream.

Sequence analysis has shown that PspC, CbpA, Hic, PbcA (= *C*3-*b*inding *p*rotein *A*) and SpsA are variants of the same CBP (Brooks-Walter *et al.* 1999). Under different names several properties have been attributed to this surface protein. Its abilities to bind C3 and secretory IgA were described under the names PbcA (= *C*3-*b*inding *p*rotein *A*) and SpsA (= *S. pneumoniae s*ecretory Ig*A* binding *p*rotein), respectively (Hammerschmidt *et al.* 1997; Hostetter *et al.* 1997). Under the name CbpA (= *c*holine-*b*inding *p*rotein *A*), the protein has been shown to interact with human epithelial and endothelial cells (Rosenow *et al.* 1997). CbpA was the first Pnc surface adhesin to be described (Weiser *et al.* 1996). A CbpA-deficient mutant showed a >50% reduction in adherence to cytokine-activated human cells and failed to bind to immobilized sialic acid or lacto-N-neotetraose, the known targets for Pnc adherence on eukaryotic cells (Rosenow *et al.* 1997). Under the name Hic (= factor *H*-binding *i*nhibitor of *c*omplement) the protein was found to absorb factor H, an inhibitor of complement, from human plasma (Janulczyk *et al.* 2000). The complement inhibitory function of factor H was not

impaired in the presence of Hic. This suggests that by accumulating an active complement inhibitor at the Pnc surface, Hic may act to block the deposition of C3b and concomitant opsonophagocytosis (Janulczyk *et al.* 2000; Jarva *et al.* 2002). The term PspC is preferred over the other designations for two reasons. First, it was used for the first sequence deposited in GenBank (accession number: U72655). Second, it is a generic name referring only to the surface location of the molecule (Iannelli *et al.* 2002).

Analysis of the deduced amino acid sequence of different PspC variants has revealed 11 major groups of PspC proteins (PspC1-11) (Iannelli *et al.* 2002). Single proteins within a group display only minor variations in the amino acid sequence. A common organization of the PspC molecules was shown to be: (i) a 37-amino-acid leader peptide, (ii) an amino-terminal segment, which is essentially α-helical, and (iii) a carboxy-terminal anchor, responsible for the cell surface attachment (Iannelli *et al.* 2002). Unexpectedly, the mechanism for surface attachment for different PspC molecules is not uniform, since some of the PspC allelic variants (PspC7-11) do not show the choline-binding domain, but rather the LPXTGE motif instead (Iannelli *et al.* 2002). The presence of different anchor regions in different allelic variants is a unique trait of PspC.

When used as an immunogen in the mouse model, PspC has proved a good candidate for a Pnc protein vaccine (Briles *et al.* 2000b). Immunization of mice with a proline-rich domain of PspC molecule results in protection against challenge with Pnc strains expressing both PspA and PspC, or only PspA suggesting cross-protection (Brooks-Walter *et al.* 1999).

3.1.3. IgA1-protease

Pneumococci produce IgA1-protease, which functions to impair host defense at the mucosal surfaces and supports colonization of the nasopharynx. (Kilian *et al.* 1986; Weiser *et al.* 2003). These proteases are thought to be important for the ability of the bacteria to colonize human mucosal surfaces in the presence of a predominant secretory immunoglobulin (Ig) isotype, specific secretory IgA (sIgA). Nasopharyngeal carriage of pneumococcus is common despite of mucosal IgA against the capsular PS, which is the immunodominant surface antigen of *S. pneumoniae* (Opstad *et al.* 1995; Virolainen *et*

al. 1995). This indicates that IgA1-protease may impair the function of anti-Pnc PS IgA antibodies. In a recent report, it has been shown that IgA1 antibody modified by IgA1-protease, rather than inhibiting Pnc adherence, markedly enhances bacterial attachment to host epithelial cells in a cell-culture colonization model (Weiser *et al.* 2003).

3.2 Invasion

From the nasopharynx, pneumococci may spread locally, either upward into the Eustachian tube and middle ear cavity, or downward into the alveoli. Invasion of the lower respiratory tract is an important event in Pnc infection. Pnc pneumonia itself is a life-threatening illness, but it also serves as a focus for invasion to the bloodstream (Gillespie *et al.* 2000). Pneumococci are also able to establish systemic invasion even in the absence of a clinically evident focus of infection (Balakrishnan *et al.* 2000). The MLST method has shown that some sequence types define strains with an increased capacity to cause invasive disease (Enright & Spratt 1998). These apparently successful clones may have gathered a collection of certain genes or may have enhanced expression of certain genes facilitating the transition from Pnc carriage to invasion

3.2.1. Capsule

For a long time, Pnc PS capsule has been considered unquestionably crucial for Pnc virulence based on its capacity to confer resistance to complement-mediated opsonophagocytosis (Wood *et al.* 1949; Austrian 1981; Bruyn *et al.* 1992; Watson *et al.* 1995). The encapsulated Pnc strains have been found to be at least 10^5 times more virulent in mice than the strains lacking the capsule (Avery *et al.* 1931; Watson *et al.* 1990). Antibodies against capsular components are highly protective (MacLeod *et al.* 1945; Austrian *et al.* 1976), and in the absence of antibodies to capsular PS phagocytosis and killing are low. At the moment, 90 different capsular PSs have been described on the surface of the pneumococcus.

The capsule consists of high-molecular weight polymers made up of units of repeating oligosaccharides, which can contain 2 to 8 monosaccharides (AlonsoDeVelasco *et al.* 1995). The capsule itself lacks inflammatory potential and it does not cause toxicity to animals and humans. The most important mechanism by which the capsule promotes Pnc virulence is the protection of the bacteria against ingestion by resident pulmonary macrophages or recruited polymorphonuclear neutrophils (PMNs) (Tuomanen *et al.*

1985; Tuomanen *et al.* 1987). The capsule forms a physical barrier that separates bound, fixed complement components from complement receptors on host phagocytes (Brown *et al.* 1982; Winkelstein 1984). It may also function in the electrostatic repulsion of phagocytes from bacteria (Kasper 1986). Furthermore, the capsule may cover protective epitopes of Pnc surface proteins so that they are not exposed to antibodies.

The association between capsular type and disease is well documented. Pnc virulence and invasiveness depend on both the composition and quantity of the capsule produced, the chemical composition however, being a more important factor than the thickness (Knecht *et al.* 1970; Lee 1987; Lee *et al.* 1991). For example, Pnc strains of type 3 and 37 both produce large amounts of capsule, but they are different in virulence. Type 3, which is composed of a polymer of glucose and glucuronic acid, is among the most invasive and virulent types, whereas type 37, with a homopolymer of glucose, is rarely associated with Pnc pathogenesis (Knecht *et al.* 1970).

There is considerable cross-reactivity among certain of the 90 recognized serotypes, and a single antigen may provide protection against several types within a given serogroup (Robbins *et al.* 1983; Butler *et al.* 1993). This is the case for example with serotypes 6A and 6B (Robbins *et al.* 1983), while with serotypes 19A and 19F the cross-immunogenicity is more limited (Penn *et al.* 1982). Some immunological cross-reactivity has been observed between Pnc PSs and PSs from other bacteria, such as *Klebsiella* and non-groupable streptococci (Lee *et al.* 1991). PS of *Klebsiella* K2 induces cross-reactive antibodies to Pnc 19F PS (Lee *et al.* 1984). Furthermore, the PS of streptococcal strain 14636/74 has an identical composition to that of Pnc 19F PS (Lee *et al.* 1984).

The genes responsible for the synthesis of capsular substances are arranged in cassettes comprising all the genetic material necessary for capsular synthesis (Dillard *et al.* 1995; Garcia *et al.* 1997). Pneumococcus is naturally transformable which means that genetic material may be exchanged between different Pnc strains. Thus, the capsular type of pneumococcus can be exchanged by transformation of capsular gene cassettes *in vitro* and *in vivo* (Dillard *et al.* 1995; Garcia *et al.* 1997). Capsule transformation has been known since as early as 1928 (Austrian 1981), and it has been previously found on several occasions with antibiotic resistant clones (Coffey *et al.* 1991; Barnes *et al.*

1995). A few highly virulent Pnc clones appear to be circulating around the world with the ability to change capsular type *in vivo* (Nesin *et al.* 1998). Given the readiness of pneumococcus for horizontal transfer of resistance, capsule and virulence genes (Dowson *et al.* 1997; Caimano *et al.* 1998), the threat of rapid emergence and dissemination of strains circumventing antimicrobial drugs and capsule-based vaccines is very real (Campbell *et al.* 1998).

3.2.2. Pneumococcal surface protein A (PspA)

Pnc surface protein A (PspA) is a surface protein with variable molecular size ranging from 67 to 99-kDa in different Pnc strains. It is present on the surface of all clinically important pneumococci and is required for full virulence (McDaniel *et al.* 1987; Crain *et al.* 1990). PspA is immunogenic and elicits protective antibody response in mice (McDaniel *et al.* 1994).

Based on sequence analyses, PspA molecule has four distinct domains (Figure 3): (i) an amino-terminal, highly charged α-helical coiled-coil structure (amino acids 1 to 288 in strain Rx1), (ii) a proline-rich domain (amino acids 289 to 370), (iii) a stretch of 10 highly conserved repeats of 20 amino acids comprising the choline-binding component (amino acids 371-571), and (iv) a slightly hydrophobic tail of 17 amino acids at the carboxy-terminus (amino acids 572-588) (Yother *et al.* 1992a). The choline-binding component of PspA is homologous with other Pnc CBPs, and is responsible for the attachment of PspA to the Pnc surface (Yother *et al.* 1992b). This orientation results in the amino-terminal α-helical domain of the molecule being exposed on the surface and thus available to interact with the human host (Gray 1995; Briles *et al.* 1998). All protective monoclonal antibodies bind to the amino-terminal half of the molecule and this part also exhibits more antigenic variability due to the accumulation of mutations under selective pressure (McDaniel *et al.* 1994).

PspA is a serologically highly variable molecule and the structure of the *pspA* gene has been shown to be mosaic (Hollingshead *et al.* 2000). The term “mosaic” refers to the pattern of interspersed blocks of nucleotide sequence, which have different evolutionary histories, but are found combined in the resulting gene allele subsequent to recombination events (Milkman *et al.* 1988). The high degree of variability exhibited by PspA may indicate the importance of this surface protein as a natural target for host

defense. Based on the relatedness of the nucleotide and amino acid sequences, the different PspA molecules are grouped into three families, which are further subdivided into clades (Hollingshead *et al.* 2000). A clade is a group of PspA molecules that vary less than 20% of their amino acids. Over 98% of the PspA molecules typed to date are members of families 1 and 2 (Table 1). The clade-defining region is constituted by the amino acids 192-260 of the α-helical domain.

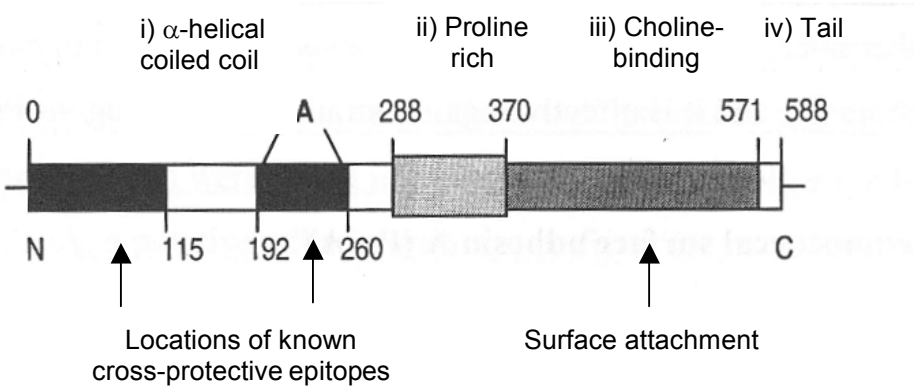

Figure 3. Amino-acid-sequence domains of PspA. **A**, clade defining region (clade = group of PspA molecules that vary less than 20% of their amino acids). Figure modified from Briles *et al.* 2000d and Hollingshead *et al.* 2000.

family 1 (~50%)	clade 1	98% of the PspAs typed
	clade 2	
family 2 (~50%)	clade 3	
	clade 4	
	clade 5	
family 3	clade 6	

Table 1. Families and clades of PspA molecules.

The mechanism of action of PspA is not fully understood. Hammerschmidt *et al.* (1999) showed that PspA functions as a specific receptor for lactoferrin, the amino-terminal part being responsible for lactoferrin binding. Lactoferrin is an iron-sequestering glycoprotein, which predominates in mucosal secretions. It has been suggested that by binding lactoferrin pneumococci are able to interfere with the host's immune functions (Håkansson *et al.* 2001). PspA has also been reported to inhibit complement activation by *S. pneumoniae*. Tu *et al.* (1999) showed that PspA blocks the C3 convertase of the alternative complement pathway and/or accelerates its dissociation. This leads to an inhibition of the downstream events of the complement pathway, which would result in opsonization of pneumococci and chemotaxis. In addition, mutant strains lacking PspA have been shown to fix more complement than isogenic parent strains expressing PspA (Briles *et al.* 1997). The interference of PspA with complement activation is suggested to facilitate Pnc survival and host invasion.

PspA is considered as a Pnc protein vaccine candidate. PspA has been shown to elicit protective immunity against Pnc infection in mice (McDaniel *et al.* 1991; Tart *et al.* 1996; Briles *et al.* 1996b, 1998). It can elicit protection against Pnc carriage and subsequent invasive disease in mice following intranasal immunization (Wu *et al.* 1997). In a recent study utilizing an experimental human colonization model, serum antibodies against PspA have been shown to correlate with protection against Pnc carriage (McCool *et al.* 2002). Animal models have shown that PspA has the ability to elicit cross-protection against heterologous strains (Crain *et al.* 1990; McDaniel *et al.* 1991; Briles *et al.* 1996b). In addition, human antisera from a phase I vaccine trial were competent for protecting mice against challenge wth Pnc strains of various PspA types (Briles *et al.* 2000c). However, a study using DNA vaccination of mice showed that the cross-reactivity of the induced anti-PspA antibodies was not reflected in cross-protection (Miyaji *et al.* 2002). Because of the variability of the PspA protein, a potential PspA-based vaccine may need to contain PspA's of more than one Pnc strain to be able to protect against all pneumococci.

3.2.3. Pneumolysin (Ply)

Ply is a 53-kDa intracellular toxin produced by all clinical isolates of pneumococcus. It is released upon lysis of Pnc cells at the stationary phase of growth. The virulence properties of Ply are therefore directly dependent on the action of the cell wall

degrading enzyme autolysin, Lyt A. Mutants negative for Ply are less virulent than their parental strains (Berry *et al.* 1989b). Immunization with Ply prolongs the survival of mice after challenge with pneumococci of different serotypes (Paton *et al.* 1983b; Lock *et al.* 1992; Alexander *et al.* 1994).

Ply belongs to a family of toxins known as thiol-activated toxins, which lose their activity on oxidation but regain full activity following addition of reducing agents (Smyth *et al.* 1978). Ply shares amino acid homology with other thiol-activated cytotoxins, such as those produced by *Streptococcus pyogenes* (streptolysin O), *Clostridium perfringens* (perfringolysin) and *Listeria monocytogenes* (listeriolysin). The presence of cholesterol in target membrane is a critical determinant of the susceptibility of cells to these toxins (Smyth & Duncan 1978). Thiol-activated toxins bind to the target membrane as a monomeric toxin. Upon binding to cholesterol, certain regions of the toxin monomer change their structural conformation, which exposes a hydrophobic part of the monomer that can undergo an insertion process into the host cell membrane. Toxin monomers then move laterally on the host membrane and oligomerize to form a high molecular weight transmembrane pore. This results in leakage of intracellular solutes and an influx of water, resulting in lysis of the host cell. By this mechanism, Ply is able to damage a wide range of eukaryotic cells.

Ply is capable of inhibiting several functions of host defense that are known to be important in protection against Pnc disease. Ply inhibits neutrophil bactericidal activity by reducing chemotaxis, phagocytosis and the respiratory burst (Paton *et al.* 1983a). It also inhibits the lymphocyte proliferation response to mitogens, the synthesis of all Ig classes (Ferrante *et al.* 1984) and the beating of cilia on the human respiratory tract epithelium (Steinfort *et al.* 1989; Feldman *et al.* 1990). Since the beating of cilia is an important aspect in the non-specific host defenses, this may promote the appearance of pneumococci in the lower respiratory tract. Ply is able to induce separation of the tight junctions of the alveolar epithelial cells (Rayner *et al.* 1995). This, with its direct cytotoxic effect on the cells of the alveolar capillary barrier, serves to facilitate invasion of pneumococci into the bloodstream.

Ply has the ability to activate the classical complement pathway in the absence of anti-toxin antibodies and consume the limited supply of complement factors in the alveoli.

The activation of complement may allow pneumococci to evade opsonophagocytosis, the essential mechanism for clearance of pneumococci from the lung (Winkelstein 1984). In addition, Ply released during Pnc autolysis activates the complement at a distance from the organism, an activity thought to contribute to virulence by reducing serum opsonic activity (Mitchell & Andrew 1997). The mechanism by which Ply activates the classical complement pathway remains controversial. It has been suggested that this might be related to sequence similarity between Ply and C-reactive protein (CRP). CRP is a human acute phase protein, which binds to bacterial surfaces. It is capable of activating the classical complement cascade in the absence of antibodies by direct binding of the C1q component of complement. However, molecular structural studies have shown, that despite the sequence similarity between Ply and CRP, there is no structural homology. Thus, an alternative mechanism must exist.

Ply is considered as one of the Pnc protein vaccine candidates. Immunization with Ply prolongs the survival of mice after challenge with pneumococci of different serotypes (Paton *et al.* 1983b; Lock *et al.* 1992; Alexander *et al.* 1994). Mice immunized with combinations of Pnc virulence proteins, including Ply, have significantly longer survival times than those immunized with any of the antigens alone (Ogunniyi *et al.* 2000). Conjugation of pneumolysoid toxoids or recombinant Ply with Pnc capsular PSs have shown that conjugates using Pnc protein carriers may confer broader protective immunity than conjugates using non-Pnc proteins (Paton *et al.* 1991; Lee *et al.* 1994; Kuo *et al.* 1995; Michon *et al.* 1998; Lee *et al.* 2001).

3.2.4. Hyaluronidase

Hyaluronidase facilitates Pnc invasion by degrading hyaluronic acid, a ubiquitous and important component of connective tissues. It has been suggested that hyaluronidase allows greater access of organisms to the tissue and may contribute to the translocation of pneumococci between tissues, for instance from the lung to the bloodstream. Kostyukova *et al.* (1995) demonstrated that Pnc strains with higher hyaluronidase activity could breach the blood-brain barrier and disseminate more effectively. Hyaluronidase contributes also to the generation of the inflammatory response. In *S. pneumoniae* cultures, the hyaluronidase is found in both the culture and the cell-associated fractions. This may suggest that at least part of the enzyme is released by the pathogen to the surrounding host tissues during infection to facilitate the bacterial invasion (Berry *et al.* 1994). At the same time, hyaluronidase (along with

neuraminidase) activity may serve to increase the substrate availability for Pnc growth by converting larger polymers to products that can be transported into the cell (Tettelin *et al.* 2001).

3.2.5. Neuraminidase enzymes (NanA and NanB)

Neuraminidase enzymes cleave sialic acid residues from host glycolipids and gangliosides causing damage to host tissues. This action may reveal new receptors for Pnc adhesins, facilitating both adhesion and invasion (Krivan *et al.* 1988). Pneumococci have at least two enzymes with neuraminidase activity, NanA and NanB. Both proteins are exported proteins with typical signal peptides, but NanB lacks the typical surface anchorage domain (LPXTGE) present in NanA. It is still uncertain, why *S. pneumoniae* produces two distinct neuraminidases. It is likely, that they specialize to be most efficient in different environmental conditions during Pnc infection or invasion of the host. This possibility is supported by the different molecular sizes as well as the widely different pH optima of the two proteins. NanA and NanB possess very little amino acid homology and have molecular masses of ~108-kDa and ~75-kDa, respectively (Camara *et al.* 1994; Berry *et al.* 1996a). NanA has maximum activity at ~pH 5, whereas NanB is most active at ~pH 7 (Berry *et al.* 1996a).

3.2.6. Binding to the platelet-activating factor (PAF) receptor

It has been proposed that progression to invasive Pnc disease involves the local generation of inflammatory factors (e.g., cytokine production), which change the number and type of receptors available by activated human cells for Pnc binding (Cundell *et al.* 1995b). Cell wall components released from dividing Pnc or a concurrent viral respiratory infection may function as factors inducing the inflammation. Lung and endothelial cells that are activated by inflammatory factors express the platelet-activating factor (PAF) receptor, which enhances Pnc adherence to the host cells (Cundell *et al.* 1995b) and facilitates the uptake of pneumococci (Tuomanen 1999). Pneumococci adhere srongly to and invade endothelial and epithelial cells activated by inflammatory factors, as well as PAF-receptor-transfected cells (i.e., the cells that harbor cDNA of the PAF-receptor) (Cundell *et al.* 1995b). Pneumococci cannot enter resting vascular endothelial cells *in vitro*, which is shown by the survival of only 0.1% of a Pnc inoculum upon exposure to exogenous gentamicin (Cundell *et al.* 1995b). The invasion rates of classically “invasive” bacteria such as *Salmonella* and *Shigella*

represent >2-3% of the inoculum (Isberg 1991). Thus, *S. pneumoniae* cannot be considered invasive for naïve cells. On the contrary, activation of cells results in the entry of 2-3% of the pneumococci within 30 minutes (Cundell *et al.* 1995a). This internalization of pneumococci by activated cells can be largely prevented by treatment with PAF-receptor antagonists.

Pneumococci adherent to the PAF-receptor may translocate across the alveolar and vascular epithelium into the bloodstream or across the blood-brain barrier to the meninges. Thus, the PAF-receptor has been suggested to serve as a gateway for Pnc invasion. The interaction between pneumococci and the cytokine-upregulated PAF-receptors is mediated by the phosphorylcholine of the Pnc cell wall (Cundell *et al.* 1995c). Phosphorylcholine is a determinant shared between the Pnc cell wall and the natural ligand PAF (Cabellos *et al.* 1992). Also, PspC has been shown to be an absolute requirement for Pnc traffic across the monolayers of rat and human brain microvascular endothelial cells (Ring *et al.* 1998). The opaque and transparent Pnc variants adhere to a similar degree to nonactivated epithelial and endothelial cells, but enhanced adherence to cytokine-stimulated cells or PAF-receptor-transfected COS-cells is limited to the transparent variants (Cundell *et al.* 1995c). This is consistent with the fact that transparent pneumococci produce readily invasive disease *in vivo* (Tuomanen 1997). The PAF-receptor is rapidly internalized after interaction with its natural ligand, and accordingly, pneumococci seem to invade the endothelial cell in a vacuole together with the PAF-receptor. Within the endothelial cell, the pathogen is either killed intracellularly or it transmigrates through the cell. Only transparent pneumococci seem able to transcytose through the endothelial cell monolayers in a significant proportion (Ring *et al.* 1998).

3.3. Inflammation and shock

3.3.1. Pneumolysin (Ply)

The properties of Ply, along with its function as a pore-forming hemolysin, also include a role as a general inflammatory agonist (Braun *et al.* 1999). The ability of Ply to activate phopsholipase A in pulmonary artery endothelium has been suggested to mediate both the inflammatory response and direct lung damage. Once activated, phospholipase A breaks down a wide variety of cell-membrane phospholipids (Rubins *et al.* 1994). This results in the release of free fatty acids and lysophosphatides. These

metabolites of phospholipase activity are directly cytotoxic and potent neutrophil chemotaxins. The recruitment and activation of neutrophils further contributes to the inflammatory response and lung damage. The ability of Ply to activate the classical complement pathway without the need for specific antibody further enhances the inflammation. Ply stimulates the production of inflammatory cytokines such as TNF-α and IL-1β by human monocytes (Houldsworth *et al.* 1994). Furthermore, Ply is the main inducer of nitric oxide (NO) production in macrophages. NO production during inflammation is an essential element of antimicrobial immunity, but it may also contribute to the host-induced tissue damage (Braun *et al.* 1999).

3.3.2. Autolysin (LytA)

The major enzyme responsible for Pnc cell wall turnover is autolysin, *N*-acetyl-muramoyl-L-alanine amidase (LytA). It is a cell wall-associated protein that belongs to the family of Pnc CBPs, which are held on the Pnc surface by docking them non-covalently with the choline of the cell wall. LytA is responsible for the degradation of the peptidoglycan backbone of pneumococcus, which leads to cell lysis (Tomasz 1984). Thus, activity of LytA allows the release of intracellular toxins (e.g., Ply) and highly inflammatory cell wall fragments. The enzyme is activated under conditions in which biosynthesis stops, such as nutrient starvation, the end of logarithmic phase of growth or penicillin treatment (Tuomanen *et al.* 1990; Mitchell 2000). Several studies have demonstrated the role of LytA in Pnc virulence. LytA-negative mutants are less virulent than wild-type pneumococci in a mouse model after intranasal challenge (Berry *et al.* 1989a). Human lysozyme released upon infection and inflammation has been shown to trigger LytA thereby enhancing the inflammation (Bruyn *et al.* 1992). Immunization of mice with LytA confers limited protection against intranasal challenge with wild-type pneumococci (Berry *et al.* 1989a).

3.3.3. Cell wall and cell wall polysaccharide (CPS)

The pneumoccoccal cell wall is composed of a sugar backbone consisting of alternating molecules of *N*-acetyl-muramic-acid and *N*-acetyl-glucosamine, which are connected to a three-dimensional network by pentapeptide side chains (Sande & Tauber 1999). In addition, the cell wall contains teichoic acid and lipoteichoic acids, the PSs covalently linked to peptidoglycan.

Teichoic acid is found in many gram-positive cell walls, but Pnc teichoic acid is unique in that it contains phosphorylcholine (Tomasz 1967; Mosser *et al.* 1970). This phosphorus-containing teichoic acid is designated as a cell wall polysaccharide (CPS), which is the major cell wall component of the pneumococcus. CPS is covalently linked to the peptidoglycan (Tomasz 1981) and is uniformly distributed on both the inside and outside of the cell wall. CPS functions as a recognition site for activation of the alternative complement pathway (Winkelstein *et al.* 1977, 1978) as well as for the binding of CRP (Mold *et al.* 1981) and LytA (Mosser & Tomasz 1970; Giudicelli *et al.* 1984). The removal of CPS diminishes complement activation by cell wall components (Tomasz *et al.* 1989).

Another important structure of Pnc cell wall is the lipoteichoic acid, the Forssman antigen (F-antigen). It is a teichoic acid similar to CPS containing phosphorylcholine and an additional covalently attached lipid material. Via its lipid moiety, this antigen is inserted into the plasma membrane. The F-antigen inhibits the function of LytA. During the stationary phase of growth Pnc cells release the F-antigen enabling the unrestrained autolytic activity of LytA and the destruction of the cell wall (Horne *et al.* 1985).

The activity of LytA releases the components of the Pnc cell wall as fragments. The cell wall fragments induce release of proinflammatory cytokines from mononuclear macrophages (e.g., TNF-α, IL-1 and IL-6) (Tuomanen *et al.* 1986). The induction of proinflammatory cytokines triggers a complex network of additional inflammatory mediators. In animal models, injection of cell wall preparations has been shown to generate a strong inflammatory response and to recreate many features of Pnc pneumonia, meningitis and AOM (Tuomanen *et al.* 1986, 1987; Giebink *et al.* 1988). The phosphorylcholine-containing cell wall pieces directly activate the alternative complement pathway (Winkelstein & Tomasz 1977) and bind the acute-phase reactant CRP (Horowitz *et al.* 1987).

4. Host defense

4.1. Mucosal immunity

The first-line defense against pneumococci is the mucosal surface. In healthy individuals, the mucosae of the upper respiratory tract form a mechanical barrier against the spread of pneumococci from nasopharynx into surrounding tissues or lungs. However, if the mucosal surface is injured by a previous viral infection or by chemical or physical agents, it may not be able to withstand bacterial invasion. Depending on their specificity and the timing of their appearance, the defense mechanisms of the body surfaces are divided into two general categories: nonspecific constitutive mechanisms and specific induced mechanisms.

4.1.1. Unspecific, constitutive mucosal defenses

The nonspecific, innate mucosal defense mechanisms are continuously present and effective against most invading micro-organisms. Since it takes 5 to 7 days for the specific inducible defenses to appear, the nonspecific defenses are particularly important in cases where a person encounters the pathogen for the first time. Nonspecific mucosal immunity includes several different mechanisms, such as the physical adhesive barrier composed by the layer of mucus, cough reflexes, mucociliary transport, washing action of secretions (e.g., saliva and urine), and various antibacterial substances. The special antibacterial substances on mucosal surfaces that either kill the invading bacteria or inhibit their growth include lysozyme, lactoferrin and lactoperoxidase. Lysozyme is an enzyme, which is able to digest the peptidoglycan of bacterial cell walls. It is effective mainly against gram-positive bacteria, because the outer membrane of gram-negative bacteria protects the peptidoglycan of their cell wall. Lactoferrin is a protein that binds iron with high affinity and prevents bacterial growth by limiting the amount of free iron. Lactoperoxidase is an enzyme that produces toxic superoxide radicals, which are reactive forms of oxygen and harmful to many bacteria.

The Pnc surface protein PspA has been found to specifically recognize and bind the iron-carrier protein lactoferrin (Hammerschmidt *et al.* 1999). Interaction of PspA with lactoferrin was observed in 88% of the clinical Pnc isolates (Hammerschmidt *et al.* 1999). Both family 1 and family 2 PspAs bind lactoferrin (Håkansson *et al.* 2001).

Interestingly, lactoferrin binds to the same region of PspA that is most important in eliciting cross-protective immune responses (McDaniel *et al.* 1994; Tart *et al.* 1996; Håkansson *et al.* 2001). Thus, although PspA is highly variable between strains, there are apparently conformationally conserved regions of the molecule that are responsible for lactoferrin binding (Håkansson *et al.* 2001). The conservation of lactoferrin binding may be interpreted so that it is important and beneficial to the bacteria. The interaction between PspA and lactoferrin has been suggested to be a mechanism used by pneumococcus to overcome the iron limitation at mucosal surfaces. It has earlier been shown though, that lactoferrin does not support the growth of *S. pneumoniae* in an iron-deficient medium (Tai *et al.* 1993; Brown *et al.* 2001). If pneumococci do not use lactoferrin for the acquisition of iron, it must play some other role in human infections. Lactoferrin inhibits complement activation and several other immune mechanisms (Broxmeyer *et al.* 1978; Kijlstra *et al.* 1982; Veerhuis *et al.* 1982; Kievits *et al.* 1985; Mattsby-Baltzer *et al.* 1996). Furthermore, lactoferrin receptors are known to exist on host cells. They may play a role in Pnc adherence by allowing lactoferrrin to form a bridge between the bacteria and host cells (Håkansson *et al.* 2001). Further studies are needed to clarify the importance of the interaction between PspA and human lactoferrin in the pathogenesis of Pnc infection.

Normal nasopharyngeal, as well as intestinal and vaginal bacterial flora, may also be seen as part of the nonspecific mucosal defense. The bacterial species of normal flora compete with the pathogenic species for the same nutrients and available colonization sites. Resident oropharyngeal flora such as viridans streptococci are capable of antagonizing colonization with other streptococci (Johanson *et al.* 1970). Inhibition of Pnc growth by viridans streptococci has been demonstrated *in vitro* (Johanson *et al.* 1970). The role of bacterial antagonism in preventing Pnc colonization and subsequent disease is unknown. It appears that episodes of AOM in children are preceded by colonization with large numbers of respiratory pathogens, such as *S. pneumoniae, H. influenzae* and *M. catarrhalis*. At the same time, the number of nonpathogens of the resident flora, in particular viridans streptococci, decrease (Faden *et al.* 1990). These findings suggest that an alteration in the normal bacterial colonization patterns may predispose to local Pnc disease (Faden *et al.* 1990).

4.1.2. Specific, induced mucosal defenses

4.1.2.1. Common mucosal immune system (CMIS)

The mucosal surfaces of the respiratory, gastrointestinal and genito-urinary tracts have a specialized common mucosal immune system (CMIS), which functions independently from the systemic immune system (McGhee *et al.* 1990, 1992). The CMIS consists of an integrated network of lymphoid cells, which work in concert with innate host factors to promote host defense. It has been suggested that about half of the number of human lymphocytes are found in association with mucous membranes and exocrine glands. The major effector function of the mucosal immunity is production and transport of antibodies into external secretions. IgA is the predominant isotype in most mucosal secretions. Other Ig isotypes are also found in secretions, but in substantially lower concentrations than IgA. In addition to the mucosa-associated antibody response, there is also a mucosa-associated cell-mediated response. It includes macrophages, mucosa-specific mast cells, cytotoxic T-cells, as well as cytokines, chemokines and their receptors. This part of the mucosal immunity is, however, poorly understood.

The organized accumulations of lymphoid and non-lymphoid cells situated directly underneath the epithelium at several sites of the mucosal lining form the so-called mucosa-associated lymphoid tissue (MALT). An important activity of the MALT is the production of special type of antibody, secretory IgA (sIgA). In the respiratory and digestive tracts the MALT is covered by a specialized epithelium, the follicle-associated epithelium. The follicle-associated epithelium comprises so-called M-cells, which are specialized for the uptake and transcytosis of macromolecules and micro-organisms from the luminal surface (Figure 4). The M-cells pass antigens from the mucosal surface to the underlying cells of the MALT, which leads to antigen processing and presentation, and the stimulation of specific B- and T-lymphocytes. The primed, IgA-committed B-cells migrate to local lymph nodes and enter systemic circulation where they selectively home to the mucosal sites where the antigen was first encountered, but also to distant mucosal sites. After this, local synthesis and secretion of IgA occurs. Thus, exposure of one mucous membrane to a microbial antigen results in the production of sIgA also at distant mucosal surfaces.

Over the past few years, it has become apparent that dendritic cells have an important role in mucosal immunity. They have been shown to be capable of mediating an

alternative, M-cell-independent mechanism for bacterial uptake across the intestinal mucosal surfaces. In this mechanism dendritic cells open the tight junctions between epithelial cells, extend dendrites from the epithelium and sample bacteria directly from the gut lumen (Granucci & Ricciardi-Castagnoli 2003). They have also been suggested to have a regulatory function in the control of mucosal immunity via producing regulatory cytokine IL-2 (Granucci & Ricciardi-Castagnoli 2003).

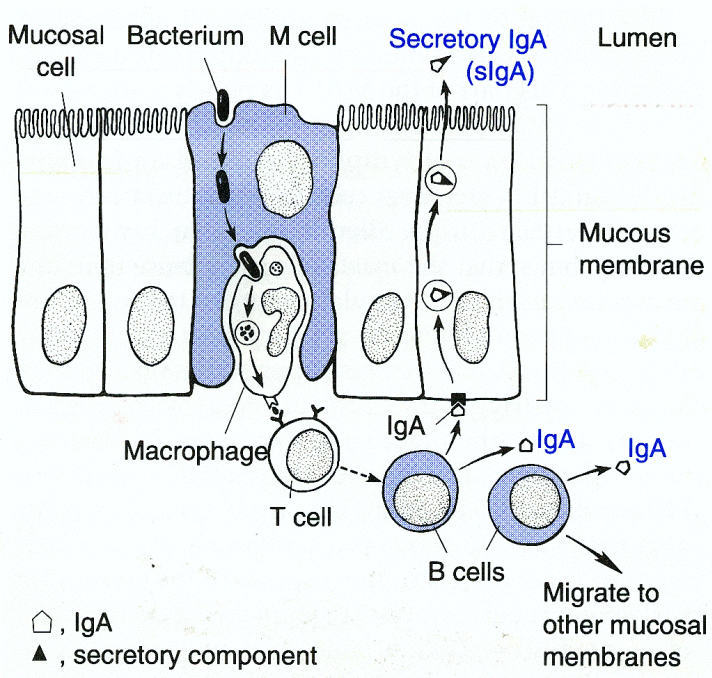

Figure 4. Cells of the mucosa-associated lymphoid tissue (MALT) of the gut (Salyers *et al*. 1994, with permission).

4.1.2.2. Secretory IgA (sIgA)

The significance of IgA in the host defense is highlighted by the fact that the daily production of total IgA is considerably greater than that of the other Ig classes combined (Conley & Delacroix. 1987). Approximately 2/3 of the total Igs produced in humans are IgA (Conley & Delacroix 1987; Mestecky & McGhee 1987). In serum, IgA occurs in monomeric form and the proportion of IgA among all Ig classes is minor, being only approximately 13% (Stokes 1984). In external secretions, by contrast, the majority of antibodies belong to the IgA class. The IgA antibodies in secretions occur

predominantly in dimeric or tetrameric forms containing four to eight antigen-binding sites, respectively. This multivalence of IgA increases its avidity and enables it to agglutinate bacteria better than corresponding IgA monomers. IgA functions in an environment rich in proteolytic enzymes (Kilian *et al.* 1988). However, IgA is intrinsically resistant to proteolysis by common microbial and intestinal enzymes, futher enforced by association with SC (Almogren *et al*. 2003). This provides functional advantages to IgA when compared with Igs of other isotypes (Mestecky & McGhee 1987; Kilian *et al.* 1988).

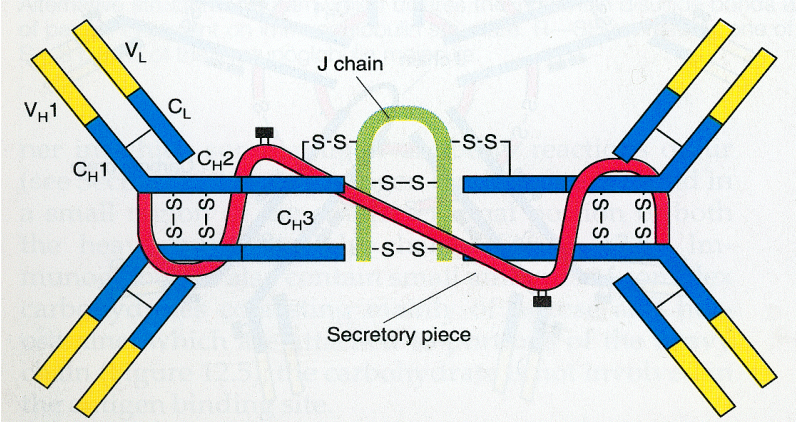

Figure 5. Structure of human secretory Ig A (sIgA). (Brock TD, Madigan MT, Martinko JM *et al:* Biology of Micro-organisms. Copyright© 1994 Prentice-Hall Inc. Reprinted with permission of Pearson Education, Inc., Upper Saddle River, NJ, p. 444.)

MALT B-cells synthesize polymeric IgA covalently linked with a joining peptide (J chain) (Figure 5). The J chain is required for the binding of IgA to a special basolateral epithelial cell receptor, polymeric Ig receptor (pIgR) (Figure 6). The complex of IgA and pIgR traverses the epithelial cell in an endocytic vesicle by transcytosis and reaches the apical surface of the epithelium. At the apical surface proteolysis cleaves the pIgR between its extracellular and transmembrane domains releasing sIgA. Thus, the SC bound to the sIgA molecule, is an extracellular domain of the pIgR. In addition to sIgA, secretory form of IgM (sIgM) transported by the pIgR is also present in external secretions. However, the concentration of sIgM is considerably lower than that of sIgA because of the lower proportion of IgM-producing cells in mucosal tissues (Mestecky *et*

al. 1999). Furthermore, pentameric IgM may not be transported as well as polymeric IgA because of a molecular weight restriction in SC-dependent transport (Schiff *et al.* 1983).

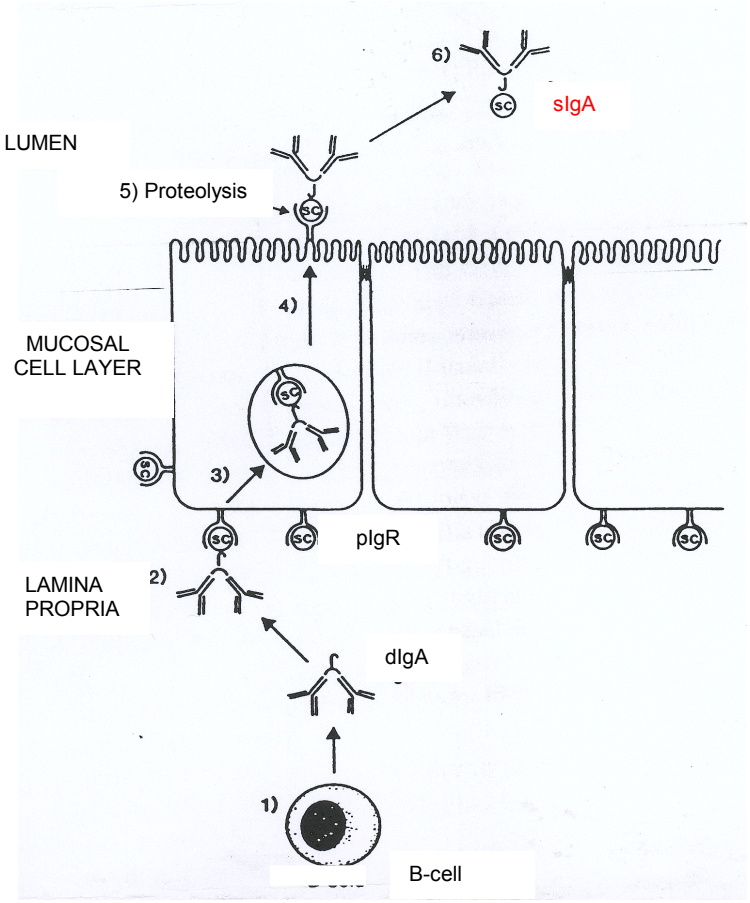

Figure 6. The transcytosis of dimeric IgA through epithelial cell layer to secretions. 1) The dimeric IgA (dIgA) is produced by the B-cells of the MALT. 2) dIgA binds IgA to polymeric Ig receptor (pIgR) via its J-chain. 3) The complex of dIgA and pIgR traverses the epithelial cell in an endocytic vesicle by transcytosis 4) The complex reaches the apical epithelial surface. 5) Proteolytic enzymes cleave the pIgR between its extracellular and transmembrane domains. 6) Secretory IgA (sIgA) is released in secretions. Figure modified from Kantele 1992.

Previously, it was suggested that pneumococcus might have found a way of using pIgR to its own benefit: a laboratory strain of *S. pneumoniae*, R6x, was shown to be capable of invading a human nasopharyngeal cell line (Detroit 562) in a human pIgR-dependent manner (Zhang *et al.* 2000). However, when the study was extended to other Pnc strains and other cell lines, the generalization of such a mechanism was challenged (Brock *et al.* 2002). The human pIgR-dependent enhanced invasion of epithelial cells by

pneumococcus seems to be a limited phenomenon that occurs in a strain-specific and cell type-specific manner. Under real-life conditions, it is probable that factors such as cleavage of pIgR from the apical surface, the relative inefficiency of apical-to-basolateral transport and the presence of free SC and sIgA in the respiratory secretions should prevent pIgR-mediated internalization of pathogens (Kaetzel 2001).

4.1.2.3. Function of sIgA

The Fc region of sIgA is wrapped within the SC molecule, which renders the associated chains resistant to most endogenous proteases (Mestecky & Russell 1986) (Figure 5). The functional capacity of the sIgA molecule is further increased by its dimeric (or tetrameric) status (Karush *et al.* 1973). The important functions of mucosal sIgA antibodies are to help trapping bacteria in mucus and to prevent microbial adherence to mucosal cells by binding to proteins on the bacterial surface that mediate adherence. sIgA antibodies are able to neutralize microbial and other environmental toxins and enzymes, as well as viruses, by sterically blocking their binding to target cells or substrates (Gilbert *et al.* 1983; Mansa *et al.* 1986; Childers *et al.* 1989). Also, sIgA prevents the absorption of antigens from mucosal surfaces in the intestinal tract, a phenomenon called immune exclusion (Stokes *et al.* 1975; McGhee & Mestecky 1990). Prior enteric exposure to alimentary antigens diminishes the absorption of subsequent doses of the same substances in immunologically reactive form (Walker *et al.* 1972; Andre *et al.* 1974). During their passage through the mucosal epithelial lining, IgA antibodies may also have opportunity to neutralize intracellular pathogens, such as viruses (Mazanec *et al.* 1992). sIgA antibodies have no pro-inflammatory effect, and they do not efficiently activate complement or initiate phagocytosis (Russell *et al.* 1997; Stenfors 1999). By contrast, opsonization of bacteria with serum IgA has been shown to enable efficient phagocytosis (van Egmond *et al.* 2000).

4.1.2.4. IgA subclasses

Human IgA occurs in serum and secretions as two subclasses, IgA1 and IgA2. These subclasses differ from each other in several aspects including minor differences in the primary structure, carbohydrate composition, antigenic properties, and sensitivity to the proteolysis by bacterial IgA1-proteases. A major difference between the two human subclasses occurs in the hinge region: IgA2 molecules lack a 13-amino-acid segment

found in the hinge region of IgA1 molecules that contains five carbohydrate moieties O-linked to serin (Underdown *et al.* 1994).

The IgA1- and IgA2-secreting cells are distributed in different ratios in the different lymphoid tissues of the human body. In general, most lymphoid tissues show a predominance of IgA1-producing cells. However, in secretory lymphoid tissues the IgA2 production is relatively enhanced when compared to the nonsecretory lymphoid organs, such as peripheral lymph nodes and spleen (Kett *et al.* 1986). Correspondingly, the IgA1 and IgA2 antibodies are characteristically distributed in body fluids: serum IgA is mainly of subclass IgA1, while IgA2 is more prominent in external secretions (Delacroix *et al.* 1982). The proportion of IgA2 of the total IgA in serum is usually 11 to 23% and in secretions 26 to 41% (Delacroix *et al.* 1982).

The extended hinge region of IgA1 molecules renders them highly susceptible to the IgA1-proteases produced by several important mucosal pathogens, including *S. pneumoniae*, *H. influenzae, Neisseria meningitidis* and *Neisseria gonorrhoeae* (Brandtzaeg 1985; Mestecky & Russell 1986; Conley & Delacroix 1987; Mestecky & McGhee 1987) as well as some members of resident oral or pharyngeal normal bacterial flora (Kilian *et al.* 1989; Frandsen *et al.* 1991). Bacterial IgA1-proteases enable mucosal pathogens to evade Fc-dependent functions of the predominant Ig isotype on mucosal surfaces. These proteases are thought to be important for the ability of the bacteria to colonize human mucosal surfaces in the presence of specific sIgA1 antibodies. Since the IgA2 antibodies are resistant to bacterial IgA1-proteases, the pronounced production of IgA2 antibodies in secretions may offer a functional advantage for the defense of mucosal surfaces against the IgA1-protease-producing bacteria.

4.1.2.5. Early maturation of the mucosal immune system

There is a contradiction between the relatively early appearance of sIgA in external secretions and the much later appearance of IgA in serum. Although adult levels of IgA in serum are reached only in early adolescence, the sIgA system has in several studies reported to have a remarkably rapid maturation pattern (Allansmith *et al.* 1968; Selner *et al.* 1968; Gleeson *et al.* 1982; Mellander *et al.* 1984; Taubman *et al.* 1989). At the same time, there are other studies that have suggested a slower maturation of secretory immunity. Burgio *et al.* (1980) reported that the concentrations of total salivary sIgA

increased gradually during infancy, but attained adult levels in unstimulated saliva only by the age of 6 to 8 years and in stimulated saliva at the age of 2 to 4 years. Furthermore, Fitzsimmons *et al.* (1994) found that sIgA can be detected in the saliva of infants within the first week after birth, but the levels of sIgA remain low during the first 18 months of life, at the very time when the infant is susceptible to infections by mucosal pathogens. In the case of *S. pneumoniae*, mucosal immunity has been suggested to be immature in young children compared to adults who show significantly higher specific IgA activity against pneumococcus than children (Lindberg *et al.* 1993). This could partly explain the high incidence of Pnc carriage and Pnc AOM in infants. Nasopharyngeal carriage of Pnc serotypes that are relatively poor in inducing immunity in children tends to last a longer than the carriage of more immunogenic serotypes (Gray *et al.* 1980). This suggests that local antibodies are important in limiting the duration of Pnc carriage.

The mucosal immunity particularly to bacterial PSs has been suggested to mature earlier than the systemic immunity (Pichichero *et al.* 1981). Serum IgG to Pnc PSs is rarely detected in children before 18 months of age, whereas sIgA to Pnc PSs can be detected in nasopharyngeal secretions of children as early as at 6 months of age (Virolainen *et al.* 1995; Nieminen *et al.* 1996). The suggested more rapid appearance and development of anti-PS IgA in secretions than anti-PS IgG in serum could be related to a greater antigenic stimulation initially occurring at the mucous membranes (Tomasi *et al.* 1972). This important potential of infants to respond by production of sIgA should be taken into consideration in the design of new vaccines.

4.1.3. Investigation of mucosal immunity

Different immunohistochemical methods have been used to study the cellular types of CMIS and the secretion of Igs (Brandtzaeg 1989). Polyclonal and monoclonal antibodies have been used in direct and indirect immunofluorescence for the detection of IgA and IgA subclasses in cells and tissues (Crago *et al.* 1984; Brandtzaeg *et al.* 1986; Conley & Delacroix 1987).

The measurement of specific antibodies in secretions with enzyme immunoassay (EIA) has been widely adopted. Saliva, breast milk and colostrum are probably the most studied fluids because of their easy accessibility, while other secretory fluids are less

accessible. In contrast to working with serum, most mucosal secretions are difficult to collect, standardize and assay, because of factors such as viscosity, presence of bacteria, desquamated cells and bacterial proteases (Jackson *et al*. 1999). In addition, the stability of different secretions during long-term storage is variable.

Specific antibody-secreting cells (ASCs) can be enumerated in peripheral blood with an enzyme-linked immunospot assay (ELISPOT) (Czerkinsky *et al.* 1983; Sedgwick *et al.* 1983). The B-cells are constantly circulating through the lymphatics and blood back to the peripheral tissues (Gowans *et al.* 1964). The B-cells committed to mucosal sites are present in the peripheral blood for a limited period of time after an antigen challenge, before homing to different exocrine tissues. The appearance of these antigen-specific ASCs in blood can be measured. In the ELISPOT method, cell suspensions containing ASC are incubated on solid phase to which a specific antigen has been conjugated. The antibody attaches to the latter within the immediate microenvironment of the ASC, producing localized zones of bound antibody, which are subsequently developed as visual 'spots' in the EIA.

4.2. Systemic immunity

4.2.1. Antibody-independent clearance of pneumococci

The spleen plays a major role in antibody-independent Pnc sequestration and contributes to resistance against Pnc infections in several ways. It is thought to be important in the synthesis of antibodies, in phagocytosis and clearance of blood-borne antigens in the non-immune host (Wara 1981). The spleen also plays a role in the activation of the alternative complement pathway (Wara 1981). Patients who have undergone splenectomy face a greatly increased risk of invasive bacterial diseases, *S. pneumoniae* being responsible for >50% of these episodes (Gillespie 1989). After IgM and IgG have appeared, the liver preferentially clears pneumococci (Schulkind *et al.* 1967).

The intact complement system plays a biologically significant role *in vivo* in the host's defense against *S. pneumoniae* (Winkelstein 1981, 1984). Complement deficiencies have been associated with an increased susceptibility to Pnc diseases. The activation of the alternative complement pathway takes place in the absence of specific antibodies. Pneumococci are potent activators of the alternative pathway, CPS being the active cell

wall component (Winkelstein & Tomasz 1978). Also the Pnc capsule can activate the alternative pathway. However, the complement activation by the Pnc capsule is 50-100 times less efficient than with CPS with variation among different serotypes (Fine 1975). The complement is not able to produce bacterial lysis in gram-positive pneumococci: although the membrane attack-complex forms normally, it is inserted only 10-15nm into the peptidoglycan (Joiner *et al.* 1983). Thus, opsonization for phagocytosis by the deposition of the complement component C3b on the bacterial surface is the main way in which complement proteins act in the defense against *S. pneumoniae* (Gillespie 1989).

The non-antibody-mediated defense mechanisms against pneumococcus may also involve phagocytosis mediated by lectins (lectinophagocytosis) (Ofek *et al.* 1988). Lectins are host glycoproteins that specifically recognize certain carbohydrate structures. Serum components that belong to the family of lectins include pulmonary surfactant proteins, such as surfactant proteins SP-A and SP-B, CRP and mannose-binding lectin (MBL) (Ofek *et al.* 1995; Tino *et al.* 1996; Schagat *et al.* 1999). They combine with complementary carbohydrates on micro-organisms and special receptors on phagocytes, thereby enhancing phagocytosis in the absence of opsonins, such as antimicrobial antibodies, C3b and C3bi fragments (Ofek *et al.* 1995). However, lectinophagocytosis is dependent on the Pnc serotype and is thus not likely a general defense mechanism against all pneumococci (Alonso de Velasco *et al.* 1994).
In the absence of specific antibodies, CRP may facilitate the clearance of pneumococci (Boulnois 1992). CRP was first identified by its ability to bind to the CPS from the Pnc cell wall. The classical complement pathway is activated via direct binding of the C1q component of complement to ligand-fixed CRP (Volanakis *et al.* 1974). In this regard CRP acts as a component of nonspecific defense against bacterial disease in the preimmune phase of infection.

4.2.2. Antibody-dependent clearance of pneumococci

The humoral and cellular arms of the immune system act in concert to defend the host against Pnc infection. Once an immunocompetent individual has been colonized or infected by pneumococcus, an immune response against capsular PS, CPS and Pnc proteins will be mounted (Gray *et al.* 1981, 1983; Briles *et al.* 1987; Renneberg *et al.* 1991; Rapola *et al.* 2000; Soininen *et al.* 2001). The importance of serum antibodies in

protection against systemic disease is clear, while it becomes less clear in infections restricted to the mucosae. Invasive diseases caused by encapsulated bacteria occur frequently in infants and children. Infants become susceptible to these infections after the loss of maternally transferred serum antibodies, since they are not yet able to mount a sufficient systemic immunity to limit the spread of bacteria.

In the presence of anti-capsular antibodies, pneumococci are rapidly cleared from the blood, mainly by the liver and to a lesser extent by the spleen. Complement components are necessary for effective clearance. Antibodies to capsular PSs and CPS activate the classical complement pathway. Opsonization of pneumococci by type-specific antibodies and/or complement components is crucial for phagocytosis by macrophages or neutrophils. The binding of type-specific anticapsular antibodies to the capsule changes the structure of the Pnc surface so that phagocytosis is facilitated. Once ingested and entrapped in a phagosome pneumococci are readily killed by phagocytic cells (Johnston 1991).

The bacterial PSs induce production of anti-capsular antibodies by a thymus-independent or T-cell-independent (TI) mechanism. They can interact with B-cells and stimulate clonal expansion of B-cells by direct binding and cross-linking the B-cell receptor, the membrane-bound Ig. The result is the production of a clonal population of short-lived terminally differentiated antibody-producing plasma cells. The measurable effect in adults is a small rise in IgM but a more substantial increase in IgG (Barrett *et al.* 1986; Chudwin *et al.* 1987). The B-cell response is enhanced by the presence of opsonins bound to the PS antigen, in particular the cleavage products of complement factor C3 that acts as a ligand for complement receptor 2 (CR2 or CD21) (Griffioen *et al.* 1991). Young children express CR2 poorly and this in part explains their poor response to Pnc PS antigens (Griffioen *et al.* 1992). Once a B-cell becomes an antibody-producing plasma cell, it has a short lifespan and will undergo cell death in the space of only a few days, and immunologic memory is not generated.

Protein antigens are referred to as thymus-dependent or T-cell-dependent (TD) antigens, since the immune response to them uses the co-operation of B-cells and stimulated T-cells (Noelle *et al.* 1991). For the proper activation and differentiation of protein antigen-specific B-cells to memory cells or antibody-producing plasma cells, the

interaction between B-cells and helper T-cells and cytokine-mediated events are required (Noelle *et al.* 1990). TD responses are characterized by the induction of memory, which is shown by a booster effect upon subsequent immunizations, affinity maturation, and extensive Ig class switching (AlonsoDeVelasco *et al.* 1995).

4.2.3. Investigation of systemic immunity

EIA is the currently recommended technique for determining the concentration of serum antibodies to Pnc capsular PS and protein antigens (Siber *et al.* 1989). The EIA techniques may also be used to measure the relative antibody avidity to different types of antigens. The binding of an antibody to an antigen conjugated in solid phase may be prevented either by competitive inhibition using decreasing concentrations of a free antigen (Devey *et al.* 1988; Goldblatt 1997) or by eluting the antibody from the antigen by a dissociating agent, such as thiocyanate (Pullen *et al.* 1986; Goldblatt *et al.* 1993, 1997), urea (Hedman *et al.* 1989) or diethylamine (Devey *et al.* 1988; Goldblatt *et al.* 1993, 1997).

Since opsonin-dependent phagocytosis is the primary defense mechanism against *S. pneumoniae*, a variety of techniques measuring the opsonophagocytic activity of serum antibodies against Pnc capsular PSs have been developed (Esposito *et al.* 1990; Lortan *et al.* 1993; Vitharsson *et al.* 1994; Romero-Steiner *et al.* 1997; Jansen *et al.* 1998; Vidarsson *et al.* 1998; Martinez *et al.* 1999). These include radioisotopic, flow cytometric, microscopic, and viability (or killing) assays. Most of them are performed by using human polymorphonuclear leukocytes as the effector cells. Some assays have also been adapted to utilize culturable phagocytes (differentiated HL-60 cells) (Romero-Steiner *et al.* 1997; Martinez *et al.* 1999). In addition to these *in vitro* assays, many studies use animal models to investigate, for instance, the immunogenicity and protective efficacy of Pnc conjugate vaccines in mice, rats, chinchillas, or infant monkeys (Paton *et al.* 1991; Peeters *et al.* 1992; Vella *et al.* 1992; Giebink *et al.* 1993; Lee *et al.* 1994; Rodriguez *et al.* 1998; Jakobsen *et al.* 1999; van der Ven *et al.* 1999).

5. Pneumococcal vaccines

The search for an efficient vaccine against pneumococcus has continued for a long time. The vaccine development began originally at the beginning of the 20th century (year 1914), when attempts to induce protective immunity against pneumococcus in humans by vaccination with whole-cell killed pneumococci turned out to be unsuccessful. In the 1930s, the immunogenicity of purified capsular PSs was demonstrated and the first Pnc PS vaccine was developed. However, along with the demonstration of the therapeutic efficacy of antibiotics, the enthusiasm for Pnc vaccine development ceased for some decades. Despite the use of antibiotics, the mortality rate of systemic Pnc diseases remained high (Gillespie 1989). This, with the emergence of the first penicillin-resistant pneumococci in 1965 (reviewed in: Appelbaum 1992) led to renewed efforts to develop improved Pnc vaccines. At the moment, increasing antibiotic-associated resistance complicates disease management and highlights the importance of effectively preventing Pnc diseases.

The clinical efficacy of the current Pnc vaccines is based on the production of opsonizing anti-capsular antibodies, which have proved to be important in the host defense against Pnc disease (Bruyn *et al.* 1992). The data on the pathogenesis of Pnc infection and development of new vaccination strategies in animal models (including immunization with Pnc proteins and mucosal immunization) have accumulated during the past few years. This has opened up new possibilities for the prevention of Pnc infections by immunization. Furthermore, clinical trials on combination vaccines including Pnc vaccine are underway with the aim of decreasing the number of shots administered during early childhood (Choo *et al.* 2000a; Dennehy 2001).

5.1. Systemic immunization

5.1.1. Pneumococcal polysaccharide vaccines

Purified Pnc capsular PSs used as a vaccine can induce type-specific anticapsular antibodies, which are protective against Pnc disease in healthy adults. The efficacy of the first Pnc PS vaccine against bacteremic Pnc disease was demonstrated in the 1930s and 1940s. In 1977, a 14-valent vaccine containing 50μg of capsular PSs of each 14 serotypes was licensed. In 1983, the valency was increased to 23 serotypes. The vaccine contains 25μg of each capsular PS of the 23 serotypes most frequently causing disease

in the U.S. The 23-valent vaccine remains the current preparation of Pnc PS vaccine. Pneumocccal PS vaccine protects healthy non-elderly, immunocompetent adults against pneumonia, invasive disease and death (reviewed in: French 2003).

Although safe and efficacious among healthy adults, the current Pnc PS vaccine has some apparent shortcomings. First, young children respond poorly to Pnc PS vaccines. A number of clinical trials on the Pnc capsular PS vaccines have demonstrated limited or no evidence of efficacy among children less than 2 years of age (Douglas *et al.* 1983; Koskela *et al.* 1986; Leinonen *et al.* 1986). Second, the PS molecules are of TI nature and the important features of these antigens are poor immunogenicity, as well as a lack of ability to induce affinity maturation, isotype switch and immunologic memory (Stein 1992). Thus, revaccination with a PS vaccine does not result in booster responses but instead leads to a similar or even reduced serological response in comparison to the response to primary vaccination (Mufson *et al.* 1991; Musher *et al.* 1993). Consequently, the antibody concentrations and eventually the protection diminish in the course of time. Third, the Pnc PS vaccine does not protect certain high-risk groups such as immunologically incompetent individuals and elderly against Pnc pneumonia or invasive disease (reviewed in: French 2003). Finally, the Pnc PS vaccine does not provide significant protection against mucosal Pnc diseases, such as AOM in young children, or against the spread of resistant strains from person to person (reviewed in: Eskola *et al.* 1999).

5.1.2. Pneumococcal conjugate vaccines

In order to obtain improved vaccines for infant use, Pnc capsular PSs of the epidemiologically most important Pnc serotypes have been covalently coupled with various carrier proteins, such as diphtheria toxoid, tetanus toxoid and the outer mebrane protein complex (OMPC) of *N. meningitidis* group B. Covalent coupling of PS antigen with a protein carrier converts the nature of the vaccine into TD and increases the immunogenicity of the hapten molecule. The antigen-presenting cells take up the conjugated PS-protein vaccine molecule, internalize it via the membrane-bound Ig and present the peptides of the protein to the helper T-cells in association with the major histocompatibility complex class II (MHC II) molecules. This induces the helper T-cells to stimulate PS-specific B-cells to mature either into antibody-producing plasma cells or into memory cells (Schneerson *et al.* 1980; Lanzavecchia 1985; Siber 1994).

T-cell help associated with Pnc conjugate vaccines leads to several benefits. Long-lived memory B-cells are produced, which leads to the induction of a strong antibody response after revaccination (Granoff *et al.* 1993; O'Brien *et al.* 1996; Åhman *et al.* 1998). Additionally, T-cell signaled rearrangements of the Ig variable region leads to affinity maturation of the antibody response, improved antibody-antigen 'fit' and an increased opsonising function (Goldblatt 1998). Finally, mucosal immune responses are enhanced with the production of mucosally active IgG (Choo *et al.* 2000b; Nurkka *et al.* 2001b).

The first Pnc conjugate vaccine, a 7-valent vaccine PncCRM7, was licensed in the U.S. in 2000 (Prevnar®, Wyeth-Ayerst Laboratories, Philadelphia, USA) and in Europe in 2001 (Prevenar®). This vaccine includes Pnc serotypes 4, 6B, 9V, 14, 18C, 19F and 23F conjugated to a nontoxic mutant diphtheria toxin known as CRM_{197}. Another investigational 7-valent vaccine, PncOMPC, contains same serotypes as PncCRM7, but the carrier protein is the meningococcal OMPC. The Pnc conjugate vaccines with higher valencies are now in ongoing clinical trials (Wuorimaa *et al.* 2001; Dagan *et al.* 2002; Huebner *et al.* 2002; Obaro *et al.* 2002; Puumalainen *et al.* 2002). In the 9-valent vaccine serotypes 1 and 5 are added, while the 11-valent vaccine includes also serotypes 3 and 7V (Fedson *et al.* 1999; Hausdorff *et al.* 2000b; Wuorimaa *et al.* 2002). In practise, vaccine valency has proved technically difficult to increase, the limiting factor being the quantity of carrier protein that can be incorporated whilst maintaining immunogenicity (reviewed in: Wuorimaa & Käyhty 2002). Use of multiple carriers or alternate carrier proteins may provide a solution to this problem (Nurkka *et al.* 2002; French 2003).

The Pnc conjugate vaccines have been shown to be safe and immunogenic already in early infancy (Käyhty *et al.* 1995; Dagan *et al.* 1996a; Mbelle *et al.* 1999; Puumalainen *et al.* 2002). They induce high concentrations of serum antibodies (Rennels *et al.* 1998; Åhman *et al.* 2001; Eskola *et al.* 2001) and reduce nasopharyngeal carriage of vaccine serotypes (Obaro *et al.* 1996; Dagan *et al.* 1996a, 1997, 1998; Mbelle *et al.* 1999). PncCRM7 has been shown to be highly efficacious in preventing vaccine-serotype invasive disease in young children (Black *et al.* 2000) and modestly beneficial against AOM (Black *et al.* 2000; Eskola *et al.* 2001). Because Pnc conjugate vaccines reduce carriage and disease in the vaccinated population, transmission to the nonvaccinated

population may also be decreased, thus reducing the overall burden of Pnc disease on a population level (Pelton *et al.* 2003). This effect is often referred to as "herd immunity" or the "indirect" effect of immunization. The impact since the licensure of a 7-valent Pnc conjugate vaccine on invasive disease epidemiology has been recently determined in the U.S. A notable fall in disease incidence was seen both in vaccinated and unvaccinated children under 5 years of age, but also in older children and adults. These findings suggest a herd immunity effect in non-vaccinated individuals (Shinefield *et al.* 2002; Whitney *et al.* 2003).

Studies on the impact of Pnc conjugate vaccines with different valencies and protein carriers on Pnc nasopharyngeal carriage have shown that immunization with these vaccines (4-, 7- and 9-valent) results in a decrease of carriage by vaccine serotypes but in an increase by non-vaccine serotypes (Obaro *et al.* 1996; Mbelle *et al.* 1999; Dagan *et al.* 1996a, 1997, 1998, 2002). This effect may be due to colonization with new pneumococci or through capsular transformation of pneumococci *in vivo* (Barnes *et al.* 1995; Nesin *et al.* 1998). However, it has not been definitively determined if the increase in carriage by non-vaccine types is due to true replacement or just an unmasking of already present nasopharyngeal serotypes (Pelton *et al.* 2003). In a study evaluating PncCRM7 in the American Indian population using a highly sensitive immunoblot assay to determine the effect of conjugate vaccine on nasopharyngeal carriage, an association of PncCRM7 with true replacement carriage was detected (Pelton *et al.* 2003). Furthermore, in two clinical trials conducted in Finland evaluating the effect of 7-valent Pnc conjugate vaccines on Pnc AOM, replacement disease was observed. In the first trial with PncCRM7, an increase of 33% in nonvaccine serotype AOM was shown (Eskola *et al.* 2001). With another investigational 7-valent vaccine, PncOMPC, the rate of AOM episodes due to all other Pnc serotypes was 27% higher among the vaccine recipients than in the control group (Kilpi *et al.* 2003). Whether nasopharyngeal replacement is associated with invasive replacement disease is not yet definitively clear. In the trial on invasive Pnc disease in the U.S., significant replacement disease was not observed (Black *et al.* 2001; Whitney *et al.* 2003).

Current information is insufficient to suggest whether Pnc conjugate vaccines would be indicated to other target groups than infants. Although Pnc conjugate vaccines are able to induce better antibody responses than a Pnc PS vaccine in healthy adults (Nieminen

et al. 1998; Wuorimaa *et al.* 2001), they do not seem to offer any significant advantages to the PS vaccine in an adult population (Ahmed *et al.* 1996; Powers *et al.* 1996). With the substantially greater cost of the Pnc conjugate vaccines in comparison with the Pnc PS vaccine, it will be important to clearly demonstrate efficacy of the conjugate vaccine in the target populations along with safety and acceptability. Future clinical trials of Pnc conjugate vaccines will include elderly to find out whether conjugates prove to be efficacious in protection against Pnc pneumonia (Pelton *et al.* 2003).

The main problem with the Pnc conjugate vaccines is that only a limited number of types may be included in the conjugated formulation due to logistic difficulties in the manufacturing process, and the attendant high cost. Thus, the choice of antigens to be included in a Pnc conjugate vaccine has to be based primarily on the predominant serotypes causing disease in the target population. It is apparent that a vaccine, which is based on the most prevalent serotypes among children in one country, may not be appropriate for adults in the same region or children in a different region of the world. This is because of the differences in Pnc serotype prevalence in various age groups and localities. In accordance with this, the serotypes included in the 7-valent conjugate vaccine provide different levels of coverage in different geographic regions (Hausdorff *et al.* 2000b). Adding serotypes may increase coverage, especially in developing countries, where serotypes not included in PncCRM7 are more common (e.g., serotypes 1 and 5). It appears that especially the 9-valent and 11-valent conjugate vaccines might have the potential to prevent a large portion of the cases of Pnc pneumonia and meningitis in the developing world (Hausdorff *et al.* 2000a). However, when the number of serotypes is increased, production costs of the vaccine also increase. Regardless of its efficacy, an expensive vaccine may be unlikely to be used on a large scale in the developing world.

5.1.3. Pneumococcal protein vaccine candidates

The problems with the Pnc PS and conjugate vaccines have stimulated an interest in alternative Pnc vaccination strategies. A promising complementary or alternative approach for prevention of Pnc infections is to develop vaccines directed against an antigenic moiety common to all Pnc serotypes, such as a Pnc protein antigen. The use of Pnc proteins would have several advantages. As TD antigens they are expected to be immunogenic even in young children and to induce immunological memory. Pnc

proteins would provide protection against pneumococci regardless of the serotype. Thus, the development of Pnc protein vaccines may overcome the problem of serotype replacement. Pnc protein vaccines could be used to fill the gaps in protection provided by the Pnc PS or conjugate vaccines and, if highly successful, they might be even able to act as stand-alone vaccines. They have potential to cover the high-risk target groups who may not be covered by the current conjugate vaccine formulations, such as young infants in the developing countries and the elderly. Protein antigens can be produced with low expenses by recombinant technology. However, before Pnc protein antigens can be considered for large-scale human trials, their protective efficacy has to be clearly established in animal models.

Diversity among protein antigens is an important consideration in the selection of vaccine candidates. Non-variable protein antigens could potentially protect against a whole population of bacteria, provided that they have a critical function. However, many of the proteins required for critical bacterial functions show diversity among the strains. Despite the diversity, the proteins may still offer cross-protection. A number of proteins that act at different stages of the pathogenic process contribute to the virulence of *S. pneumoniae*. It has been suggested that vaccination with a mixture of different Pnc virulence proteins might provide a higher degree of protection than any antigen alone (Briles *et al.* 2000a; Ogunniyi *et al.* 2001).

Several Pnc proteins have been considered as potential vaccine candidates, including PspA, PsaA and inactivated Ply (Paton 1998). These proteins may be used either as a pure protein (Alexander *et al.* 1994; Talkington *et al.* 1996; McDaniel *et al.* 1997), conjugated with Pnc PSs (Kuo *et al.* 1995; Michon *et al.* 1998) or as fusion proteins combined with immunomodulating molecules (Wortham *et al.* 1998). Immunization of mice with inactivated or recombinant Ply toxoid induced enhanced survival against intraperitoneal challenge with several Pnc strains of different serotypes (Alexander *et al.* 1994; Lee *et al.* 1994). Similarly, mice immunized with PspA or with truncated PspA molecule were protected against challenge with virulent pneumococci (McDaniel *et al.* 1991; Tart *et al.* 1996; Briles *et al.* 1996b, 1998). Also, administration of PsaA together with an appropriate adjuvant protected mice against Pnc challenge (Talkington *et al.* 1996).

The novel Pnc vaccine candidates include several proteins, such as PspC, PpmA (putative proteinase maturation protein A) (Overweg *et al.* 2000b), Pht (Pnc histidine triad)-protein family (Adamou *et al.* 2001), BVH-3 and BVH-11 (Hamel *et al.* 2002). Immunization of mice with PspC protected against challenge with a Pnc strain that expressed PspA but not PspC. The PspA- and PspC-cross-reactive antibodies were directed to the proline-rich domain present in both molecules (Brooks-Walter *et al.* 1999). PpmA is a Pnc surface protein possibly playing a role in the maturation of surface proteins or secreted proteins. It is able to elicit protective, cross-reactive antibodies in rabbits (Overweg *et al.* 2000a). The immunization of mice with PhtA, PhtB or PhtD from the Pht-family has been shown to induce protection against invasive Pnc disease with diverse Pnc serotypes (Adamou *et al.* 2001). The function of these proteins, however, is still unknown. BVH-3 and BVH-11 are ubiquitous, conserved surface proteins of pneumococcus that are immunogenic and show protective efficacy in animal models against Pnc pneumonia and sepsis (Hamel *et al.* 2002). Both proteins have been well characterized and may be produced by recombinant technology.

Human trials on immunization with Pnc proteins are sparse at the moment. Nabors *et al.* (2000) immunized healthy adults with a single recombinant PspA variant and were able to stimulate broadly cross-reactive antibodies to heterologous PspA molecules. These antibodies have been shown to protect mice passively from fatal infection with *S. pneumoniae* strains bearing heterologous PspAs (Briles *et al.* 2000c).

5.2. Mucosal immunization

Pneumococci enter the body via mucosal surfaces of the upper respiratory tract and mucosal immunization represents an attractive alternative for current systemic immunization strategies. The protection against Pnc acquisition or carriage and local Pnc diseases, such as AOM, are thought to depend on mucosal antibodies. Already in early studies, intranasal immunization of rabbits with pneumococci was shown to produce resistance to a subsequent challenge with the live organism in the absence of a detectable serum antibody (Bull *et al.* 1929). Following nasal immunization with killed pneumococci, the rabbits were also resistant to an intravenous challenge with a live organism, suggesting that local immunization may induce also systemic immunity (Walsh *et al.* 1936).

It appears that nasal-associated lymphoid tissue (NALT) and upper airway mucosal epithelium are able to process and present antigens and mount a specific immune response locally, as well as in distant mucosal sites, via distinct homing mechanisms. Available evidence based on the vaccine antigens appropriate for the preventing AOM has suggested that oral immunization can induce effective immune responses in the middle ear cavity and nasopharyngeal tissues. Immunization via the nasal route appears to be as effective as the oral route, may require a smaller antigen dose, and can be effective even with non-replicating agents. Human experience with intranasal immunization is limited at this time, but recent studies with live attenuated influenza virus vaccine have been encouraging (Gruber *et al.* 1996; Belshe *et al.* 1998, 2001).

Several advantages have been linked to mucosal immunization. Mucosal immune responses induced by systemic immunization with the Pnc conjugate vaccines have been modest in infants and children (Choo *et al.* 2000b; Korkeila *et al.* 2000; Nurkka *et al.* 2001a, 2001b). However, immunization by mucosal (e.g. intranasal) route might possibly induce substantially stronger mucosal secretory IgA responses. Based on animal studies, mucosal immunization may induce antibody responses simultaneously in mucosal surfaces and serum (VanCott *et al.* 1996; Flanagan *et al.* 1999; Jakobsen *et al.* 1999; Seong *et al.* 1999). Local administration of Pnc vaccines would also be attractive due to its easiness, particularly in the developing world. Furthermore, the sIgA immune system has a potent immunological memory that is stimulated repeatedly by renewed contact with the antigen; this leads to a high level of production of specific IgA (McGhee & Mestecky 1990). Finally, mucosal immune responses have been suggested to develop early in life (Gleeson *et al.* 1982) and still function well in the elderly (Szewczuk *et al.* 1981; Garg *et al.* 1996). The possibility of exploiting the potential of young infants to respond to PS antigens by the early production of sIgA should be considered in the design of vaccines, particularly because of the high risks associated with infection by encapsulated bacteria. Similarly, elderly people are more susceptible to various infections, among these Pnc pneumonia. Aging has a generally negative effect on the immune system (Makinodan *et al.* 1991; Solana *et al.* 1998; Ginaldi *et al.* 2001). The mucosa-associated lymphoid system, however, is suggested to remain immunologically vigorous even during old age (Szewczuk *et al.* 1983; Smith *et al.* 1987; Russel *et al.* 1989). Garg and Subbarao (1992) have shown in a mouse model

that the response to a 23-valent PS-vaccine in the mucosa-associated mesenteric lymph nodes does not decline with age, but remains constant over the entire age range.
Encouraging results regarding mucosal vaccination against pneumococcus have been obtained in animal models. Both oral and intranasal immunization of mice with PspA has elicited protective immunity against Pnc carriage and systemic disease (Wu *et al.* 1997; Yamamoto *et al.* 1997). Intranasal immunization of mice with a mixture of the PsaA and PspA has proved to be highly protective against Pnc carriage (Briles *et al.* 2000a). Mucosal immunization of mice with the Pnc capsular PS antigens or conjugate vaccine and an appropriate mucosal adjuvant induce both mucosal and systemic antibody responses and can protect against intranasal challenge with live bacteria (VanCott *et al.* 1996; Jakobsen *et al.* 1999; Seong *et al.* 1999). These data suggest that the mucosal vaccination is able to reduce Pnc carriage and disease.

The experience with mucosal vaccination in humans is so far largely restricted to the use of the oral attenuated live viral vaccine against polio and bacterial vaccines against cholera and typhoid fever. At the moment, a viral intranasal vaccine against the influenza virus and an oral vaccine against the rotavirus are subjects of active investigation. Before a mucosal vaccination against Pnc disease can be considered in humans, more basic work will be necessary. The leap from animal studies to human trials requires a demonstration that antibodies to Pnc vaccine candidates protect humans against Pnc disease. Studies on natural immunity may support this idea and an understanding of additional aspects of antibody-mediated immunity in secretions may enable us to develop new methods of local protection against pathogens. However, because vaccine induced immunity may be better than natural immunity, these studies will not disprove the potential efficacy of vaccines.

5.3. Other immunization strategies

Other options of immunization to induce antibody responses against *S. pneumoniae* include the delivery of Pnc protein antigens either in recombinant carrier bacteria or in a form of nucleic acid vaccine (DNA vaccine). Protective responses against Pnc challenge have been obtained in mice with a recombinant Bacille Calmette-Guerin (rBCG) vaccine (Langermann *et al.* 1994) and an oral recombinant *Salmonella* vaccine (Nayak *et al.* 1998), both expressing PspA. In addition, protection against fatal Pnc infection

has been elicited in mice after intramuscular injection of a plasmid expressing PspA (McDaniel *et al.* 1997).

AIMS OF THE STUDY

An active search for novel Pnc vaccine strategies is under way. Mucosal antibodies are expected to be relevant in the defense against Pnc carriage and Pnc AOM. In the future, mucosal vaccination may be a potential alternative for current Pnc immunization strategies. Understanding the development and significance of natural immunity is an important part of the vaccine development process. To this end, the aim of this thesis was to describe the natural development of salivary antibodies to Pnc protein and PS antigens in relation to Pnc carriage and AOM. The specific objectives were:

- to determine the natural development of salivary IgA antibodies to three Pnc protein antigens (PsaA, Ply and PspA) and six Pnc capsular PS antigens (types 1, 6B, 11A, 14, 19F and 23F) in children by age, and in relation to Pnc carriage and Pnc AOM (I, II).
- to confirm the secretory origin of natural salivary IgA antibodies (I, II).
- to study the subclass distribution of natural salivary IgA antibodies to one Pnc protein (PsaA) and one Pnc PS antigen (capsular PS of type 14) (III).
- to evaluate if natural salivary antibodies to PsaA, PspA and Ply affect the risk of subsequent Pnc carriage and Pnc AOM (IV).

MATERIALS AND METHODS

1. Study cohort and samples

1.1. Study design and subjects

This thesis consists of four studies. Studies I and II describe the natural development of salivary antibodies to three Pnc protein and six Pnc PS antigens after Pnc carriage or Pnc AOM in children of the FinOM Cohort Study. Study III describes the IgA1 and IgA2 distribution of natural salivary anti-protein and anti-PS antibodies. Study IV evaluates the associations of salivary anti-PspA and -PsaA antibodies with the risk of subsequent Pnc carriage and AOM.

The FinOM Cohort Study was initially designed to examine the natural course and epidemiology of Pnc carriage, and the risk factors leading to Pnc carriage and subsequent Pnc AOM. Altogether 329 children were enrolled in the FinOM Cohort Study at two months of age at their second routine visit to the Hervanta child health center, in Tampere, and followed prospectively up until their second birthday. During the study, the children were immunized following the Finnish general vaccination schedule, which included the following vaccines: Bacille Calmette-Guérin (BCG) vaccine against tuberculosis, PDT vaccine against pertussis, diphtheria and tetanus, Hib vaccine against invasive infections caused by *H. influenzae* type b, inactivated poliomyelitis vaccine (IPV) against polio, and MMR vaccine against measles, mumps and rubella. The vaccination schedule does not include any Pnc vaccine. A special study clinic with a study doctor and one to three study nurses was established for the purposes of this study for a time period from April 1994 to July 1997. The children were scheduled to make altogether ten healthy visits to the study clinic at 2, 3, 4, 5, 6, 9, 12, 15, 18 and 24 months of age for an interview and nasopharyngeal swabs (NP) for bacterial culture. The saliva and serum (5 ml of venous blood) samples were collected at the 6 (± 2 weeks), 12 (± 2 weeks), 18 (± 4 weeks) and 24 (± 4 weeks) month healthy visits. In case of respiratory illness or symptoms suggesting AOM, the parents were asked to bring their child to the study clinic and this event was recorded as a sick visit. During the sick visit, the study physician obtained the history of the current illness from the parent, performed a physical examination of the child, and collected a

nasopharyngeal aspirate (NPA) for bacterial culture. In case of AOM, myringotomy with aspiration of the middle ear fluid (MEF) was performed for etiologic diagnosis. AOM was diagnosed, if pneumatic otoscopy suggested effusion in the middle ear cavity and the child had concomitant signs or symptoms of acute infection. The resolution of each AOM was followed four weeks after the diagnosis at a check-up visit.

Saliva samples from 17 healthy adults (15 females and two males, the mean age of 35 years, resident in the Helsinki area) were obtained in order to measure the concentrations of natural antibodies in the saliva of adults.

1.2. Definitions

Acute otitis media (AOM) was defined as a visually abnormal tympanic membrane with regard to color, position and/or mobility suggesting middle ear effusion, with at least one of the following symptoms of acute infection: fever, earache, tugging at or rubbing of the ear, irritability, restless sleep, loss of appetite, acute gastrointestinal symptoms or other symptoms of respiratory infection.

Any Pnc contact was defined as any NP, NPA and/or MEF culture positive for *S. pneumoniae.*

Pnc AOM was defined as an AOM event with *S. pneumoniae* cultured from at least one MEF sample.

Asymptomatic Pnc carriage was defined as NP culture obtained at the 6, 12, 18 or 24 months' healthy visit positive for *S. pneumoniae*.

Pnc carriage without Pnc AOM was defined as NP or NPA but not concomitant MEF culture (if obtained) positive for *S. pneumoniae.*

1.3. Subsets of the Finnish Otitis Media (FinOM) Cohort Study children

Due to the fact that not all four saliva samples were available from each child, the number of children varied from one time point to another. Furthermore, because of the small volume of some of the saliva samples, we could not analyze concentrations of all antibody specificities in all of the samples.

<u>*Study I*</u>: The concentration of IgA antibodies against PsaA, Ply and PspA were measured at 6, 12, 18 and 24 months of age in all available saliva samples. In addition, anti-PsaA, -Ply and -PspA IgA concentrations were determined in the saliva of 17

adults. The concentration of sIg antibodies against PsaA, Ply and PspA were measured in a subcohort of 168 children. The anti-PsaA, -Ply and -PspA IgG was determined in the saliva samples of 104 children and 16 adults.

To evaluate the association between previous Pnc history and the development of salivary IgA antibodies, the children were divided at each time point into two categories on the basis of the NP, NPA or MEF cultures positive for *S. pneumoniae* on healthy or sick visits before the indicated age. The antibody concentrations were compared between children with and without culture confirmed Pnc findings. The categories were as follows:

1. Pnc-negative children (Pnc-): children with no NP, NPA, or MEF cultures positive for *S. pneumoniae* on healthy or sick visits up to the age in question.
2. Pnc-positive children (Pnc+): children with one or more NP, NPA, or MEF cultures positive for *S. pneumoniae* on healthy or sick visits up to the age in question. The Pnc+ children were further divided into two groups according to the type of Pnc contact they had experienced by that age: *Pnc+ carrier children* had one or more NP cultures (but no NPA or MEF cultures) positive for *S. pneumoniae* up to the time point, and *Pnc+ sick children* had one or more NPA or MEF cultures (regardless of the result of NP cultures) positive for *S. pneumoniae* up to the time point.

The category of the child could change from one time point to another from a Pnc- to a Pnc+ category or from the Pnc+ carrier category to the Pnc+ sick category according to the Pnc culture findings, but never vice versa.

Study II: The concentration of IgA antibodies against six Pnc capsular PSs were measured in subgroups of the FinOM Cohort Study saliva samples at 6, 12, 18 and 24 months of age. Anti-1, anti-6B, anti-11A, anti-14, anti-19F and anti-23F IgA were analyzed from the saliva samples of 87, 241, 99, 225, 246, and 93 children, respectively. The types 6B, 14 and 19F were prioritized on the grounds of results from our earlier studies. In addition, anti-1, anti-6B and anti-14 IgA concentrations were determined in the saliva of 17 adults. The concentrations of anti-6B, anti-14 and anti-19F sIg antibodies were measured in a subcohort of 168 children. The anti-1, anti-6B and anti-14 IgG were determined in saliva of 51 children and anti-11A, anti-19F and anti-23F IgG in saliva of another 59 children. In addition, anti-1, anti-6B and anti-14 IgG concentrations were measured in the saliva of 17 adults.

To evaluate the association between previous Pnc history and salivary anti-Pnc PS production, we analyzed the data at each time point in three categories, according to the Pnc culture results before the indicated age. When analyzing type-specific antibody data, the categories were established separately for each of the Pnc types 1, 6B, 11A, 14, 19F and 23F. The categories were as follows:

1. Pnc-negative children (Pnc-): children with no NP, NPA or MEF cultures positive for any Pnc type up to the age in question (this was a common category, regardless of which serotype antibodies were analyzed).
2. Pnc-positive, serotype negative children (Pnc+ ST-): children with at least one NP, NPA or MEF culture positive for *S. pneumoniae* up to the age in question, but the Pnc type(s) did not include the type being analyzed. Thus, six different Pnc+ ST- categories were formed: Pnc+ 1-, Pnc+ 6B-, Pnc+ 11-, Pnc+ 14-, Pnc+ 19F- or Pnc+ 23F-.
3. Pnc-positive, serotype-positive children (Pnc+ ST+): children with at least one NP, NPA or MEF culture positive for *S. pneumoniae* up to the age in question; the cultured Pnc types included the type being analyzed. Thus, five different Pnc+ ST+ categories were formed: Pnc+ 6B+, Pnc+ 11+, Pnc+ 14+, Pnc+ 19F+, Pnc+ 23F+ (Pnc type 1 was not found in any of the FinOM Cohort Study samples).

The category of the child could change from one time point to another from Pnc- to either one of the Pnc+ categories or from Pnc+ ST- category to the Pnc+ ST+ category according to the Pnc culture findings, but never vice versa.

Study III: The IgA subclass distribution of natural anti-PsaA and anti-PS14 antibodies was analyzed in 39 saliva samples found to contain anti-PsaA (38/39) and/or anti-PS14 (32/39) IgA antibodies in previous measurements (I, II).

Study IV: The association between the presence of salivary antibodies against PspA and PsaA, and the risk of subsequent Pnc carriage and AOM was evaluated in all children with saliva samples obtained at 6, 12 and 18 months of age.

1.4. Clinical samples

Clinical samples were collected as described in detail previously (Rapola *et al.* 2000; Kilpi *et al.* 2001; Soininen *et al.* 2001; Syrjänen *et al.* 2001). The NP swabs were

obtained by the study nurses through a nostril by using a sterile swab with a flexible aluminum wire shaft and a dry calcium alginate tip (Galgiswab; Spectrum Laboratories, Dallas, TX). The NPAs were obtained with a sterile pediatric mucus extractor (UNO sterile EtO; UnoPlast A/S, Hundested, Denmark) by guiding the catheter through the nostril to the nasopharynx and applying a gentle suction with an electric suction device. The study doctor performed myringotomy to all children with AOM to confirm the diagnosis. Prior to myringotomy, the external ear canal was cleaned and the tympanic membrane was anesthetized with 70% liquid phenol. A myringotomy knife was inserted through the tympanic membrane, and MEF was aspirated with an electronic suction apparatus into a sterile collector. From there the fluid was rinsed into a polypropylene microtube containing 0.5 to 1ml of phosphate-buffered saline (PBS). For the antibody measurements, unstimulated saliva samples and venous blood samples (5ml) were collected. The saliva samples were collected by placing a plastic pipette in the cheek area and applying a gentle suction. The saliva samples were immediately frozen and stored at -70°C for further analysis. Samples were thawed only once (exception: study III) and centrifuged at 15 000 rpm for 10 minutes prior to assays. The supernatants were used for the measurement of antibodies.

2. Bacteriological methods

The NP, NPA and MEF samples were collected for the detection of Pnc carriage and the diagnosis of the etiology of AOM. They were immediately plated on enriched chocolate agar and selective sheep blood agar (containing 5μg gentamicin) plates, which were incubated overnight at the study clinic at +36°C with 5% CO_2. On the next day, the plates were transported to the Laboratory of Bacterial Respiratory Infections at KTL, in Oulu, where they were further incubated for 24 hours. Identification of pneumococcus was carried out by using standard methods (Kilpi *et al.* 2001). Serotyping of Pnc isolates was performed by counterimmunoelectrophoresis (CIEP) or, for neutral serogroup 7 or serotype 14, by latex agglutination. The uncertain results were confirmed with the capsular swelling test (Quellung test). All antiserum pools and group- or type-specific antisera for serotyping, as well as factor antisera for subtyping within groups 6, 9, 18, 19 and 23, were purchased from Statens Seruminstitut (Copenhagen, Denmark).

3. Serological methods

3.1. Protein antigens (I)

Certain practicalities dictated in the beginning of this study which Pnc protein antigens were selected for the measurement of salivary antibodies. On the grounds of information at that moment, PsaA, Ply and PspA were considered as the most important Pnc protein vaccine candidates. Natural antibodies to these three proteins had never been analyzed in saliva samples earlier. Furthermore, these antigens were readily available for the purposes of this study.

3.1.1. Pneumococcal surface adhesin A (PsaA)

The recombinant PsaA antigen prepared with the Qiaexpress^{TM}-system (Qiagen, Inc. Chatsworth, CA) was kindly provided by Drs Jacquelyn Sampson and Edwin Ades, CDC, Atlanta, Georgia. The expression host *Escherichia coli* SG 13009 (pREP4) was transformed with pAB247, which is the recombinant plasmid carrying *psaA* gene from the strain D39 of serotype 2 cloned into pQE30. The His-tagged recombinant PsaA was purified by Ni-NTA chromatography as described previously (Pilling *et al.* 1998).

3.1.2. Pneumolysin (Ply)

The recombinant Ply antigen was a kind gift from Prof. James C. Paton, Department of Molecular Biosciences, Adelaide University, Adelaide, Australia. The Ply antigen was a derivative of Ply with a Trp433-Phe mutation, which reduces the hemolytic activity without affecting antigenicity. This pneumolysoid antigen was purified from the recombinant *E. coli* as described previously (Paton *et al.* 1991).

3.1.3. Pneumococcal surface protein A (PspA)

The recombinant PspA product representing the 315-amino-acid amino-terminal half of the Rx1 PspA was used. It had been produced in *E. coli* by Aventis-Pasteur (Swiftwater, PA). PspA is variable in structure, but different PspAs share cross-protective epitopes. By the time of the antibody measurements of this study, the 315-amino-acid fragment of Rx1 was believed to contain the sufficient epitopes for cross-reactivity of antibodies produced against different PspAs (Briles *et al.* 1996a; Tart *et al.* 1996), as well as the protection-eliciting epitopes (McDaniel *et al.* 1994).

3.2. Polysaccharide antigens (II)

Pnc serotypes 6B, 11A, 14, 19F and 23F were found frequently in both carriage and AOM in the study cohort. Therefore, the capsular PSs of these Pnc types were selected for the measurement of natural salivary antibodies. The Pnc type 1 was not detected in any of the bacterial cultures during the study. Capsular PS of this Pnc type was included as a control PS antigen in the antibody measurements.

Pnc capsular PSs of types 1, 11A, 14, 19F and 23F were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Capsular PS of serotype 6B was received via collaboration with The National Institute of Public Health and the Environment (Bilthoven, The Netherlands). The CPS antigen used for absorbing anti-CPS antibodies from the saliva samples was obtained from Statens Seruminstitut (Copenhagen, Denmark).

3.3. Enzymeimmunoassays (EIA)

3.3.1. IgA specific assay (I, II)

The salivary IgA antibodies against PsaA, Ply and PspA, and the Pnc PSs of types 1, 6B, 11A, 14, 19F and 23F were measured by EIA. The microtiter plates (Costar 3591, Cambridge, MD) were coated with Pnc proteins in concentrations of 5μg of PsaA and Ply, and 150ng of PspA in 1ml of PBS by incubating the PsaA and PspA plates overnight at +4°C, and the Ply plate overnight at +37°C. For the Pnc PSs, the coating concentrations of 5-10μg in 1ml of PBS were used and the plates were incubated overnight at +22°C. The plates coated with PBS only were used as blank plates for each sample for subtraction of the non-specific binding. The PBS-blank plates were treated in the same way as the antigen plates during the assay. The plates were washed between each step four times with PBS containing 0.05% Tween 20 (Tween-PBS) by SkanWasher 300 (Skatron Instruments, Norway), except before the substrate, when they were washed three times with Tween-PBS and twice with distilled water. The plates were blocked with 10% fetal bovine serum (FBS; Gibco, BRL, Karlsruhe, Germany) in PBS (FBS-PBS) by incubating for 1 hour at +37°C. FBS-PBS was further used as a dilution buffer for the saliva samples, the monoclonal and the polyclonal antibodies. The saliva samples were diluted 1:10 and analyzed in triplicate. The saliva samples were thawed only once and thus none of the assays could be repeated (exception: study

III). The diluted samples and reagents were pipetted 50µl per well. Monoclonal antibodies to human Igs were used to determine specific IgA (M26012; Bionostics, Bedford, MA and Skybio, Bedfordshire, UK). The incubations of samples and monoclonal anti-human IgA antibodies were performed on a horizontal rotator (200rpm) for 2 hours at room temperature (RT). The polyclonal alkaline phosphatase-conjugated rabbit anti-mouse IgG antibodies (H&L 315-055-045, Jackson Immunoresearch Laboratories, West Grove, PA) were incubated without rotation overnight at +22°C. The substrate solution containing 1mg of *p*-nitrofenyl phosphate disodium (Sigma104®; Sigma Immuno Chemicals, St. Louis, MO) in 1ml carbonate buffer, pH 9.8, was pipetted 50µl per well and the plates were incubated for 1 hour at +37°C. The optical densities (OD) were measured on the 405nm wavelength with an EIA-reader (Titertek; Labsystems, Helsinki, Finland). The OD value of each well on the PBS-blank plate was subtracted from that on the plate coated with the antigen.

An OD value of 0.05 for anti-PsaA, 0.04 for anti-Ply and 0.03 for anti-PspA was set up as a cut off limit for positivity. These cut off limits were calculated from the OD readings given by the blank wells on the plates coated with the antigen (>2 x SD of daily assays). The results were expressed as units of IgA per ml of saliva (U/ml) calculated by comparing the mean OD readings of sample triplicates to a reference curve drawn on the basis of OD values given by the serial dilution of the in house reference serum (pool of adult serum). The reference serum was assigned to contain 100 U/ml of IgA anti-PsaA, anti-Ply and anti-PspA antibodies. The cut off OD values corresponded to the concentrations of 0.20 U/ml for anti-PsaA and 0.16 U/ml for anti-Ply and -PspA. If the concentration of the sample was less, the concentration was assigned as one half of the cut off concentration.

An OD value of 0.03 for anti-1, anti-11A, anti-19F and anti-23F, and 0.04 for anti-6B and anti-14 was set up as a cut off limit for positivity (the cut off limit calculated as described above). The results were expressed as ng of IgA to Pnc PS in 1 ml of saliva (ng/ml) calculated on the basis of the officially assigned IgA concentrations in the 89-SF reference serum (Quataert *et al.* 1995, 1998). The cut off OD readings corresponded to the concentrations of 1.7 ng/ml for anti-1, 1.2 ng/ml for anti-6B, 1.2 ng/ml for anti-11A, 1.7 ng/ml for anti-14, 1.5 ng/ml for anti-19F and 1.0 ng/ml for anti-23F antibodies.

If the concentration was less, the concentration was assigned as one half of the cut off concentration.

The day-to-day variation of the assay was followed by including into every daily assay an adult saliva sample containing antibodies to all used antigens. The coefficient of variations (CV) for anti-PsaA, anti-Ply and anti-PspA were 22, 27 and 19, respectively. The CVs for the anti-1, anti-6B, anti-11A, anti-14, anti-19F and anti-23F were 30, 26, 22, 24, 27 and 26, respectively.

3.3.2. sIg specific assay (I, II)

The sIg antibodies against PsaA, PspA, Ply, and Pnc PSs of types 6B, 14 and 19F were analyzed by determining the presence of SC in the anti-PsaA, -Ply, -PspA, -6B, -14 and -19F in a subpopulation of 168 children by EIA as described above. Monoclonal antibody to human SC (I-6635, Sigma) was used. The use of this monoclonal antibody was expected to reflect concentration of the predominant secretory Ig isotype, sIgA, in the saliva samples. The concentrations of possible salivary sIgM antibodies were presumed to be marginal. An OD value of 0.03 was set up as a cut off limit for positivity for all antibody specificities. Results were expressed as OD values. If the OD value of the sample was less than the cut off limit, it was assigned as one half of the cut off limit. The day-to-day variation of the sIg specific assay was followed with an adult saliva sample. The CVs for anti-PsaA, -Ply and -PspA were 19, 28 and 19, respectively. The CVs for the anti-6B, anti-14 and anti-19F were 26, 24, and 27, respectively.

3.3.3. IgG specific assay (I, II)

The IgG antibodies against PsaA, Ply and PspA, and Pnc PSs of types 1, 6B, 11A, 14, 19F and 23F were measured by EIA as described earlier (Käyhty *et al.* 1995) with one exception: the alkaline phosphatase-conjugated anti-human IgG (A3188, Sigma Immuno Chemicals, St Louis) was used. An OD value of 0.03 for anti-PsaA, -Ply and -PspA was set up as a cut off limit for positivity. An OD value of 0.02 for anti-1 and anti-6B, 0.03 for anti-11A, anti-14 and anti-23F, and 0.01 for anti-19F antibodies was set up as the cut off limit. Serum IgG anti-Pnc PS antibodies had been determined at the same ages (Soininen *et al.* 2001).

3.3.4. IgA and IgA subclass specific assay (III)

The IgA, IgA1 and IgA2 antibodies against PsaA and Pnc PS14 were measured by EIA as described above. The monoclonal antibodies to human IgA (M26012; Skybio), IgA1 and IgA2 (A89-036 and A89-038, Nordic Immunological Laboratories, Tilburg, The Netherlands) were used. An OD ≥0.04 for all measurements was considered to be positive. Samples with undetectable anti-PsaA or anti-PS14 IgA1 or IgA2 were assigned a value equivalent to half of the detection limit. The results are given as OD-units (OD x 1000), which were calculated from the mean OD readings of triplicate samples after subtracting the OD readings of the PBS blank plates.

4. Statistical methods

Studies I and II: The concentrations of IgA and sIg antibodies were reported as geometric mean concentrations (GMC) and geometric mean (GM) OD readings, respectively. Log-transformed data was used in the analyses of antibody concentrations. Statistical comparisons of antibody concentrations were performed using Student's *t* test and one-way analysis of variance (ANOVA), followed by a post hoc test (Tukey honestly significant difference test) when appropriate (I, II). Yates's corrected chi square-test or Fisher's two-tailed exact test were used when proportions of variables were compared (I, II). Pearson's correlation was used to evaluate the correlation between the antibody concentrations of different specificities (I), and the IgA- and sIg-specific assays (I, II). P values of less than 0.05 were considered to be statistically significant.

Study III: The results were reported as OD-units. Pearson's correlation was used to evaluate the correlation between the OD-unit values of previous and present IgA measurements, and the correlation between IgA and the sum of IgA1 and IgA2 concentrations. Wilcoxon signed rank test was used to compare the GMCs of IgA1 and IgA2 antibodies to PsaA and PS14 and the GMs of the anti-PsaA and anti-PS14 IgA1/IgA2 ratios. P values of less than 0.05 were considered to be statistically significant.

Study IV: The results were examined by using dichotomous antibody variables: the anti-PspA and -PsaA concentrations going under the detection limit were denoted 0, and the concentrations exceeding the detection limit were denoted 1. Logistic regression models were used to evaluate the presence of salivary anti-PspA and anti-PsaA antibodies as the risk factors for asymptomatic Pnc carriage. Cross-tabulation was used in the preliminary analysis of association between the presence of salivary anti-PspA and -PsaA and Pnc AOM. Yates's corrected chi square test or Fisher's two-tailed exact test were used to compare the proportions of the individuals with Pnc AOM. An extended version of the Cox proportional hazard model was used to estimate the risk of Pnc AOM in the age intervals from 6 to 12 months, from 12 to 18 months, and from 18 to 24 months in relation to the presence of anti-PspA and -PsaA IgA antibodies in saliva at the beginning of each age interval (that is: at 6, 12 and 18 months of age). This gave the average effect of the covariate on Pnc AOM episodes. In univariate analysis, the presence of salivary anti-PspA and anti-PsaA antibodies was used as a covariate modifying the individual risk of Pnc AOM. In multivariate analysis, besides the presence of salivary antibodies, the information on previous Pnc exposure before the indicated time period, i.e. any Pnc contact, Pnc carriage without Pnc AOM, and Pnc AOM, were used as covariates modifying the individual risk of Pnc AOM. P values of less than 0.05 were considered to be statistically significant.

5. Approval of Ethics Committees

The FinOM Cohort Study protocol and consent form were evaluated and approved prior to the start of the trial by the Ethics Committees of the National Public Health Institute (KTL), Tampere University Hospital, and the Department of Social and Health Care of Tampere City. Written informed consent was obtained at the time of enrollment from the parents of all children participating in the FinOM Cohort Study.

RESULTS

1. Natural development of salivary antibodies to pneumococcal protein and polysaccharide antigens in relation to age and pneumococcal contacts (I, II)

1.1. Development of salivary antibodies by age

1.1.1. PsaA, Ply and PspA

Antibody concentrations to the three Pnc protein vaccine candidates PsaA, Ply and PspA were analyzed in saliva samples collected during scheduled healthy visits at the ages of 6, 12, 18 and 24 months (I). Natural IgA antibodies to Pnc protein antigens were detected already in the saliva of infants at six months of age and the antibody production increased with age. This was indicated by the increase in the proportion of antibody positive samples and in the GMCs of anti-PsaA, -Ply and -PspA IgA. The kinetics of antibody production to distinct protein antigens was slightly different. At six months of age anti-PsaA and -Ply IgA was found in the saliva of 57% and 85% of the children, respectively, whereas anti-PspA IgA was found in the saliva of only 9% of the children (Table 1). By the age of 24 months, 74% of the children had IgA antibodies to PsaA and 93% to Ply, but still only 23% to PspA. In adults, salivary anti-PsaA and anti-Ply IgA were detected in all individuals and anti-PspA in 88%.

Further, an age-dependent increase was seen in the GMCs of the salivary anti-PsaA, -Ply and -PspA IgA (Table 1). Like the GMCs, the medians of the salivary anti-PsaA and -Ply IgA concentrations increased by age. Overall, the antibody concentrations remained lower than in adults (Table 1; I: Figure 1). The variation in the antibody concentration between individuals was broad both in children and adults. The majority of the anti-PspA values in the children were below the detection limit.

The anti-PsaA, -Ply and -PspA IgG antibody concentrations were measured in saliva samples of 104 children and 16 adults. IgG to only Ply, but not to PsaA or PspA, was detected in the saliva of children and adults. In children, the proportion of saliva samples positive for anti-Ply IgG decreased steadily by age: at the age of 6 months approximately 90% of children had measurable IgG anti-Ply in their saliva, while at 24

months of age the proportion of positive samples was approximately 60% (data not shown). Less than one half of the adults (7/16) had anti-Ply IgG in their saliva. The correlation between salivary and serum anti-Ply IgG antibodies was evaluated from saliva and serum samples of the children. No correlation was detected between salivary and serum anti-Ply IgG (data not shown). In approximately one third of the cases, the serum sample didn't contain a measurable concentration of anti-Ply IgG, while anti-Ply IgG was detected in the saliva sample collected at the same age.

1.1.2. Pneumococcal polysaccharides of types 1, 6B, 11A, 14, 19F and 23F

Antibody concentrations to the five most common Pnc serotypes/groups (6B, 11A, 14, 19F and 23F) cultured from the clinical samples (NP, NPA and/or MEF samples) in the study population were analyzed in saliva samples collected during scheduled healthy visits at the ages of 6, 12, 18 and 24 months (II). In addition, a serotype that was not detected in any of the clinical samples was included in the antibody measurements (type 1).

As with Pnc protein antigens, natural IgA antibodies to Pnc capsular PSs were detected already at six months of age and the antibody production increased by age. The kinetics of anti-6B production was different from the other serotypes: at six months of age anti-6B IgA antibodies were already found in the saliva of 62% of the children, whereas anti-1, anti-14 and anti-19F IgA were each found in approximately 20%, and anti-11A and anti-23F IgA in approximately 10% of the samples (Table 1). The proportion of positive samples increased by age for all serotypes: at the age of 24 months positive findings ranged from 45 to 78% of the samples. Among the adult samples, ten out of 17 (59%) were positive for anti-1 and the majority for anti-6B or anti-14 IgA (both 94%). Correspondingly, the GMCs of anti-Pnc-PS IgA increased with age throughout the follow-up (Table 1). The only exception was anti-6B, which was found often already at 6 months of age and whose concentration increased modestly only between the ages of 6 and 12 months.

The saliva samples of 51 children and 17 adults were analyzed for anti-1, anti-6B and anti-14 IgG. The saliva samples of another 59 children were analyzed for anti-11A, anti-19F and anti-23F IgG. None of the studied samples of children contained anti-1, anti-6B or anti-19F IgG antibodies. Salivary IgG antibodies to the serotypes 11A, 14 and 23F

were found in 2-8% of the samples in children. The IgG concentrations in the respective serum samples were examined, but no relation between the concentrations of salivary and serum IgG was detected. None of the 17 saliva samples from adults contained detectable anti-1 or anti-6B IgG, but 2 samples had anti-14 IgG.

1.1.3. Maturation of mucosal immunity

Several studies have reported that the sIgA system would have a rapid maturation pattern. In the present study, the GMCs of anti-PsaA, -Ply, -PspA, -1, -6B, and -14 did not reach the antibody concentrations detected in adults by the age of 24 months despite the increase in antibody concentrations by age (Table 1).

Table 1. The proportions of samples with detectable salivary anti-PsaA, -PspA, -Ply, -1, -6B, -11A, -14, -19F and -23F IgA antibodies (% positive) and the geometric mean anti-PsaA, -PspA, -Ply, -1, -6B, -11A, -14, -19F and -23F IgA concentrations (GMC; ng/ml) with age in all children and adults.

Antigen	N	6mo		12mo		18mo		24mo		Adults	
		% positive	GMC, ng/ml	% positive	GMC, ng/ml	% positive	GMC, ng/ml	% positive	GMC, ng/ml	% positive	GMC, ng/ml
PsaA	260-300	57	0.33	57	0.40	70	0.63	74	0.62	100	1.96
Ply	260-300	85	0.77	89	0.91	92	1.71	93	2.01	100	6.11
PspA	235-272	9	0.10	14	0.11	22	0.13	23	0.13	88	0.79
1	74-87	20	1.19	43	1.69	45	2.05	59	2.14	59	2.89
6B	200-241	62	2.24	70	2.71	71	2.60	73	2.60	94	3.72
11A	86-99	10	0.72	52	1.38	70	2.75	67	2.80		
14	189-225	21	1.31	28	1.49	34	1.96	45	2.46	94	17.40
19F	205-246	20	1.04	58	2.32	72	4.11	78	4.88		
23F	80-93	8	0.62	40	0.92	56	1.56	60	1.73		

1.2. Development of salivary antibodies in relation to pneumococcal contacts

To evaluate the relationship between Pnc contacts and salivary IgA production, the children were grouped at each time point into two or three categories according to their Pnc culture findings before the indicated age. When analyzing the salivary antibodies to Pnc protein and PS antigens two (Pnc- and Pnc+ children) and three categories (Pnc-, Pnc+ ST- and Pnc+ ST+ children) were used, respectively.

1.2.1. PsaA, Ply and PspA

The increase of antibody production by age was associated with the previous Pnc contacts of the children. The respective proportions of the anti-PsaA, -Ply and -PspA IgA positive samples were at all ages higher in the Pnc+ than in the Pnc- children (I: Table 1). The proportion of the anti-PsaA positive samples in the Pnc+ children was at all ages approximately 1.5–2 times greater than in the Pnc- children ($P<0.001$ for all comparisons). The proportion of the anti-Ply positive samples was at 12 and 18 months higher in the Pnc+ children when compared to Pnc- children ($P<0.01$ at 18 months of age) (I: Table 1). The proportion of the anti-PspA positive samples increased steadily with age in the Pnc+ children, but remained the same or decreased in the Pnc- children. At six months of age the proportion of the anti-PspA positive samples was two times greater in the Pnc+ than in the Pnc- children ($P<0.05$) and at 24 months of age the difference between these categories was already eight-fold ($P<0.001$).

The respective GMCs of anti-PsaA, -Ply and -PspA IgA antibodies were at all ages significantly higher in the Pnc+ than in the Pnc- children (I: Table1). The GMCs of anti-PsaA were at all ages three to four times higher in the Pnc+ than in the Pnc- children ($P<0.001$ for all comparisons). Among the Pnc- children the GMCs remained close to the detection limit. The GMC of anti-Ply increased both in the Pnc+ and Pnc- children, but in the former category the increase was more pronounced. The GMC of anti-Ply was significantly higher in the Pnc+ than in the Pnc- children at all ages ($P<0.05–0.001$). The GMC of anti-PspA increased slightly with age in the Pnc+ children and remained constant or decreased in the Pnc- children, the difference between the two categories being significant at all ages ($P<0.05–0 .001$).

The association between the detected Pnc+ culture findings and salivary anti-PsaA, -Ply and -PspA concentrations in selected individuals has been illustrated in Figure 3 of study I. In the Pnc- children (*Panel A*), the antibody concentrations mainly remained unchanged or decreased during the follow-up. In the Pnc+ children (*Panel B*), a culture finding positive for pneumococcus was associated with a salivary antibody response in many, but not in all cases. The kinetics of the anti-PspA production differed from the production of anti-PsaA and -Ply antibodies, which seemed to occur often simultaneously and with similar intensity.

1.2.2. Pneumococcal polysaccharides of types 1, 6B, 11A, 14, 19F and 23F

Children with a previous positive culture(s) for the indicated serotype (Pnc+ ST+; note that for type 1 there was no Pnc+ ST+ children) had a larger proportion of saliva samples positive for antibodies than the children without known previous Pnc contacts (Pnc-) or the children with positive cultures for other Pnc types than the one being analyzed (Pnc+ ST-). By the age of 24 months saliva samples positive for anti-6B, anti-11A, anti-14, anti-19F and anti-23F IgA antibodies were found for 88%, 93% 100%, 89% and 74% of the Pnc+ ST+ children, respectively. The corresponding proportions for anti-6B, anti-11A, anti-14, anti-19F and anti-23F IgA among the Pnc+ ST- children were 69%, 64%, 37%, 75% and 51% (respectively) and among the Pnc- children 58%, 63%, 30%, 62% and 67% (respectively). The proportions of anti-6B, anti-14 and anti-19F IgA were significantly higher in the Pnc+ ST+ than in the Pnc- and Pnc+ ST- children ($P<0.05$). The proportions of anti-1 IgA antibodies in the Pnc+ ST- and Pnc- children were equal: 57% and 56%, respectively.

The mean antibody concentrations in the Pnc-, Pnc+ ST- and Pnc+ ST+ children have been plotted in Figure 1 of study II. For all serotypes examined, the GMC in the Pnc+ ST+ category was at most, if not all, ages significantly higher than in the Pnc- category (II: Figure 1). The difference was most notable for serotypes 11A and 14 (at all ages), and weakest for serotype 23F. In addition, for some serotypes (6B, 11A, 19F) the antibody concentrations were modestly higher in the Pnc+ ST- category than in the Pnc- category.

The effect of contacts with the cross-reacting types of serogroup 6 and 19 on salivary anti-6B and -19F concentrations was also evaluated. The contacts with the cross-reacting types 6A and 19A did have an effect on the anti-6B and anti-19F concentrations, respectively: a clear tendency of higher concentrations in Pnc+ 6A+ 6B- children was seen when compared to Pnc- and Pnc+ 6A- 6B- children, and in Pnc+ 19A+ 19F- children when compared to Pnc- and Pnc+ 19A- 19F- children (II: Figure 2).

2. The origin of salivary IgA (I, II)

To ascertain that the salivary antibodies to the Pnc protein and PS antigens were locally produced, the correlations between the IgA and the presence of SC in the anti-PsaA, -Ply, -PspA, -6B, -14 and -19F were determined at 6, 12, 18 and 24 months of age in a subpopulation of 168 children. The correlations were highly significant at all ages ($P<0.01$ for all comparisons) indicating that the origin of IgA was secretory. The correlations between salivary anti-PsaA, -Ply and -PspA IgA and SC, and between salivary anti-6B, -14 and -19F IgA and SC have been illustrated in figure 2 of study I and in figure 4 of study II, respectively.

3. The salivary IgA1 and IgA2 antibodies in relation to the antigen's nature (III)

To evaluate the potential effect of the antigen's nature on the IgA subclass distribution (III), IgA1 and IgA2 antibodies were analyzed in saliva samples found to contain anti-PsaA and/or -PS14 IgA in the previous IgA measurements (I, II). Anti-PsaA and anti-PS14 IgA antibodies were re-measured in the present study, and the results correlated well with the previous measurements ($r=0.87$ and $r=0.84$, respectively). Saliva samples found to contain anti-PsaA and/or anti-PS14 were available from 39 children. Anti-PsaA IgA was detected in 38 saliva samples, of which 3, 7, 11 and 17 samples were collected at 6, 12, 18 and 24 months of age, respectively. Anti-PS14 IgA was detected in 32 saliva samples, of which 4, 7, 8 and 13 samples were collected at 6, 12, 18 and 24 months of age, respectively.

Since the anti-PsaA and anti-PS14 IgA1/IgA2 ratios were not dependent on age, the results at different ages were combined. For both antigens, IgA1 was the predominant subclass. Anti-PsaA IgA1 was found in 97% and anti-PS14 IgA1 in 88% of the samples, whereas IgA2 to PsaA and PS14 was found in 47% and 56% of the samples, respectively (III: Table 1). Salivary IgA1 and IgA2 antibodies to both PsaA and PS14 were found in 26 samples.

The IgA1 and IgA2 concentrations were given semiquantitatively as arbitrary OD-units based on OD-values. To see if it was appropriate to compare the OD-units of IgA1 and

IgA2 and calculate their ratios, the sum of the OD-units of anti-PsaA and -PS14 IgA1 and IgA2 of each sample was correlated with the corresponding value of specific IgA (III: Figure 1). Irrespective of the proportions of the subclasses in the individual samples, there was a significant linear correlation between the sum of OD-units of specific IgA1 and IgA2 and anti-PsaA and -PS14 total IgA (r = 0.98 and r = 0.97, respectively). This allowed us to compare the IgA1 and IgA2 concentrations.

The salivary anti-PS14 IgA2 concentrations seemed to be relatively higher than those for anti-PsaA IgA2. This was seen both in higher median anti-PS14 IgA2 (50 OD-units) when compared to median anti-PsaA IgA2 (20 OD-units) (III: Figure 2), and in the significantly lower anti-PS14 IgA1/IgA2 ratios when compared to anti-PsaA (III: Figure 3, P=0.001). The proportionally higher anti-PS14 IgA2 concentrations were also seen in 26 individual saliva samples, which were found to contain simultaneously anti-PsaA and anti-PS14 IgA1 and IgA2 antibodies (III: Figure 4). In 20 (77%) of the 26 saliva samples the IgA1/IgA2 ratio was lower for anti-PS14 than for anti-PsaA, which indicated proportionally more anti-PS14 IgA2 than anti-PsaA IgA2.

4. Association between the salivary antibodies to PspA, PsaA and Ply, and the risk of pneumococcal carriage and AOM (IV)

Anti-PspA IgA was detected quite rarely in the saliva samples of the FinOM Cohort Study, while anti-PsaA and -Ply antibodies were found more frequently (I). From the samples collected at 6, 12 and 18 months of age only 9, 14 and 22% were positive for salivary anti-PspA, while 57, 57 and 70% were positive for salivary anti-PsaA, and 85, 89 and 92% were positive for salivary anti-Ply, respectively.

4.1. Prediction of asymptomatic pneumococcal carriage

Logistic regression models were used to evaluate the presence of salivary anti-PspA, -PsaA and -Ply at 6, 12 and 18 months of age as a predictor of asymptomatic Pnc carriage six months later. No sign of protection against Pnc carriage was found. In contrast, the presence of detectable salivary anti-PspA seemed to increase the odds of becoming an asymptomatic Pnc carrier, the result being statistically significant at 18 months of age (OR, odds ratio, 1.44 [95% CI 0.43-4.81], 1.43 [0.63-3.25] and 2.18

[1.08-4.38] at 6, 12 and 18 months, respectively). A similar risk-increasing effect was found with salivary anti-PsaA antibodies as well, the result being statistically significant at 6 and 18 months of age (OR 2.09 [1.13-3.88], 1.59 [0.92-2.75] and 2.03 [1.11-3.74] at 6, 12 and 18 months, respectively). The detectable salivary anti-Ply antibodies seemed to have no effect on the risk of subsequent Pnc carriage (OR 1.06 [0.46-2.46], 0.76 [0.33-1.75] and 1.29 [0.46-3.61] at 6, 12 and 18 months, respectively).

4.2. Prediction of pneumococcal AOM

4.2.1. Preliminary analysis

To characterize the association between salivary anti-PspA, -PsaA and -Ply and subsequent Pnc AOM, the presence or absence of these antibodies at 6, 12 and 18 months of age was cross-tabulated with the occurrence of Pnc AOM during the following six months. Children having anti-PsaA in their saliva at 6 months of age tended to have more often Pnc AOM during the following six months than those without salivary anti-PsaA (P=0.01) (IV: Table 1). At this age, similar tendencies were also seen for anti-PspA (IV: Table 1) and anti-Ply (Table 2), but the results were not statistically significant.

After 12 months of age, however, the association between detectable anti-PspA and the occurrence of subsequent Pnc AOM appeared to differ from that of anti-PsaA and anti-Ply. At 12 and 18 months of age, the Pnc AOM episodes during the following six months were as frequent in children with and without detectable salivary anti-PsaA (IV: Table 1), and more frequent in children with detectable salivary anti-Ply than in those without salivary anti-Ply, though the result was not statistically significant (Table 2). By contrast, children having anti-PspA in their saliva at 12 or 18 months of age seemed to have a lower frequency of Pnc AOM during the following six months (IV: Table 1).

Table 2. Presence of salivary anti-Ply IgA antibodies in children at 6, 12 and 18 months (mo) of age in relation to pneumococcal AOM during the following six months. The proportions of the individuals with Pnc AOM were compared with Yates's corrected chi square test or Fisher's exact test.

		Anti-Ply			
			Pnc AOM next 6 mo		
Age (mo) of sample	Antibody	N	No	Yes	% with Pnc AOM
6	Not detected	41	38	3	7
	Detected	250	214	36	14
12	Not detected	31	29	2	7
	Detected	246	198	48	20
18	Not detected	18	17	1	6
	Detected	238	211	27	11

4.2.2. Univariate analysis using the Cox proportional hazard model

To confirm the associations suggested by the preliminary analysis, an extended version of the Cox proportional hazard model was applied using the presence of salivary anti-PspA, -PsaA and -Ply as explanatory variables. The relative risk (RR) of Pnc AOM was analyzed over three time intervals, from 6 to 12, from 12 to 18 and from 18 to 24 months of age (IV: Table 2; Table 3). In this model, the presence of salivary anti-PsaA antibodies at six months of age was associated with an increased risk of Pnc AOM during the following six months (RR 3.13 [95% CI 1.50-6.55]) (IV: Table 2). Similar, though not statistically significant, tendencies were also seen with salivary anti-PspA (RR 2.06 [0.63-6.75]) (IV: Table 2) and anti-Ply (RR 2.44 [0.77-7.69]) (Table 3) at this age.

At 12 and 18 months of age, detection of salivary anti-PsaA did not appear to have any clear-cut effect on the risk of subsequent Pnc AOM (IV: Table 2). The presence of detectable anti-Ply antibodies at the same ages seemed to be associated with an increased risk of subsequent Pnc AOM (not statistically significant) (Table 3). By contrast, the presence of salivary anti-PspA at 12 and 18 months of age seemed to decrease the risk of subsequent Pnc AOM, the point estimates for RR being as low as

0.29 [0.07-1.16] and 0.16 [0.02-1.13], respectively (IV: Table 2). However, the results did not reach statistical significance.

Table 3. Relative risks (RR) of pneumococcal AOM with 95% confidence intervals (CI) in the uni- and multivariate Cox proportional hazard models determined by the presence of salivary anti-Ply IgA and pneumococcal history in 6-month (mo) intervals.

	Risk of Pnc AOM in the following 6 mo			
	Anti-Ply	Any Pnc contact	Pnc carriage	Pnc AOM
Age (mo) of sample	RR (95% CI)	RR (95% CI)	RR (95% CI)	RR (95% CI)
6 mo	2.44 (0.77, 7.69)			
	2.25 (0.69, 7.38)	3.88 (2.08, 7.23)		
	2.40 (0.76, 7.60)		1.51 (0.75, 3.02)	
	2.31 (0.70, 7.63)			6.73 (3.39, 13.37)
12 mo	3.67 (0.93, 14.49)			
	4.01 (0.99, 16.13)	0.67 (0.38, 1.18)		
	4.38 (1.11, 17.20)		0.32 (0.16, 0.64)	
	3.63 (0.93, 14.10)			1.86 (0.99, 3.51)
18 mo	2.27 (0.32, 16.08)			
	2.03 (0.30, 13.90)	1.39 (0.55, 3.49)		
	2.88 (0.41, 20.30)		0.30 (0.13, 0.73)	
	2.00 (0.27, 14.97)			3.54 (1.71, 7.35)

4.2.3. Multivariate analysis using the Cox proportional hazard model

To find out, whether early exposure to pneumococci could be the underlying factor increasing the risk of Pnc AOM, explanatory variables indicating previous Pnc exposure were included in the subsequent multivariate analyses. Thus, the information on any previous Pnc contact, previous Pnc carriage without Pnc AOM, and previous Pnc AOM were used as covariates besides the presence of salivary antibodies. Inclusion of these factors in the model left, however, the relations between anti-PspA, -PsaA and -Ply and the risk of Pnc AOM essentially unchanged (IV: Table 2; Table 3). The results were comparable to those described earlier in connection with serum anti-PsaA antibodies

(Rapola *et al*. 2003) indicating that previous Pnc AOM increases the risk of subsequent Pnc AOM, while previous Pnc carriage without AOM decreases the risk of Pnc AOM after the age of 12 months.

5. Association between the serum antibodies to PspA and Ply, and the risk of pneumococcal AOM

The relation between the salivary anti-PspA, -PsaA and -Ply antibodies and the risk of subsequent risk of Pnc AOM was described above. In a previous study by Rapola *et al.* (2003), the relation between the serum anti-PsaA concentrations at 6, 12 and 18 months of age and the risk of subsequent Pnc AOM was evaluated. At that time, the results for serum anti-PspA and anti-Ply IgG were not published. To enable the comparison between the salivary and serum findings, the association between the serum anti-PspA and anti-Ply IgG and the risk of subsequent Pnc AOM is described below (unpublished data).

5.1. Prediction of pneumococcal AOM

5.1.1. Univariate analysis using the Cox proportional hazard model

An extended version of the Cox proportional hazard model was applied using the serum anti-PspA and -Ply IgG concentrations (log transformed) at 6, 12 and 18 months as explanatory variables. As in salivary models, the RR of Pnc AOM was analyzed over three time intervals, from 6 to 12, from 12 to 18, and from 18 to 24 months of age. The higher the serum anti-PspA or -Ply IgG concentration at six months of age, the higher was the risk of Pnc AOM during the following six months. More precisely, at six months of age, an increase of one log unit in serum anti-PspA concentration increased the risk of subsequent Pnc AOM by 93% (RR 1.93 [95% CI 1.45-2.56]). Correspondingly, an increase of one log unit in serum anti-Ply concentration increased the risk of subsequent Pnc AOM by 57% (RR 1.57 [95% CI 1.26-1.95]). From the age of 12 months onwards, higher serum anti-PspA or -Ply concentrations either had neutral or slightly decreasing effect on the risk of subsequent Pnc AOM: at 12 and 18 months of age the RRs for serum anti-PspA were 1.03 [95% CI 0.84-1.25] and 0.75 [95% CI 0.52-1.09], respectively. The RR for serum anti-Ply at 12 months of age was 0.97 [95% CI 0.84-1.12] and at 18 months of age 0.86 [95% CI 0.71-1.05].

5.1.2. Multivariate analysis using the Cox proportional hazard model

Inclusion of the information on previous Pnc contacts (any Pnc contact, Pnc carriage without Pnc AOM, and Pnc AOM) in the model left the relations between serum anti-PspA and -Ply, and the risk of subsequent Pnc AOM essentially unchanged (data not shown).

DISCUSSION

1. Study design

This thesis is a summary of four studies (I-IV), which were conducted to evaluate the natural development and role of mucosal immunity against pneumococcus in children in relation to age and Pnc contacts. In studies I and II, the salivary IgA antibodies to several Pnc protein and PS antigens were measured and the origin of these salivary antibodies was addressed. In study III, the distribution of IgA1 and IgA2 antibodies to one Pnc protein and one Pnc PS antigen was determined. In study IV, the relation of salivary anti-protein antibodies to subsequent Pnc carriage and AOM was evaluated.

The FinOM Cohort Study was initially designed to examine the natural course and epidemiology of Pnc carriage, and the risk factors leading to Pnc carriage and subsequent Pnc AOM. Altogether 329 children were followed from 2 to 24 months of age from April 1994 to July 1997 at a special study clinic. The overall compliance in the study was high: 85% (281/329) of children initially enrolled in the study completed the follow-up (Syrjänen *et al.* 2001).

The Pnc cultures in the FinOM Cohort Study were performed on a regular basis at one to six months' intervals, with additional cultures during respiratory infections and AOM episodes. The time between the NP samples was one month at 2 to 6 months of age, 3 months at 6 to 18 months of age, and 6 months at 18 to 24 months of age. During the first 6 months of age, the majority of Pnc carriage was most probably detected. Thereafter, however, some carriage may have gone undetected due to the longer sampling intervals. Furthermore, the bacterial culture methods, though standardized and performed in an expert laboratory, may have failed to detect small numbers of bacteria in the clinical samples. The PCR test for Pnc antigens has been shown to increase the numbers of positive samples compared to conventional bacterial culture methods (Virolainen *et al.* 1994). Thus, the study design makes it possible that some Pnc contacts may have been missed at some time points during the study, which could explain those inexplicable increases in antibody concentrations, which were detected at some occasions in the absence of culture-confirmed Pnc contacts.

Because of the small volume of many of the saliva samples, all antibody specificities could not be analyzed in all of the samples. Antibodies to PsaA, Ply and PspA were prioritized and these antibodies were measured in study I in all available saliva samples. These data were applied in study IV that evaluated the association between salivary antibodies against Pnc proteins and subsequent Pnc carriage and AOM. In study II, the selection of available saliva samples was made when analyzing salivary anti-PS antibodies; the types 6B, 14 and 19F were prioritized on the basis of earlier experience. Thus, anti-1, anti-6B, anti-11A, anti-14, anti-19F and anti-23F IgA concentrations were analyzed from the saliva samples of 87, 241, 99, 225, 246 and 93 children, respectively. Furthermore, in studies I and II, anti-PsaA, -PspA, -Ply, -1, -6B and -14 IgA concentrations were determined in saliva samples of 17 adults. These persons were considered to form a representative sample of the healthy Finnish adult population. In studies I and II, one of the aims was to determine the origin of salivary IgA antibodies, i.e., are these IgA antibodies locally produced or have they transduced from the serum. This was done by determining the presence of SC in salivary anti-PsaA, -Ply, -PspA, -6B, -14 and -19F antibodies in a subcohort of 168 children. These children were picked randomly from the study children and the sample was thus expected to be unbiased. In study III, IgA1, IgA2 and IgA antibodies against PsaA and Pnc capsular PS type 14 were measured in 39 saliva samples known to contain anti-PsaA and/or anti-PS14 IgA antibodies based on previous measurements (I, II). Due to the small number of children in the study IV, the results should be considered descriptive.

To evaluate the relationship between previous Pnc contacts and natural salivary antibody production, the children were grouped into different categories in studies I and II. When analyzing the antibodies to Pnc protein and PS antigens, two and three categories were used, respectively. The children were grouped at each age according to their previous Pnc culture findings before the indicated age, irrespective of the time between the contact and antibody measurement. Thus, a child once assigned, for example, in the Pnc+ or Pnc+ 6B+ category stayed there through the whole follow-up, even if the Pnc contact had been detected in the beginning of it. Therefore, the GMCs of salivary antibodies may have been affected by the decrease of antibodies from the peak concentration over time. Since the onset and duration of Pnc carriage is difficult to define, and because some Pnc carriage may have gone undetected, the antibody concentrations may have differed from peak concentrations before the saliva samples

were collected. However, we did not try to find peak responses but to study the age-specific development of salivary antibodies and to determine associations between the salivary antibody concentrations and the culture-confirmed Pnc contacts.

2. Methodological aspects

In the present study, saliva samples were used as a proxy of the mucosal immunity of the upper respiratory tract and in particular of the nasopharynx. Any studies evaluating the correlation between the antibody concentrations of nasopharyngeal samples and saliva has not been reported to our knowledge. Although antibody activity in the nasopharynx may be more relevant to Pnc, collecting saliva is a noninvasive and easily repeatable sampling method. Because of its easiness, collection of saliva is superior to such methods as nasopharyngeal washes, especially when repeated samples from infants or children are required. The results of this study show clear differences in the concentration of anti-Pnc IgA among exposed and apparently unexposed individuals, which suggests that saliva indeed serves as a proxy for local immune responses. Since the consecutive saliva samples used in the present study were collected at 6, 12, 18 and 24 months of age, it was not possible to evaluate the immediate antibody response to a current Pnc contact, as has been done in studies of serum antibodies of the FinOM Cohort Study (Rapola *et al.* 2001; Soininen *et al.* 2002).

Sample storage and enzymatic digestion have been suggested to have an adverse effect on the concentrations of IgA in saliva samples of some individuals (Brandtzaeg *et al.* 1970). This instability of saliva causes problems in the transport and storage of the samples. The addition of enzyme inhibitors and glycerol, as well as storage of saliva samples at -70°C, have been shown to protect against IgA degradation (Butler *et al.* 1990). However, Nurkka *et al.* (2003) have recently questioned the protective effect of the enzyme inhibitors. In the FinOM Cohort Study the collection of samples was tried to be made as simple as possible, and the addition of preservatives was not found practical. Furthermore, this would have diluted the samples and thus made the detection of low concentrations of natural anti-Pnc antibodies in saliva even more difficult. The saliva samples were immediately put into -70°C and stored at this temperature for further analysis. The samples were principally thawed only once, just before the assays

(exception: III). All these procedures were done to assure minimal degradation of antibodies in the saliva samples.

Some of the saliva samples had to be stored up to three to four years before the measurement of the antibodies. In a study by Cripps *et al.* (1989), the salivary IgA molecules were shown to be stable for periods up to 2.5 years even at -20°C. In addition, repeated freeze-thawing of saliva samples did not cause degradation of sIgA in adult or infant saliva samples. In the present study, an adverse effect of long-term sample storage or freezing-thawing on salivary IgA concentrations was not detected. This was indicated in study III by the significant correlation between the repeated measurements of anti-PsaA and anti-PS14 IgA antibodies with a few years of interval.

An EIA method used for analyzing serum samples (Rapola *et al.* 2000) was modified for the saliva samples on the basis of previous experience (Kauppi *et al.* 1995). The plates coated with PBS were used as background plates to control non-specific binding usual in EIAs analyzing saliva. Because of the heterogeneity of saliva, the samples were analyzed in triplicates, from which the means were calculated. The monoclonal antibody reagents were used to assure the specificity of the assays (Mestecky *et al.* 1996). A good correlation was found between IgA anti-Pnc antibodies and SC in anti-Pnc antibodies (I, II), as well as between IgA and the sum of IgA1 and IgA2 specific for PsaA or PS14 (III).

PspA is a structurally and antigenically highly variable molecule. Different PspA molecules are grouped into three different families on the basis of the relatedness of their nucleotide and amino acid sequences (Hollingshead *et al.* 2000). Over 95% of the PspA molecules typed to date are members of families 1 and 2 (Nabors *et al.* 2000). At the time when the FinOM Cohort Study was started, the different PspA types were considered to be immunologically highly cross-protective, based on studies in animal models (McDaniel *et al.* 1991; Tart *et al.* 1996). Thus, a recombinant PspA fragment from Pnc strain Rx1 representing only family 1 was used as an antigen in the antibody measurements. Since then, it has turned out that cross-protection between different PspA types is minor (Miyaji *et al.* 2002). Our research group has recently measured antibodies in part of the FinOM Cohort Study serum and saliva samples using two different PspA antigens, from families 1 and 2. The results suggest that anti-PspA

responses after Pnc contacts in children are family-specific (Melin *et al.*, data to be published). In study I, measurable amounts of salivary anti-PspA antibodies were detected in only 9 to 23% of the samples, and the anti-PspA concentrations remained low up to the age of 24 months. Thus, if both family 1- and family 2-specific PspA antigens had been used in the original antibody measurements, more anti-PspA positive saliva samples would probably have been detected.

Several reports have stated that a considerable amount of anti-Pnc PS IgG antibodies measured by EIA in serum samples of unimmunized subjects are polyspecific, i.e. these antibodies are reactive also with other than the type-specific PS (Coughlin *et al.* 1998; Yu *et al.* 1999b; Soininen *et al.* 2000). The capsular PS of type 14, however, is an exception: serum anti-14 antibodies measured by EIA seem to be type-specific (Coughlin *et al.* 1998; Soininen *et al.* 2000). The EIA specificity can however be improved when the test sera are absorbed with an irrelevant Pnc capsular PS (Yu *et al.* 1999b; Concepcion *et al.* 2001). Serotype 22F has been chosen for this purpose because the capsular PS of this type is readily available, but is not likely to be included in any future Pnc conjugate vaccine (Concepcion & Frasch 2001). In study II, the specificity of salivary anti-Pnc PS IgA antibodies could be evaluated. The data suggested that the salivary anti-Pnc PS antibodies would be mostly type-specific: the type-specific GMCs showed a clear increase with age in children with documented Pnc contact, but did not increase markedly in the saliva of Pnc+ ST- or Pnc- children. For instance, for Pnc types 6B, 19F and 23F significant differences between children with homologous Pnc contacts (Pnc+ ST+) and children with heterologous Pnc contacts (Pnc+ ST-) were detected in the saliva at several time points (I: Figure 1). This is opposite to serum anti-Pnc PS antibodies (IgG) that seem to be highly polyspecific (Soininen *et al.* 2001). Some of the saliva samples used in this study have been tested previously with 22F-neutralization EIA (unpublished data). The results suggest that the salivary anti-14 IgA antibodies are type-specific, while the salivary anti-6B and anti-19F IgA antibodies in Pnc- children are to some extent polyspecific but in Pnc+ children they are type-specific. This suggests that the 22F-neutralization step would not have a significant influence on the present salivary data.

3. Effect of age on salivary antibody production

3.1. Development of salivary antibody production by age

Natural antibodies to the Pnc protein and PS antigens were detected already in the saliva of infants at six months of age and the antibody production increased by age. This was seen by the increase in the proportion of antibody positive samples and in the GMCs. The kinetics of antibody production to various antigens was slightly different.

3.2. Maturation of mucosal immunity

The sIgA system has been reported to have a rapid maturation pattern in a number of studies, while other studies have suggested a slower maturation for secretory immunity (See: Review of literature; 4.1.2.6. Early maturation of mucosal immune system). Particularly, mucosal immunity to pneumococcus has been suggested to be immature in young children compared to adults. In accordance with this, our results showed that the specific IgA concentrations in saliva samples of children by the age of 24 months did not reach the antibody concentrations detected in adults, in spite of the increase in the salivary anti-PsaA, -Ply, -PspA, -6B, -14 and -19F concentrations with age.

The mucosal immunity to bacterial PSs has been suggested to mature earlier than the systemic immunity. The samples collected during the FinOM Cohort Study (Rapola *et al.* 2000; Soininen *et al.* 2001, I and II) offered us an opportunity to evaluate this issue (See: Discussion; 5. Comparison of the development of salivary and serum antibodies to Pnc protein and PS antigens). The salivary IgA production to Pnc PS antigens seemed to start earlier than the serum IgG development. Significant differences between the antibody concentrations of children in different categories (Pnc-, Pnc+ ST- and Pnc+ ST+) were detected at earlier ages and more frequently in saliva (II) than in serum (Soininen *et al.* 2001) during the follow-up.

4. Effect of previous pneumococcal contacts on salivary antibody production

4.1. Anti-protein antibodies

The increase of salivary antibody concentrations by age was associated with Pnc encounters (I). A larger proportion of samples positive for specific IgA and clearly higher mean antibody concentrations were detected in the saliva of children, in whom pneumococci were found by culture of nasopharyngeal or MEF samples on healthy or sick visits before the indicated age (Pnc+), than of children without positive Pnc culture findings (Pnc-). The higher antibody concentrations in the Pnc+ children seemed to be largely independent of whether the pneumococci were found during asymptomatic carriage or illness, although a tendency for higher anti-protein antibody concentrations was observed in the sick children. This might indicate a longer or more intense contact with the bacteria during illness.

4.2. Anti-polysaccharide antibodies

Previous contacts (carriage or AOM episodes) with the Pnc types 6B, 11A, 14, 19F and 23F induced the production of salivary IgA antibodies to the homologous PSs (II). This was demonstrated by both the larger proportion of samples with detectable anti-Pnc PS IgA and higher antibody concentrations in the saliva of children with positive homologous culture findings (Pnc+ ST+) when compared to the children with respective negative cultures (Pnc+ ST- and Pnc-). The association of previous homologous Pnc contacts with the development of type-specific antibodies was also attested by the differences between the Pnc+ST+ and Pnc+ST- children, in particular with types 11A, 14 and 19F.

4.3. Anti-polysaccharide antibodies and contacts with heterologous pneumococcal serotypes

Also contacts with the cross-reactive Pnc types 6A and 19A induced the development of salivary IgA antibodies to types 6B and 19F, respectively. The members of the same serogroup are biochemically closely related and it has been shown that antibodies evoked by the type-specific PS are cross-protective (Giebink *et al.* 1996; Yu *et al.*

1999a), though less antibodies might be needed for type-specific protection than for cross-protection (Väkeväinen *et al.* 2001).

4.4. Anti-protein and anti-polysaccharide antibodies, and contacts with other bacteria with homologous surface antigens

4.4.1. Protein antigens

In individual children, the salivary IgA anti-PsaA, -Ply and -PspA responses were mainly seen in association with the Pnc positive culture findings (I: Figure 3). However, the relation was not always clear, since increases in the salivary antibody concentrations were also detected in the children in whom pneumococci had not been found (I: Figure 3). The Pnc cultures were performed on a regular basis at one to six months' intervals. Thus there is a possibility that Pnc- children may have been Pnc carriers at some stage, but this has not been detected. On the other hand, stimuli by other bacteria may account for some of the anti-PsaA and anti-PspA responses. Many Pnc surface antigens are conserved and several reports have suggested shared amino acid sequences between Pnc proteins and proteins of other streptococci belonging to the normal flora. A significant degree of homology has been reported between the primary structure of the PsaA protein and the putative lipoprotein adhesins of *S. sanguis* and *S. parasanguis* (Sampson *et al.* 1994; Paton *et al.* 1997). Also, the repeat domain of PspA has been shown to possess significant homology with the carboxy-terminal repeat regions of certain proteins of *S. mutans, S. downei* and *Clostridium difficile* (Yother & Briles 1992a). However, this repeat domain of PspA was not present in the recombinant PspA antigen that was used in this study. The role of these shared, most probably immunogenic, epitopes in the natural development of mucosal and systemic immunity against pneumococcus clearly needs to be evaluated.

4.4.2. Polysaccharide antigens

Some increases in salivary anti-PS concentrations were detected also in children who had contacts with the heterologous Pnc serotypes and even in children without positive Pnc culture findings (II: Figure 3). Antigenic structures cross-reactive with Pnc PSs have been reported in several common bacteria belonging to the normal nasopharyngeal and enteric flora (e.g. *E. coli, Klebsiellae,* and nongroupable streptococci) (Heidelberger *et al.* 1968, 1976, 1984; Lee *et al.* 1981; Robbins *et al.* 1975). The observation that cross-reactions with nonpathogenic nasopharyngeal bacteria appear to be frequent in

particular with Pnc PS of group 19 is of special interest (Lee *et al.* 1984). Thus, a potential explanation for the increases of antibody concentrations in saliva of Pnc- and Pnc+ ST- children could be that salivary anti-PS antibodies are also produced in response to the antigenic stimulus offered by other bacteria than pneumococci.

5. Comparison of the development of salivary and serum antibodies to pneumococcal protein and polysaccharide antigens

5.1. Antibodies to pneumococcal proteins

Similar differences in the kinetics of antibody production against PsaA, Ply and PspA were seen in the saliva and sera of the FinOM Cohort Study. Both the production of salivary anti-PspA IgA (I) and the production of serum anti-PspA IgG started at later age (Rapola *et al.* 2000). The proportion of samples containing detectable anti-PspA was at all ages smaller in saliva than in serum. At the age of 18 months, 45% of the serum samples and 22% of the saliva samples were positive for anti-PspA, respectively (I, Rapola *et al.* 2000).

In serum, the concentrations of natural anti-PsaA IgG reach adult levels already in early infancy (Rapola *et al.* 2000, Lindell *et al.* 2001). High concentrations of anti-PsaA antibodies were detected in serum samples of study children already at 6 months of age (Rapola *et al.* 2000). Furthermore, those young infants who had been carriers of pneumococci or had experienced Pnc AOM, developed high concentrations of serum antibodies to PsaA. At 12, 18 and 24 months of age, the GMC of serum anti-PsaA was even higher than in adults. This is opposite to the salivary anti-PsaA concentrations in children that did not reach the concentrations measured in adult saliva by the age of 24 months (I).

The production of salivary and serum anti-Ply antibodies showed similar kinetics. At the age of 24 months, the anti-Ply antibody concentration in saliva and serum of adults was 3 and 2 times higher than in children, respectively (I, Rapola *et al.* 2000).

5.2. Antibodies to pneumococcal polysaccharides

Mucosal immunity to PS antigens has been suggested to mature earlier in life than the systemic response (Pichichero *et al.* 1981, 1983). The data obtained from the present study seem to support this. Production of salivary IgA to Pnc PSs appeared to start earlier than production of corresponding serum IgG (II: Figure 1). Significant differences between the GMCs of IgA antibodies against Pnc PSs in children of the different categories (Pnc+ST+, Pnc+ ST- and Pnc-) were detected at earlier age and more frequently in saliva (II) than in sera (Soininen *et al.* 2001). The difference in antibody development between saliva and serum seemed to be clearest for the "pediatric" types, i.e. types that have been regarded as poor immunogens in children (e.g. 6B, 19F, 23F). The children having encountered one of these serotypes produced antibodies to the respective serotype in saliva (II), but corresponding serum antibody production could not be detected at any time point during the follow-up of the same children (Soininen *et al.* 2001).

By contrast, types 11A and 14 showed quite similar kinetics of salivary and serum antibodies. Development of both salivary and serum antibodies was seen at an early age, though salivary antibodies to type 14 were detected as early as at 6 months of age as compared to 12 months for serum antibodies (Soininen *et al.* 2001). The ability to respond to Pnc PS of type 14, although with modest antibody concentrations, is known to develop at an early age in both mucosa and serum (Mäkelä *et al.* 1980; Douglas *et al.* 1983; Korkeila *et al.* 2000). For type 11A this has not been described, since type 11A is not included in the current Pnc conjugate vaccines and has thus not been the subject of active research.

6. The origin of salivary IgA antibodies

The majority of mucosal antibodies belong to the IgA class, although other Ig isotypes may also be present on the mucosal surfaces. Thus, the measurement of salivary IgA antibodies was a natural choice in the present study. One of the aims in studies I and II was to determine the origin of natural salivary IgA antibodies against Pnc antigens. To attain this, the presence of SC in antigen-specific salivary antibodies was determined. The significant correlation of specific IgA with the presence of SC in anti-PsaA, -PspA,

-Ply, -6B, -14 and -19F suggested that the salivary IgA was secretory and thus locally produced.

Some of the saliva samples were detected to contain low levels of specific sIg without specific IgA. This difference in the proportions of sIg and IgA positive saliva samples could reflect the presence of sIgM. On the other hand, some of the samples contained low levels of specific IgA without sIg. These small differences most probably result from methodological factors (e.g. different sensitivities of the assays).

7. IgA subclasses of anti-PsaA and anti-PS14 antibodies

IgA occurs in two subclasses, IgA1 and IgA2. The predominant subclass both in serum and secretions is IgA1, but the share of IgA2 production has been shown to be relatively higher in secretions than in serum (Delacroix *et al.* 1982). A number of studies have shown that the ratio of IgA1 and IgA2 subclasses can depend on the nature of the antigen. Both the naturally and vaccine-induced IgA antibodies against TD bacterial protein antigens in saliva and in colostrum are predominantly of the IgA1 subclass (Brown *et al.* 1985; Conley & Delacroix 1987; Kilian *et al.* 1987; Ladjeva *et al.* 1989). By contrast, type 1 TI antigens, such as the cell wall lipopolysaccharides (LPS) of gram-negative bacteria and lipoteichoic acids of gram-positive bacteria, stimulate predominantly the production of natural IgA2 antibodies in saliva and colostrum (Brown & Mestecky 1985; Moldoveanu *et al.* 1987; Ladjeva *et al.* 1989). The typical type 2 TI antigens, such as bacterial PSs, can evoke both IgA1 and IgA2 responses in external secretions (Brown & Mestecky 1985; Ladjeva *et al.* 1989).

In the present study, the subclass distribution of natural salivary IgA antibodies was determined against two distinct types of the Pnc surface antigen: a surface protein PsaA and a capsular PS of type 14 (PS14). This was done to evaluate, whether the ratio of IgA1 to IgA2 antibodies would indicate the T cell-dependency of the immune response. Though both subclasses are believed to have the same functional effect as such, the susceptibility of IgA1 to bacterial IgA1-proteases might affect its role in the prevention of infections. The current study made it possible to compare the proportions of natural IgA1 and IgA2 antibodies against the Pnc protein and PS antigen in the same

individuals and to evaluate, whether immunization with protein or PS containing vaccines would induce a different pattern.

The predominant subclass for both salivary anti-PsaA and anti-PS14 IgA antibodies was IgA1. This was demonstrated by a larger proportion of IgA1 positive samples and a higher median concentration of specific IgA1 antibodies compared to IgA2. The median anti-PsaA IgA1/IgA2-ratio was higher than the corresponding median anti-PS14 IgA1/IgA2-ratio. Thus, PS14 seemed to induce proportionally more IgA2 antibodies in saliva than PsaA.

Several pathogenic bacteria inhabiting the mucosal membranes, including pneumococci, secrete IgA1-degrading enzymes, which may interfere with the effector functions of IgA1 on mucosal surfaces (Kilian *et al.* 1983a). Therefore, the higher mucosal levels of IgA2 antibodies may have important implications in the defense against these organisms. The IgA1-proteases cleave the IgA1 molecule in the hinge region to Fab and Fc fragments. It has been suggested that by coating themselves with functionally deficient Fab fragments, the pathogens could turn the defensive sIgA1 antibodies to their own advantage (Kilian & Reinholdt 1987). In the present study, IgA1 antibodies to PsaA and PS14 predominated in the saliva of children. This indicates that IgA1-protease produced by pneumococci and other respiratory pathogens may impair the functions of natural anti-Pnc IgA antibodies in 1- to 2-year-old children.

Bacterial IgA1-proteases have been suggested to be important for the ability of bacteria to colonize mucosal membranes in the presence of specific sIgA antibodies (Kilian *et al.* 1996). Interestingly, Weiser *et al.* (2003) have recently shown that the modification of type-specific IgA1 antibodies by Pnc IgA1-protease enhances bacterial attachment to respiratory epithelial cells in a cell-culture colonization model. The antibody-enhanced adherence was only seen when IgA1 was cleaved by bacterial IgA1-protease and it was suggested that Fab fragments bound to the bacterial surface may neutralize the inhibitory effect of the negatively charged Pnc capsule on adhesion of host cells. On the other hand, IgA1-proteases function as antigens and induce systemic and secretory antibodies, some of which have an enzyme-neutralizing activity (Gilbert *et al.* 1983; Kilian *et al.* 1983b; Devenyi *et al.* 1993). For instance, human milk contains

neutralizing antibodies to most bacterial IgA1-proteases and sIgA1 purified from human colostrum is resistant to most bacterial IgA1-proteases (Kobayashi *et al.* 1987).

8. Salivary anti-PspA, anti-PsaA and anti-Ply antibodies in relation to subsequent pneumococcal carriage and AOM

Since Pnc carriage and AOM are superficial infections on the mucosal surfaces of the nasopharynx or in their immediate vicinity, it has been hypothesized that mucosal antibodies might have a protective effect against them. To this end, the association between salivary anti-PspA, -PsaA and -Ply IgA antibodies, and the risk of subsequent Pnc carriage and Pnc AOM was evaluated during the first two years of life (IV and unpublished data). The presence of salivary anti-PsaA was associated with an increased risk of Pnc carriage throughout the follow-up and when antibodies were measured at six months of age, with an increased risk of subsequent Pnc AOM during the following six months (IV: Tables 1 and 2). Likewise, the presence of salivary anti-PspA, particularly at 18 months of age, appeared to be associated with an increased risk of asymptomatic Pnc carriage. In contrast, from 12 months onwards the low RR estimates suggested that the presence of salivary anti-PspA might be related to a decreased risk of Pnc AOM. However, since the anti-PspA antibodies were rarely detected in the saliva samples, the confidence intervals were very wide and statistical significance was not reached. The effect of salivary anti-Ply on the risk of Pnc carriage was negligible, while salivary anti-Ply seemed to be associated with an increased risk of subsequent Pnc AOM during the entire follow-up, though the results were not statistically significant (Table 3).

The relation of serum anti-PsaA antibodies to the risk of Pnc carriage and Pnc AOM has been previously explored (Rapola *et al.* 2003). As salivary antibodies to PspA, PsaA and Ply, serum antibodies to PsaA seemed to act paradoxically: the high anti-PsaA concentration in serum predicted a higher risk of Pnc carriage throughout the follow-up. Furthermore, higher serum anti-PsaA concentrations at six months of age were associated with an increased risk of Pnc AOM during the following six months. By contrast, from 12 months onwards, higher serum anti-PsaA concentrations were associated with a decreased risk of Pnc AOM, though not statistically significantly. Further, in children older than 9 months of age, high serum anti-PsaA concentration was associated with a decreased risk of Pnc involvement in AOM (Rapola *et al.* 2001).

Unquestionably, the results suggesting a risk-increasing effect of salivary and serum antibodies are unexpected and confusing. The association between serum anti-PsaA and the increased risk of Pnc carriage throughout the follow-up and Pnc AOM during the first year of life has been previously discussed and was suggested to be due frequent Pnc contacts indicating an environment with a high infection pressure (Rapola *et al*. 2003). In the present study, any new information on the specific mechanisms behind the risk-increasing effect of the anti-protein antibodies was not obtained. Seemingly the interplay between the natural anti-Pnc antibodies, Pnc carriage and Pnc AOM is too complex for any straightforward conclusions. Anyway, these data should not be interpreted so that the antibodies to Pnc proteins are harmful in early infancy.

It could be hypothesized that the immature state of mucosal surfaces during early childhood might have an influence on the risk of Pnc carriage and AOM. It is possible, that different adhesion mechanisms are used by pneumococci on mucosal surfaces of young infants than in older children. Thus, the mucosal immaturity during the first year of life might allow more efficient Pnc adherence and invasion than at older age, irrespective of the antibody responses induced by Pnc exposure. In older children, the maturation of mucosal surfaces, along with the maturation of the immunological status of the host, would reduce the chances to Pnc adherence and subsequent Pnc carriage and AOM.

Although similar statistical models were applied to the serum and salivary antibody data of the FinOM Cohort Study, the obtained risk estimates cannot directly be compared. The reason for this is that the antibody concentrations were included as continuous variables in the serum models (Rapola *et al.* 2003) and as dichotomized variables in the salivary models (IV). Dichotomous antibody variables were considered more appropriate for the analysis of salivary anti-PspA, because the majority of anti-PspA concentrations were under the detection limit and thus constant (i.e., a large fraction of the data was already dichotomous). In spite of the risk estimates of the serum and salivary models being not directly comparable, the difference in point estimates for the association of the salivary and serum anti-PspA with the decreased risk of subsequent Pnc AOM during the second year of life is distinct enough to deserve attention in future studies. It can be speculated that mucosal antibodies may be more important in the

defense against Pnc AOM than the serum antibodies, which in turn play a central role in the defense against invasive Pnc disease.

As mentioned above, the anti-PspA antibodies in the samples of the FinOM Cohort Study were measured using only one PspA antigen representing family 1. Our research group has previously determined the PspA family of the Pnc strains isolated from the clinical samples of 50 children who participated in the FinOM Cohort Study. In addition, anti-PspA antibodies in serum and still available saliva samples (n=20) of these 50 children have been measured by using PspA antigens from families 1 and 2. PspA molecules either of family 1 or of family 2 have been found on the surface of the isolated Pnc strains (Melin *et al.*; data to be published). Furthermore, the results from the serum and salivary antibody measurements suggest that the anti-PspA responses in children after Pnc contacts are mostly family-specific, i.e. children develop anti-PspA antibodies specifically against the PspA family that has been present on the surface of the encountered Pnc strain (Melin *et al.,* data to be published). Among children at 6 months of age who had detectable salivary anti-PspA antibodies, an association with an increased risk of Pnc AOM during the following six months was detected. However, there is the possibility that the salivary anti-PspA antibodies have been induced by a strain of one PspA family, and the increased AOM risk is due to a strain of another PspA family. Thus, there may be a stronger protective effect of salivary anti-PspA against Pnc AOM after the age of 12 months, if the subsequent Pnc strain belongs to the same PspA family.

The mucosal active immunization with Pnc protein and PS antigens and Pnc PS-protein conjugate antigens in animal models, as well as passive immunization, have shown that the mucosal anti-Pnc antibodies are able to reduce Pnc carriage and disease (VanCott *et al.* 1996; Wu *et al.* 1997; Malley *et al.* 1998, 2001; Jakobsen *et al.* 1999; Seong *et al.* 1999; Briles *et al.* 2000a; Saeland *et al.* 2001). The naturally acquired salivary anti-PspA, anti-PsaA or anti-Ply antibodies, however, seem not to be associated with decreased risk Pnc carriage during the first two years of life. A reason for this may be that the antibody concentrations induced by natural exposure to Pnc remain low. However, an optimally designed mucosal immunization could evoke higher and more protective immune responses than natural Pnc contacts.

CONCLUSIONS

The following conclusions were made on the basis of the studies in this thesis:

1. Even young children are capable of producing salivary antibodies to the Pnc protein and PS antigens (I, II).
2. The production of salivary IgA specific for Pnc protein and PS antigens increases by age. The production of the salivary IgA antibodies shows slightly different kinetics depending on the Pnc antigen (I, II).
3. The increase of salivary anti-Pnc antibody production with age is related to Pnc contacts (I, II).
4. Natural salivary anti-Pnc IgA antibodies are secretory, i.e. locally produced (I, II).
5. IgA1 is the predominant subclass of the natural salivary IgA antibodies to Pnc protein and PS antigens, but the proportion of IgA2 antibodies is relatively higher for anti-PS antibodies (III).
6. Natural salivary anti-PspA, anti-PsaA and anti-Ply antibodies are not associated with protection against Pnc carriage (IV).
7. The presence of salivary anti-PspA and anti-PsaA at 6 months of age seems to be associated with an increased risk of subsequent Pnc AOM during the following 6 months. Salivary anti-Ply may be associated with an increased risk of Pnc AOM during the first two years of life (results not statistically significant) (IV).
8. From 12 months of age onwards the salivary anti-PspA IgA antibodies seem to be associated with a decreased risk of subsequent Pnc AOM, though the results did not reach statistical significance (IV).

SUMMARY

The primary site of Pnc infections is the mucosal surfaces of the human upper respiratory tract. Local mucosal immunity is likely to act as an important first line defense against Pnc carriage and subsequent disease.

AOM is a mild, but extremely common disease during childhood. The most common bacterium causing AOM is *S. pneumoniae*. The efficacy of the current Pnc PS and conjugate vaccines against Pnc AOM is not optimal, which has stimulated an interest in alternative Pnc vaccination strategies. The promising complementary or alternative approaches for prevention of Pnc infections include the development of Pnc protein vaccines and Pnc mucosal vaccines. Mucosal and systemic vaccination with the Pnc protein vaccine candidates, such as PsaA, PspA and Ply, has been shown to be an effective way to offer protection against Pnc carriage and disease in animal models. However, more basic work will still be required before considering these kinds of applications in humans. This work includes characterizing the development of natural mucosal and systemic immunity against pneumococcus.

In the present study, the development of natural salivary immunity against several Pnc protein and capsular PS antigens was evaluated in 329 children who were followed from 2 to 24 months of age in the FinOM Cohort Study. For the first time, the natural development of mucosal IgA antibodies to Pnc vaccine candidates was evaluated in a longitudinal study setting. Evidence for the ability of young children to respond to Pnc carriage and AOM by producing secretory antibodies in saliva against all studied Pnc antigens was demonstrated.

The recurrent nature of Pnc AOM indicates that the antibody responses induced by natural exposure to Pnc in early childhood are not strong enough to offer effective protection against Pnc infection. In accordance, the results of this thesis suggest that the naturally developed salivary anti-PsaA, anti-PspA and anti-Ply IgA antibodies are not associated with the protection against Pnc carriage or AOM during the first year of life. However, from 12 months of age onwards the salivary anti-PspA IgA antibodies may offer some protection against Pnc AOM.

Early immunization by the mucosal route with a Pnc vaccine would probably induce higher mucosal antibody concentrations and thereby provide better protection against disease than the natural Pnc stimuli. Results from animal models suggest that optimally designed mucosal immunization is able to effectively protect against Pnc carriage and disease. Thus, further evaluation of mucosal immunization as a means of inducing protection against Pnc disease in humans is considered worthwile.

FUTURE CONSIDERATIONS

The FinOM Cohort Study material is unique in having prospective carriage and AOM data with the consecutive serum and saliva samples. At the moment, the saliva samples are no more available, but the remaining serum samples will be used for the study of antibodies to other Pnc protein antigens, which are currently considered as Pnc vaccine candidates. The FinOM Cohort Study has made it possible to investigate the development of natural mucosal immunity against pneumococcus. In future, the Pnc proteins that were used as antigens in the present study will hopefully be included in a Pnc vaccine. Then, the serological methods modified for the purposes of this study may be utilized to evaluate the differences between the natural and vaccine-induced mucosal anti-Pnc immunity and further the role of mucosal immunity in the defense against Pnc infections. Thus, we are looking forward to the future immunization studies with Pnc protein vaccines, as well as with Pnc mucosal vaccines.

As mentioned above, high concentrations of anti-PsaA IgG antibodies were detected in serum samples already at 6 months of age and in response to Pnc contacts (Rapola *et al.* 2000). Since Pnc carriage in Finnish children is rare at this age (Syrjänen *et al.* 2001), these antibodies may have been induced by some other stimulus than pneumococcus. Therefore, the potential role of cross-reacting antigens of oral and nasopharyngeal normal streptococcal flora on the development of natural immunity against pneumococcus will be evaluated. A subpopulation of 50 children has been separated from the FinOM Cohort Study for the purposes of this study. The development and composition of the normal streptococcal flora of these 50 children has been carefully followed (Könönen *et al.* 2002). The oral and nasopharyngeal streptococcal isolates have been cultured during the first two years of life, identified to species and subspecies level and stored in skimmed milk at -70°C. These streptococcal isolates will be used in the study.

The mucosal immunity to bacterial PS antigens has been suggested to mature earlier in life than the systemic immunity. To address this issue, the kinetics of serum and salivary antibody production to Pnc PS (types 6B, 14 and 19F) and protein (PsaA, PspA and Ply) antigens in the FinOM Cohort Study will be compared. This will be done to

evaluate, whether the antibody responses in saliva might appear at younger age and/or with greater intensity than in serum.

The search for an efficient vaccine against Pnc infections continues. Mucosal immunization with Pnc antigens in animal models has shown that the vaccine-induced mucosal antibodies are able to reduce Pnc carriage and disease. In future, Pnc vaccine administered via the mucosal route may thus prove to be a welcome addition or alternative for the current parenteral immunization strategies against pneumococcus.

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