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CIRCULATING ANTIBODY-SECRETING CELLS AND SALIVARY ANTIBODIES INDUCED BY THE CAPSULAR POLYSACCHARIDE OF *STREPTOCOCCUS PNEUMONIAE*

AFTER PARENTERAL IMMUNISATION AND IN ACUTE OTITIS MEDIA

by

Tea Nieminen

ACADEMIC DISSERTATION

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ABBREVIATIONS

AOM	-	acute otitis media
APC	-	antigen-presenting cell
ASC	-	antibody-secreting cell
CPS	-	C-polysaccharide
CT	-	cholera toxin
D	-	diphtheria toxoid
ELISPOT	-	enzyme-linked immunospot assay
GM	-	geometric mean
GMC	-	geometric mean concentration
Hib	-	*Haemophilus influenzae* type b
ISC	-	immunoglobulin-secreting cell
MEF	-	middle ear fluid
MHC	-	major histocompatibility complex
NPA	-	nasopharyngeal aspirate
OMPC	-	outer membrane protein complex
PBMC	-	peripheral blood mononuclear cell
Pnc	-	*Streptococcus pneumoniae*, pneumococcus
PRP	-	capsular PS of Hib; polyribosylribitol phosphate
PS	-	polysaccharide
PsaA	-	pneumococcal surface adhesin
PspA	-	pneumococcal surface protein A
RSV	-	respiratory syncytical virus
SC	-	secretory component
S-IgA	-	secretory immunoglobulin A
TD	-	thymus-dependent
TI	-	thymus-independent
T	-	tetanus toxoid

INTRODUCTION

The microbes that surround us constantly invade our body, many of them through respiratory airways, where mucosal surfaces form the first line of defence against pathogenic bacteria. This challenge by pathogens is met by both nonspecific and specific defence mechanisms of the immune system, both already functioning locally at mucosal surfaces. The specific immune system has a unique capability to recognise a specific pathogen, target the defence against it, and recognise the same pathogen even years later. This memory function has been described for both systemic and mucosal immune responses.

Each pathogen has its own strategy to gain access into the human body, but also the immune system has a specific strategy for the fight against each pathogen. It is therefore essential to study the pathogenesis of each disease and the human defence mechanisms against each pathogen, in order to develop preventive and therapeutic means, e.g., vaccines, against the disease. Acute otitis media (AOM) is an example of an infection restricted to mucosal membranes. It is common among small children; nearly all children experience at least one AOM episode before the age of two years.

The most common causative bacterium of AOM is *Streptococcus pneumoniae*, the pneumococcus (Pnc). An important virulence factor for the pneumococcus is the polysaccharide (PS) capsule, which helps it to avoid phagocytosis. Since the pneumococci are encapsulated bacteria, it is essential for the immunological defence to produce antibodies against the polysaccharide capsule in order to fight against the pathogen. Pneumococcal PS vaccines, consisting of polysaccharides of different serotypes of the pneumococcus, are immunogenic in the adult population, i.e., able to induce antibody production against the capsular PS. However, these vaccines are able to induce only poor, if any, antibody responses in infants and young children, who are the most susceptible to pneumococcal diseases, partly because of the immaturity of their immune system. The poor response to PS seen in small children is due to the T-independent nature of these antigens, which means the PS vaccines are unable to induce T-cell help for antibody production. However, coupling of the PS antigens to a protein carrier turns them into T-dependent antigens, which are recognised by helper T-cells that then stimulate antibody production by B-cells. Conjugate vaccines consisting of pneumococcal PS coupled to different carrier proteins have already been shown to be immunogenic in young children and infants.

Haemophilus influenzae type b (Hib) conjugate vaccines, and more recently also pneumococcal conjugate vaccines, have been shown to reduce colonisation with the respective bacteria in the nasopharynx, suggesting that parenteral immunisation can induce protection at mucosal surfaces. Thus the pneumococcal conjugate vaccines can be expected to prevent, in addition to serious invasive infection, mucosal infections such as pneumococcal AOM and pneumonia.

This thesis describes part of a larger series of studies, carried out to characterise the immune responses related to AOM and to study the immunogenicity of the pneumococcal conjugate vaccines. The focus of this work is on induction of mucosal antibody responses, both those seen in AOM and those induced by parenterally administered pneumococcal conjugate vaccines.

REVIEW OF THE LITERATURE

1. *Streptococcus pneumoniae*

Streptococcus pneumoniae (pneumococcus, Pnc) is a gram-positive, encapsulated, facultatively anaerobic coccus, with a distinctive asymmetric 'lancet' shape. *S. pneumoniae* was formerly known as Diplococcus, because it usually appears in pairs and consequently was believed to belong to a genus separate from Sreptococcus. These morphological characteristics help in identification of the bacteria. Three morphological layers can be distinguished in the surface of Pnc: plasma membrane, cell wall, and capsule. The peptidoglycan of the cell wall anchors the cell wall polysaccharide (CPS), and also proteins, in addition to the capsular polysaccharide.

The *S. pneumoniae* bacteria have a thick polysaccharide capsule that covers the inner structure of the bacteria (Skov Sorensen et al. 1988). Some proteins, such as the pneumococcal surface protein A (PspA), however, are exposed beyond the capsule (Gray 1996). Based on its capacity to prevent phagocytosis (Wood and Smith 1949), the capsule is established as a major virulence factor of pneumococci (Lee et al 1991), enabling the pneumococci to survive and multiply in the host. The pneumococci are classified into 90 serotypes based on the antigenic variability of the capsule (Kauffman et al 1960, Lund and Henrichsen 1978, Austrian et al 1985, Henrichsen 1995). Each serotype can be identified by its reaction with type-specific antisera. This was first done by the Quellung reaction (Neufeld 1902), in which the bacteria are mixed with antisera and methylene blue, resulting in a swelling reaction that can be visualised in microscope (Balows et al. 1991). More recently, counter-immunoelectrophoresis and latex agglutination and coagglutination tests have also been used for serotyping (Leinonen 1980, Trollfors 1983). The classification can be done according to two nomenclatures, the American and the Danish (Kauffman et al. 1960), of which the Danish nomenclature has been widely adopted since the early 1980's.

The cell wall of *S. pneumoniae* is responsible for the intense inflammatory reaction that accompanies a pneumococcal infection (Tuomanen et al.1985 and 1987, Carlsen et al. 1992). The cell-wall components peptidoglycan and teichoic acid are inflammatory mediators as potent as the lipopolysaccharide (LPS) of some gram-negative bacteria. In experimental meningitis, the peptidoglycan is shown to cause cerebral oedema and the teichoic acid to generate increased intra-cranial pressure (Tuomanen et al. 1985). This suggests that highly effective bactericidal antibiotics, such as beta-lactams, may in some cases adversely affect the outcome of pneumococcal meningitis, because they lyse pneumococci, which leads to release of cell-wall components (Bruyn et al. 1992). Thus, the inflammatory reaction of the host is responsible for most of the symptoms of pneumococcal disease, and it is suggested as being responsible for the high mortality from pneumococcal infections (Musher 1992)

The surface of *S. pneumoniae* is decorated with a family of choline-binding proteins bound to the phosphorylcholine of the teichoic acid. The major cell-wall hydrolase (or

major autolysin, LytA) was the first of these proteins characterised (Garcia et al. 1986). It is responsible for the cleavage of peptidoglycan and thus indirectly mediates inflammation and release of other non-exported virulence factors, such as pneumolysin (Mitchell et al. 1997). A second choline-binding protein characterised, the pneumococcal surface protein A (PspA) (Briles et al 1981), is an antigenically variable surface protein present in all clinically important isolates (Briles et al 1989, Crain et al. 1990). It seems to be essential for full virulence and is shown to interfere with the blood clearance, but its mechanisms of specific action remain unknown (McDaniel et al. 1987, Briles et al.1988). In addition, other choline-binding proteins have been characterised more recently (Rosenow et al. 1997). A different kind of surface protein has also been identified , the pneumococcal surface adhesin protein (PsaA) (Russell et al. 1990). It is common to all pneumococci and is highly conserved in the clinically notable serotypes (Sampson et al. 1997). No role for PsaA has yet been determined, but it has been hypothesized to be a permease (Crook et al 1998).

Pneumolysin is an intracellular protein released upon autolysis (Johnson 1977); it is produced by virtually all Pnc isolates (Walker et al. 1987, Kanclerski and Mollby 1987). The pneumolysin can interfere with the host's ability to attack the invading pneumococci by multiple effects on the host's immune system. It activates the classical pathway of complement in the absence of antibodies (Paton et al. 1984). Pneumolysin decreases the bactericidal activity and migration of neutrophils (Johnson et al. 1981, Paton and Ferrante 1983) and inhibits lymphocyte proliferation and antibody production *in vitro* (Ferrante et al. 1984). Furthermore, it inhibits the beating of the cilia of human epithelial cells, and disrupts cultured epithelial cells from the respiratory tract and pulmonal alveoli (Feldman et al. 1990, Rubins et al. 1993).

Several enzymes that are produced by Pnc (autolysin, hyaluronidase and glycosidases such as neuraminidase) also enhance its virulence. The IgA1 protease produced by pneumococcus may be an important enzyme. It is highly specific for human IgA and can thus interfere with host defences at mucosal surfaces (Kilian et al. 1979, Male 1979). In addition, fab fragments generated by this protease retain their antigen-binding capacity (Mallett et al. 1984, Mansa and Kilian 1986) and may protect the pneumococci by inhibiting the binding of intact immunoglobulin, thus preventing the Fc-dependent elimination of the opsonized pneumococci (Kilian et al. 1988, Kilian et al.1996). Furthermore, indirect evidence of cleavage of IgG and IgM has been described (Wikström et al. 1984).

2. Pneumococcus in the upper respiratory mucosa

2.1. Pathogenesis of pneumococcal disease

S. pneumoniae is a human pathogen which spreads from person to person by aerosols. It is carried in the nasopharynx without any apparent symptoms, which helps it to persist in the human population. The infection begins with colonisation in the nasopharynx by the bacteria. From there the bacteria can gain access to the lungs or Eustachian tubes. If the bacteria enter the Eustachian tubes and start growing there, they

trigger an inflammatory response that causes pain, fever, and earache. The bacteria may also enter the bloodstream directly, although the mechanisms of entry and the conditions that enable the translocation of the bacteria are unknown. Toxins secreted by the bacteria and the products liberated after breakdown (cell-wall components, pneumolysin) are suggested to play a role (Johnston 1991). The direct damage to epithelial cells by hydrogen peroxidase and the effect of pneumolysin on ciliary beating also facilitates the access of pneumococci into the bloodstream (Boulnois 1992). Furthermore, the intense inflammatory response enhanced by these by-products or concurrent viral infections may be important. Some experimental studies have suggested that conversion to invasive disease may involve generation of local inflammatory factors which change the number and type of receptors available by activating human cells (Cundell et al. 1995, Tuomanen et al. 1997).

2. 2. Mucosal carriage

Asymptomatic carriage rates of pneumococcus vary widely by age and population. In Virginia, in the USA, carriage rates reported in the early 1970 were 38% in preschool children, decreasing by age to 19% in the adult population (Hendley et al.1975). These results are very similar to those reported back in 1939 by Heffron (see Hendley et al. 1975). The most frequently isolated serogroups were, 3, 4, 6, 7, 19, and 23. The spread of pneumococci is efficient within families, and in adults, carriage rates are clearly affected by their exposure to young children at home (Hendley et al. 1975). Moreover, carriage rates of pneumococci are reported to be higher in children with AOM or other respiratory tract infections than in healthy children of the same age-group (Willard and Hanssen 1957, Herva 1980, Luotonen 1982, Faden 1990, Takala et al. 1991, Aniansson et al. 1992). Nasopharyngeal acquisition of Pnc in newborn infants is greatly affected by living conditions. In Alabama, in the USA, nasopharyngeal carriage was observed from the age of 4 days on, the mean age for acquisition being 10 months (Gray et al. 1980). By the age of two years, 96% of the children had carried Pnc at least once. In Papua New Guinea, the reported carriage rate is already 100% by the age of three months (Gratten et al. 1986). In comparison, a study in Sweden showed carriage rates of 12% by the age of three months and 67% by the age of 18 months (Aniansson 1992).

2. 3. Acute otitis media

The middle ear cavity is normally dry and free of bacteria. It is connected to the nasopharynx by the Eustachian tube, which equilibrate air pressure between the middle ear and nasopharynx. However, its function is poorer in children than in adults (Bylander 1980, Bylander and Tjernstöm 1983). In acute otitis media (AOM) the colonising bacteria gain access into the middle ear cavity through the Eustachian tube. The pathogenesis of AOM is considered to be related to the compromised middle-ear ventilation secondary to Eustachian tube dysfunction, presence of pathogenic bacteria in the nasopharynx, and biochemical and immunological host responses (Hendersson and Giebink 1986). An upper respiratory tract infection is believed to result in congestion and obstruction of the Eustachian tubes, leading to accumulation of fluid in the middle ear cavity (Giebink 1989). Especially respiratory syncytical virus (RSV)

epidemics have been shown to correlate with the occurrence of AOM (Ruuskanen et al. 1989), and RSV has been detected in both the nasopharynx and the middle ear of AOM patients (Klein et al.1982, Sarkkinen et al.1985, Chonmaitree et al.1986, Arola et al. 1988, Heikkinen et al.1999). In addition to RSV, parainfluenza and influenza viruses have frequently been detected in the middle ear of AOM patients: RSV in 74% of the children with RSV infection, and parainfluenza in 52% and influenza in 42% of the children infected with the respective viruses (Heikkinen et al.1999). Another study has suggested that besides RSV epidemics, the rhino virus is the most common virus detected in children with AOM (Arola et al.1990).

Based on bacterial cultures, Pnc is the predominant bacterial species in the middle ear fluid (MEF) in AOM patients, isolated in 30 to 40% of the cases (Karma et al. 1987). *Haemophilus influenzae* is the second most common isolate (10-20%), and *Branhamella catarrhalis* the third. *Streptococcus pyogenes and Staphylococcus aureus* are less frequent findings (Karma et al. 1987). If antigen detection is added to the diagnostic methods for AOM, Pnc has been implicated in nearly 60% of all AOM cases (Luotonen et al. 1981). In the most recent study in Finland, Pnc was isolated less frequently, in 26% of all AOM cases from which a MEF sample was obtained (Kilpi et al. 1999).

The serotypes causing acute otitis media (AOM) have been shown to differ to some extent from those causing invasive diseases (Klein 1981, Pedersen and Henrichsen 1983, Gray and Dillon 1986). However, the serotypes isolated from asymptomatic carriers are the serotypes that most frequently cause AOM (Austrian et al.1977). The Pnc serogroups most commonly involved in AOM are 19, 23, 6, and 14, in this order (Karma et al. 1987, Kilpi et al. 1999). In most cases, the infection does not occur after a prolonged carriage state, but arises from a recent acquisition of the pneumococcus (Gray et al.1980). Therefore a prolonged carriage state might even be beneficial for the host by preventing other, more virulent strains from colonising the nasopharynx and thus limiting new acquisitions (Gray et Dillon 1986).

The incidence of AOM shows great variation between populations and studies. The cumulative incidence of the first episode at 12 months of age ranged from 28% (Pukander et al. 1982) to 45% (Sipilä et al.1987) in Finnish studies, but reached 62% in studies from the USA (Teele et al.1989). The cumulative incidence at 24 months of age was 71% in Finland (Alho et al. 1990), 36% in Sweden (Lundgren and Ingvarsson 1983) and 61% in the USA (Howie et al. 1975). According to these studies, the incidence is highest in children aged from 6 to 12 months. The earlier in childhood the first AOM episode occurs, the higher is the risk of recurrent episodes (Kaplan et al. 1973, Howie et al 1975, Teele et al. 1989).

3. Host defence against pneumococcal disease

3. 1. Mucosal sites

In healthy humans the mucosal barrier in the upper respiratory tract is the first line of defence against pathogens invading the body via the airways. Intact mucosal epithelium, mucous clearance, and the ciliated cells in the airways, together with antibacterial proteins and peptides on the mucosal surfaces, prevent pathogens from spreading from the nasopharynx into the surrounding tissues and lungs. In addition to this 'innate immunity', the specific, antibody-mediated defence takes action already at the mucosal surfaces, where IgA is the predominant immunoglobulin isotype. In secretions, IgA occurs in its secretory form. Secretory IgA (S-IgA) is found in polymeric forms, with a dimeric form dominating. Monomers of IgA are connected to one another by the J-chain. The J-chain binds the complex to the secretory component (SC). The membrane-bound form of the SC (polyimmunoglobulin receptor) on the basolateral side of epithelial cells functions as the specific receptor for the polymeric J-chain containing immunoglobulins. Binding of IgA to SC mediates the specific transport into secretions (Fig 1), stabilises the polymeric immunoglobulin, and protects it against proteolytic enzymes (Russel et al. 1992).

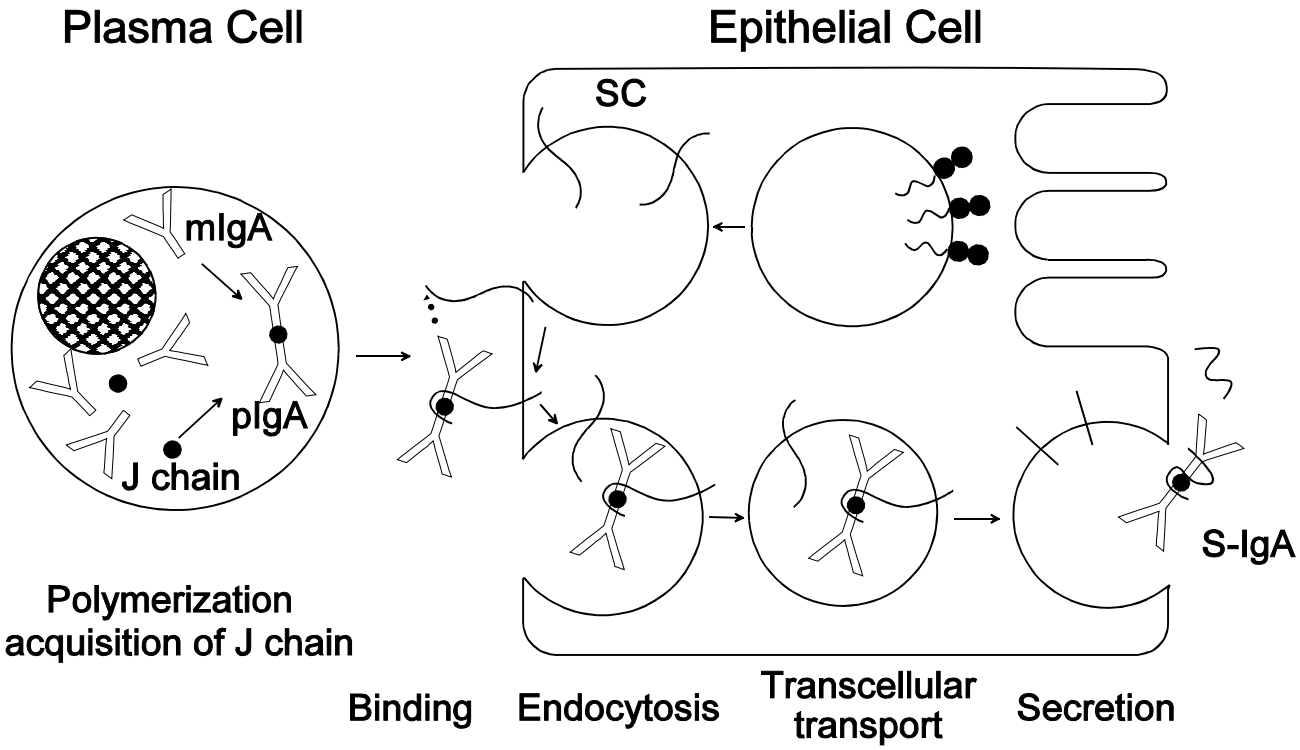

Figure 1. The selective transport of J chain containing polymeric IgA (pIgA) through epithelial cells. The membrane-bound form of secretory component (SC) on the basolateral side of epithelial cells functions as the receptor for polymeric IgA. The interaction of transmembrane SC to pIgA mediates the specific transmigration of S-IgA into secretions. The SC-pIgA complex is internalised in endoplasmic vesicles. These vesicles fuse with the apical membrane, and S-IgA is released in external secretion. (Modified from Kuhn and Krahenbuhl 1982, Trends Biochem Sci.)

Inhibition of bacterial and viral adherence is an essential function for IgA in secretions. In co-operation with innate defence factors, the S-IgA inhibits epithelial colonisation, and decreases penetration of antigens through mucosal membranes. S-IgA also neutralises viruses, bacterial toxins, and enzymes (Table 1). IgA appears in two isotypes, IgA1 and IgA2, the distribution between them varying by mucosal site (Delacroix et al. 1982). In the upper respiratory tract and upper gastrointestinal tract, the IgA1 isotype is predominant, but the proportion of IgA2 increases further along the gastrointestinal tract. Only in the lower gastrointestinal tract is the majority of the IgA of the IgA2 isotype (Mestecky and Russel 1986, Brandtzaeg et al. 1986, Kett et al. 1986).

Table 1.

Biological functions of S-IgA

direct
Neutralisation of biologically active antigens (viruses, toxins, enzymes)
Inhibition of microbial adherence
Inhibition of antigen penetration (immune exclusion)
indirect
Opsonisation for mucosal polymorphonuclears and macrophages (promotion of phagocytosis)
Antibody-dependent cellular cytotoxicity
Enhancement of antibacterial humoral factors in secretions (lactoferrin and peroxidase system)
Enhancement of monocyte and lymphocyte-dependent bactericidal activity

The B-cells responsible for local polymeric IgA production are mainly derived from organized mucosa-associated lymphoid tissue (MALT). The components of MALT in the upper respiratory tract form a structure called Waldeyer's ring that includes the palatine and pharyngeal tonsils ("adenoids") (Fig 2). In the lower respiratory tract the lymphatic tissue is referred to as the bronchus-associated lymphoid tissue (BALT). Primed B-cells and T-cells from all inductive lymphoepithelial structures of the MALT migrate via peripheral blood to exocrine tissues throughout the body (Gowans et al. 1964). The integrated mucosal immune system thus ensures that all mucous membranes are furnished with a wide spectrum of secretory antibodies. However, accumulating evidence suggests that regionalised homing mechanisms of activated cells do take place (Brandtzaeg et al. 1999). Thus the cells stimulated at the lymphoid tissues of the upper

respiratory tract would preferentially home to the upper respiratory mucosa, a hypothesis also supported by some experimental animal studies (Nadal et al. 1991).

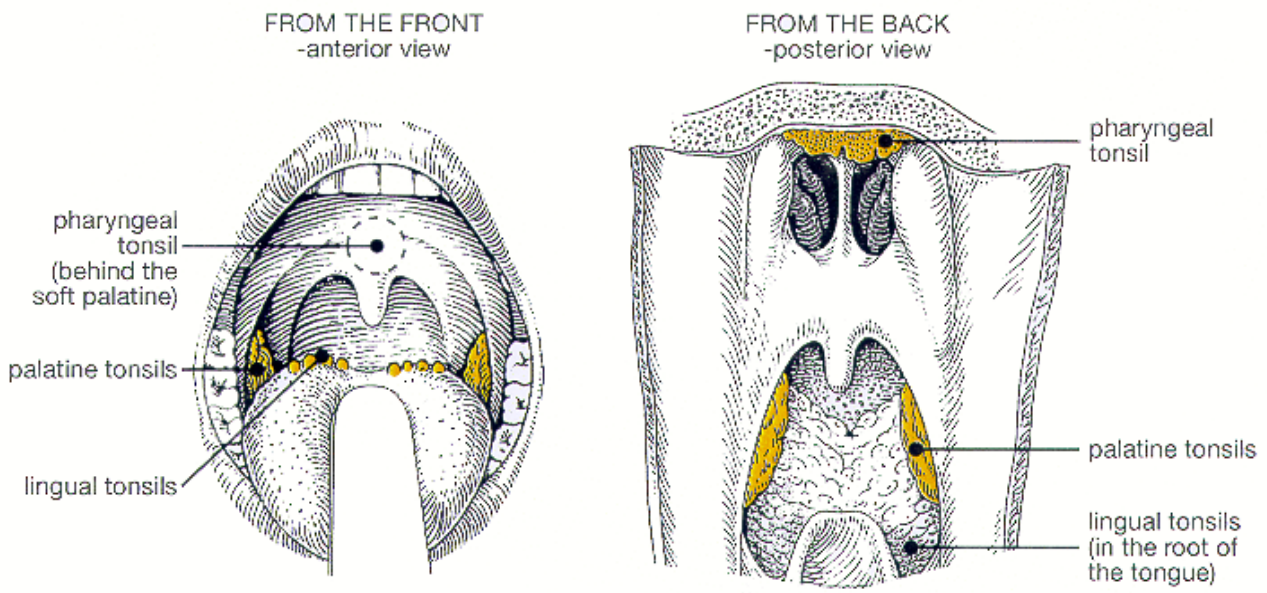

Figure 2.
Waldeyer's tonsillar ring, consisting of an unpaired pharyngeal tonsil in the roof of the pharynx, paired palatine tonsils and lingual tonsils scattered in the root of the tongue. (Modified from Kahle et al. Color Atlas and Textbook of Human Anatomy).

Whether the middle ear mucosa is a part of the integrated 'common' mucosal system still remains a matter of controversy (Kuper et al. 1992). Although there are few lymphocytes and other immunocompetent cells in the normal middle ear mucosa, antigen-specific IgA-forming cells can be induced in the middle ear mucosa during otitis media (Watanabe et al. 1988). IgA precursors homing to the middle ear can be induced in the adenoids and tonsils (Bernstein et al. 1988), but GALT or BALT can also serve as a source for precursors of the cells homing to the middle ear (Watanabe et al. 1988). However, the IgA-secreting cells are shown to be drawn to the middle ear in a relatively nonspecific manner, instead of homing by specific receptors (Ryan et al. 1990). Thus, it is rather the inflammation of the middle ear mucosa that accelerates the recruitment of the cells from the circulation (Kato et al.1994). Moreover, in children with AOM, the IgA in the nasopharynx is locally produced and is secretory in nature, whereas a significant proportion of IgA in the middle ear in the acute phase of AOM infection seems to be derived from serum (Virolainen et al. 1995a, Virolainen et al. 1995b). Nevertheless, the presence of pathogen-specific S-IgA in the MEF as early as at the onset of AOM seems to be beneficial for the resolution of the disease (Sloyer et al.1974, Sloyer et al.1976, Karjalainen et al. 1990).

In addition to polymeric IgA, pentameric IgM is likewise actively enriched in most exocrine fluids and is associated with SC, whereas IgG mostly leaks into secretions passively, although it may also be partially locally produced (Brandtzaeg et al. 1991, Bouvet and Fischetti 1999). Furthermore, leakage of IgG into exocrine fluids is enhanced by mucosal irritation. Although IgG is not considered a secretory immunoglobulin, it may contribute to immune exclusion (Brandtzaeg et al. 1987) and inhibit colonisation at mucosal sites (Kauppi et al. 1993). This can be seen especially in the respiratory tract, where the IgG is less easily subjected to proteolytic degradation than in the intestinal tract (Persson et al. 1998). On the other hand, by activating complement, IgG may actually accelerate mucosal penetration of antigens and may thus contribute to persistent immunopathology at the mucosal sites.

3. 2. Systemic sites

If the bacteria succeed in spreading into the deeper organs, a concerted action of antibodies, complement components, and phagocytic cells takes place (Gillespie 1989, Bruyn et al. 1992, Watson et al. 1995). As the polysaccharide capsule is able to disturb phagocytosis by preventing direct contact between phagocytes and pneumococcus, the opsonisation of the bacteria by antibodies or by complement components is crucial for phagocytosis (Johnston et al. 1981). If impairment in any of these functions appears, such as complement or immunoglobulin deficiencies, it will readily predispose the host to severe pneumococcal infections. The spleen has an essential role both in initiating production of antibodies to pneumococcus and in the clearance of the opsonized bacteria from the bloodstream (Wara 1981). The importance of the spleen as a filtration organ that removes pneumococci from the bloodstream is seen in those with splenectomies or splenic dysfunction. Such patients are particularly prone to rapid progress of pneumococcal disease, especially septic bacteremia following pneumonia. In the presence of anti-PS antibodies, the classical complement pathway is activated, and with its essential assistance the pneumococcus is effectively cleared from the blood by the liver and spleen (Brown et al. 1983, Holzer et al. 1984). However, phagocytosis may also be mediated by innate immunity components such as lectins and other carbohydrate-binding proteins in the liver and spleen of a non-immune host (Ofek and Sharon 1988). Nevertheless, whether or not the opsonisation is antibody mediated, an intact complement system is essential for the clearance of the bacteria (Brown et al 1983). Furthermore, the CPS and capsular PS of the pneumococcus (Winkelstein and Thomaz 1978, Hostetter 1986) are able to activate the alternative complement pathway that leads to production of opsonising complement components.

4. Antibody-mediated immunity to polysaccharide antigens

4. 1. Polysaccharides as antigens

Polysaccharide antigens differ from protein antigens in the manner in which they are processed and presented (Stein 1992, Mond et al. 1995). The responsiveness of the immune system to polysaccharide antigens develops late in ontogeny (Peltola et al. 1977, Parke et al. 1977, Käyhty et al. 1984), due to specific characteristics of polysaccharides as antigens. Protein antigens are mostly T-cell-dependent (TD)

antigens presented to T-cells by the major histocompatibility class II (MCH II) molecules on antigen-presenting cells (APC). The B-cells specific for these TD antigens require help for antibody production from stimulated T-cells. The PS antigens, by contrast, are thymus-independent, or T-cell independent (TI) antigens. Two types of TI antigens exist. Type 1 TI antigens are bacterial products such as LPS that function as mitogenic or polyclonal B-cell activators. The TI-1 response is totally independent of T-cell regulatory activity, and can be evoked in early infancy. Type 2 TI antigens are mostly high-molecular weight polymers with repeated structures, such as bacterial capsular polysaccharides (Mosier and Subbarao 1982) and virus surface capsids (Fehr et al. 1998). The antibody response to TI antigens can be stimulated without help from antigen-specific T-cells, by cross-linking the surface immunoglobulins on the B-cell surface. Although the TI-2 response can be evoked without T-cell help, the regulatory T-cells have an influence on the magnitude of the response (Rijkers and Moshier 1985). Both lymphokines and T-B cell interactions are shown to be required for an optimal antibody response to pneumococcal PS (Griffioen et al. 1992a). The TI-2 response is evoked later in life than is the TD response and shows no memory, affinity maturation, or isotype switch (Mosier et al. 1977, Baker et al. 1981). The immune responsiveness to TI-2 antigens begins during the first months of life and reaches adult levels by the age of 5 years (Pabst and Kreth 1980).

One explanation for the poor responsiveness to PS antigens in early life is suggested to be the late maturation of the B-cell subset responding to TI antigens. The cells activated by PS antigens have been shown to be the CD5- subset of the B-cell population; the appearance of these cells in ontogeny correlates with a child's responsiveness to PS antigens, both occurring at around two years of age (Barrett et al.1992). Secondly, pneumococcal PS is able to activate the alternative complement pathway and to bind the split product, C3d, of complement factor C3 in the absence of antibodies. The complement receptor 2 (CR2) is a receptor on the B-cells that binds to the PS complexed to C3d. This interaction of the CR2 to the PS-C3d complex plays a key role in B-lymphocyte activation, proliferation, and antibody production (Griffioen 1991). Neonatal B-cells in the marginal zone of the spleen (that is supposed to be the site of the initiation of the immune response to PS antigens) express significantly decreased levels of CR2 as compared to such cells in adults (Timens et al. 1989). Thus another explanation for the neonatal unresponsiveness to PS antigens may be the lower expression of this CR2 in infants than in adults (Griffioen et al.1992, Griffioen et al. 1993). Furthermore, the capacity of capsular polysaccharides to activate complement varies (Hostetter 1986), which might explain the variation in immunogenicity between different serotypes.

Another typical characteristic of PSs as antigens is that these antigens are distributed through body fluids (Spinola et al.1986, Darville et al. 1992), which may allow them to reach lymphatic tissues at distant sites. Asymptomatic carriage of encapsulated bacteria is reported to be associated with antigenuria (Murphy et al. 1989, Manary et al. 1993), and children immunised with PRP- or Hib-conjugate vaccines have been shown to

excrete PRP antigen in their urine (Spinola et al. 1985, Miller et al. 1995). This suggests that PS antigens are released into the circulation, both after mucosal invasion and after parenteral administration of the antigen.

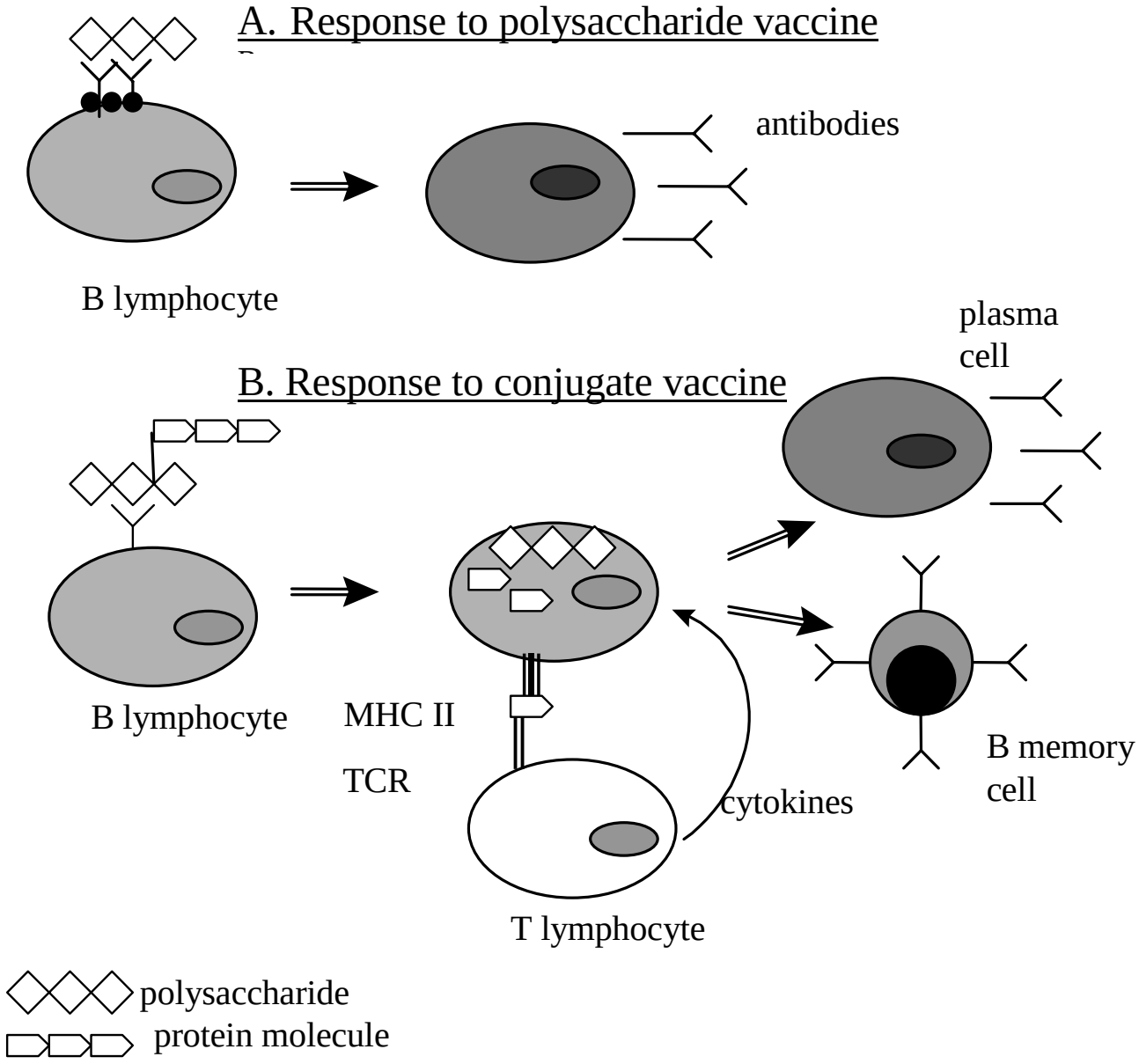

Figure 3.
T-cell independent and T-cell dependent antibody responses to PS or PS-protein conjugate antigens. (Modified from Åhman 1999, Dissertation, University of Helsinki).

The immunogenicity of PS antigens can be enhanced by coupling them to a protein carrier (Avery and Goebel 1929, Goebel 1939), which converts them into TD antigens (Schneerson et al. 1980, Schneerson et al. 1983). A hypothesis as to how conjugation of a PS to a protein carrier enhances the response is presented in Fig 3. The PS-protein complex is internalized in a B-cell via its PS-specific surface-Ig receptor. This leads to processing of the protein in the B-cell and presentation of the peptides to the Th cells in MHC complexes on the surface of the (PS-specific) B-cell (Lanzavecchia 1985,

Lanzavecchia 1986). Repeated immunisations increase the number of carrier protein-specific Th-cells that provide help to PS-specific B-cells, resulting in differentiation of the B-cells into memory or plasma cells.

4. 2. Systemic antibodies to polysaccharide antigens

In adults, parenterally administered PS antigens are able to induce systemic antibodies. The majority of the PS-specific antibodies detected in serum consist of IgG, but the fold increases of the specific IgG remain lower than the fold increase of PS-specific IgA (Heilmann et al. 1987, Lue et al. 1988, Tarkowski et al. 1989, Tarkowski et al. 1990). This is in contrast to protein antigens, which mostly enhance IgG responses. Furthermore, a marked proportion of the pneumococcal PS-specific IgG is shown to be of the IgG2 subtype (Siber et al. 1980, Barret et al. 1986, Soininen et al. 1999) differing from the IgG1 response seen to protein antigens (Seppälä et al.1984, Sarnesto et al.1985). The IgG1/IgG2 ratio of the pneumococcal PS-specific response is also altered when the PS is conjugated to a protein carrier; a higher proportion of IgG1 is seen after PS-conjugate than after pure PS (Soininen et al. 1999). A similar difference is shown to appear in IgA antibodies; the PS-specific antibodies mostly consist of the IgA2 subtype, whereas the protein-specific IgA is of the IgA1 isotype (Heilmann et al. 1988, Lue et al. 1988, Tarkowski et al.1990).

In children, the response to PS antigens is age-dependent, and maturation of the response differs with the type of PS antigen. The pneumococcal serotypes 3, 4, 8, 9N, and 18C induce good antibody responses in pre-school-age children and even in infants, whereas the responses to other Pnc serotypes tested remain poor (Koskela et al. 1982). It has also been suggested that children with recurrent AOM may have a reduced ability to respond with the IgG class antibody to the Pnc PS types most frequently causing AOM (Prellner et al. 1984). However, children with pneumococcal AOM have been shown to elicit serum antibodies to the infecting capsular serotype. Antibodies of the IgG and IgM isotypes have been detected in 25% of the children infected (Sloyer et al. 1974). Recent Finnish studies have shown serotype-specific IgG and IgM in serum even at the onset of the disease, and 36% of the children showed a twofold or greater increase in antibody concentration in the convalescent sera (Koskela et al. 1982); such responses were seen more frequently in older children. In a study by Virolainen et al. (1996) 29% of children with pneumococcal AOM showed a serum response to pneumococcal PS. In addition to AOM, pneumococcal PS-specific antibodies have been reported to be present in half the children with pneumonia five days after onset of the disease (Nohynek et al.1995). Furthermore, asymptomatic carriage of encapsulated bacteria has been shown to induce systemic PS-specific responses (Greenfield et al. 1972, Granoff et al. 1980, Gray et al. 1981). Because carriers are asymptomatic, the serum antibody response can be presumed to be a result of local antigenic stimulation of lymphocytes in the nasopharynx, i.e., mucosal immunisation, followed by clonal expansion and trafficking of antigen-secreting cells throughout the body.

The first experience with PS-protein conjugates was gained with Hib conjugate vaccines, which have proven both immunogenic and efficacious in preventing invasive Hib infections in small children and infants (Käyhty et al.1987, Lepow et al. 1987, Eskola et al.1985 and 1990, Ward et al.1990, Santosham et al.1991, Black et al. 1992, Booy et al.1994, Mäkelä et al. 1990 and 1995). Interestingly, vaccination with Hib has also been shown to reduce the nasopharyngeal carriage of Hib bacteria (Mohle-Boetani 1993, Murphy et al. 1993, Takala et al.1993), suggesting a protective activity for conjugate vaccines at mucosal surfaces.

4. 3. Mucosal antibodies to polysaccharide antigens

Parenteral administration of PS antigens also induces mucosal antibody responses (Lue et al. 1988, Kauppi et al. 1995). Mestecky has suggested that systemic administration of antigens boosts an effective S-IgA response only in situations where an immunised individual has previously encountered the same antigen, or cross-reacting antigens, via the mucosal route (Mestecky 1987). This hypothesis is supported by the studies of Svennerholm et al. (1980), who demonstrated that the S-IgA response in saliva and milk to a parenteral cholera vaccine was significantly higher in lactating Pakistani women, who were expected to be naturally exposed to cholera antigens, than in lactating Swedish women, not exposed to cholera. Enteric bacteria colonising the intestinal mucosa also have cross-reacting antigens with encapsulated respiratory pathogens and can thus give rise to antibodies cross reacting with capsular PS of Pnc and Hib (Robbins et al. 1972 and 1975). On the other hand, the PS antigens are shown to be widely dispersed in body fluids (Spinola et al. 1986, Darville et al. 1992), which would allow parenterally administered PS to reach the lymphatic inductive tissues at mucosal sites and induce local S-IgA production, even without previous exposure to the antigen at mucosal sites.

Although PS antigens are unable to induce serum antibodies in infants and small children, mucosal antibodies can be elicited even at an early age. Mucosal antibodies have been detected in children less than one year of age after systemic Hib infection (Pichichero et al.1981). Detection of the mucosal immune response, which is independent of the systemic response, suggests that functional maturation of the mucosal immune system takes place earlier in ontogeny than that of the systemic immune system, at least in respect to PS antigens (Pichichero et al.1981). Immunisation with PRP vaccine also induces greater serum antibody responses in adults than in children (>18 months of age), but induces comparable S-IgA responses in saliva and nasal washes, further suggesting earlier maturation of the mucosal immune system (Pichichero et al.1983).

PS-specific antibodies induced in secretions after immunisation are mostly IgA (Pichichero et al.1983, Lue et al.1988, Kauppi et al. 1995). In contrast to protein antigens that induce IgA1-dominant S-IgA responses, the IgA response to polysaccharide antigens is dominated by IgA2 in adults (Mestecky and Russel 1986). In

children, however, the S-IgA response to conjugated PS is mainly IgA1 (Kauppi-Korkeila et al. 1998, Korkeila et al. 1999).

It is generally believed that administration of antigens via the mucosal route results in better mucosal antibody responses than does systemic administration. Polysaccharides cannot, however, be administered mucosally as such for antibody induction. Therefore mucosal adjuvants are needed, in order to allow the PS antigen through the mucosal barrier to reach the lymphatic tissues and to enhance the release of immune mediators needed for antibody production (Mestecky et al. 1997). Very little is yet known about the immunogenicity of PS-antigen or PS-protein conjugates in humans when administered via the mucosal route. Several experimental studies, however, have demonstrated induction of both mucosal and systemic antibody responses after mucosal immunisation with these antigens. Intranasal or oral administration of bacterial PS-containing liposomes in mice has been shown to induce PS-specific S-IgA in the lung (Abraham 1992) and in the faeces when cholera toxin (CT) has been co-administered orally with PncPS (Van Cott et al.1996). Furthermore, the S-IgA response in the lung has been shown in the mouse when PncPS is conjugated to cholera toxin subunit B (CTB) and administered orally or intranasally, and the response has been enhanced by entrapping the conjugate in alginate microspheres (Seong et al. 1999). Intranasal administration of PncT in mice, together with a glyceride-polysorbate adjuvant, has also been shown to elicit S-IgA in saliva (Jakobsen et al. 1999).

Moreover, pneumococcal AOM in children has been shown to elicit a PS-specific IgA response both in MEF (Sloyer et al. 1974, Karjalainen et al. 1990) and in nasopharyngeal aspirates (NPA) (Virolainen et al. 1995). In the early phase of AOM, IgG and IgM antibodies that seem to be derived from serum appear in the middle ear, and a local S-IgA response to pneumococcal PS has been shown to develop slowly during the course of AOM (Karjalainen et al.1990). However, even more efficient induction of mucosal antibodies may be induced in children after symptomless Pnc carriage than after AOM. In a recent study, salivary antibodies were detected in 65% of the children carrying Pnc and in 53% of the children with pneumococcal AOM (Kauppi et al.1998).

4. 4. Antibody-secreting cells

The induction of antibody responses can also be studied by measuring the number of antibody-secreting cells (ASC) in various lymphatic tissues or in the peripheral blood. As the B cells are constantly circulating through the lymphatics and blood back to the peripheral tissues (Gowans et al. 1964), cells committed to mucosal sites are also present in the peripheral blood for a limited period of time, before homing to different exocrine tissues. The appearance of these antigen-specific ASC can thus be measured in peripheral blood after an antigen challenge. It is generally beliewed that antigens introduced to the host via mucosal sites may induce IgA-dominant ASC responses and S-IgA responses, whereas parenterally administered antigens may typically induce IgG-dominant ASC responses and systemic IgG in serum. However, this is not the case in

respect to PS antigens that tend to induce IgA-dominant responses even when administered parenterally. In adults, an ASC response to PS antigens has been shown to consist mostly of IgA-secreting cells (Kerl and Fauci 1983, Heilman and Pedersen 1986, Munoz and Insel 1987, Lue et al. 1988, Tarkowski et al. 1989), whereas the response detected to protein antigens is clearly dominated by IgG ASC (Lue et al. 1994).

As mentioned above, Mestecky and Russel (1986) have suggested that the IgA-dominant response seen after systemic administration of the PS antigens is due to earlier contact with the same, (or cross-reacting) antigen at a mucosal site. Thus, B-cells would be stimulated in lymph nodes near the injection site, and cells already committed to IgA production would then migrate via the circulation to mucosal sites to secrete IgA. Another explanation is that PS antigens may reach mucosal sites and lymphatic tissues in which the micro-environment favours IgA commitment of B cells (Strober et al. 1991,Weinstein and Cebra 1991).

PS conjugated to a protein carrier is shown to induce higher numbers of anti-PS ASCs in the peripheral blood than does pure PS. Furthermore, the IgG /IgA ratio of the ASC response is increased as compared to the response to PS (Lue et al. 1990). The IgA-ASC response detected in adults is mostly of the IgA2 isotype after administration of PS antigens, in contrast to the IgA1 isotype seen in response to protein antigen. Studies with pneumococcal serotype 12F showed higher IgA1 ASC responses when the PS was conjugated to a carrier protein than with PS alone (Lue et al. 1990), but the IgA-ASC response after a PS conjugated to a protein carrier is still dominated by the IgA2 isotype (Lue et a.l 1990, Tarkowski et al. 1990). In infants, the IgA response is shown to consist mostly of IgA1-secreting cells after immunisation with a PS conjugate vaccine (Barington et al. 1994).

5. Pneumococcal vaccines

5. 1. Systemic vaccines

The first immunisation studies with purified pneumococcal PS were carried out in the 1930's, after the central role of capsular PS as a pneumococcal virulence factor (Dubos and Avery 1931) and the immunogenicity of PS (Francis and Tillet 1930) were discovered (reviewed by Fedson et al.1999). The first pneumococcal vaccine was generated by MacLeod and his co-workers in 1945 (MacLeod et al.1945). Today, the only pneumococcal vaccines that are registered and in use are the 23-valent PS vaccines: Pneumovax^{R} (MSD), Pnu-immune^{R} (Wyeth Lederle vaccines), and Pneumo 23^{R} (Pasteur-Mérieux-Connaught). The types included in these comprise 90% of the serotypes causing invasive disease in developed countries. These vaccines are immunogenic and protective against invasive pneumococcal infections in adults and in older children (Austrian et al. 1976, Leinonen et al. 1982, Leinonen et al. 1986, Riley et al. 1986, Sims et al.1988, Shapiro et al. 1991). In children less than two years of age, however, they are not immunogenic (Mäkelä et al. 1983, Leinonen et al. 1986).

The first experimental studies with PS-protein conjugates were already being carried out in the late 1920's and 1930's (Avery and Goebel 1929, Goebel 1939). The present pneumococcal PS conjugate vaccines (Table 2), however, have been developed and designed during the last few decades, based on the experience acquired from conjugate technology gained with the Hib conjugate vaccines (Schneerson et al.1980). Pneumococcal conjugates have proven immunogenic in small children and infants (Käyhty et al. 1995, Åhman et al. 1996, Anderson et al.1996, Dagan et al.1997, Åhman et al.1998, Rennels et al.1998).These vaccines have also been shown to be immunogenic in high-risk children, e.g., in children with recurrent respiratory infections (Sorensen et al. 1998) and in children with recurrent AOM (Breukels et al. 1999). Recently, pneumococcal PS conjugate has been shown to be efficacious in preventing invasive infections in children and infants (Black et al.1998) and in reducing the nasopharyngeal carriage rates of pneumococci (Dagan et al.1996). In addition, some preliminary data suggest a 7% reduction in all AOM visits, irrespective of etiology (Black et al.1999). The specific data on pneumococcal AOM still remains to be studied. Finally, whether the reduced mucosal carriage is due to serum antibodies or induction of mucosal response needs elucidation.

5. 2. Mucosal vaccines

As mucosal administration of antigens is expected to be more effective in inducing protection at mucosal sites than the systemic administration, intensive studies are aimed at the development and testing of mucosal vaccines. Adjuvants that augment penetration of antigens through the mucosal layers to reach lymphatic tissues and enhance the immunological responses to vaccines administered via mucosal routes play a key role in this process. Another important advantage of mucosal vaccination would be its convenience for those immunised, not to mention the easier performance of the vaccination programs. The most practical routes of mucosal vaccination in humans would be oral and nasal administration of antigens. For prevention of respiratory infections, the most logical administration would be the intranasal route. Several experimental studies with pneumococcal PS or PS-conjugates administered intranasally or orally have been carried out, and mucosal S-IgA and systemic antibody responses demonstrated in the mouse and rat (Abraham 1992, van den Dobbelsteen et al. 1992 and 1995, Van Cott et al.1996, Flanagan et al. 1999, Seong et al. 1999, Jakobsen et al. 1999). Human studies are, however, still scarce, partly because of the lack of mucosal adjuvants acceptable for human use.

Two potent enterotoxins, cholera toxin and *Escherichia coli* heat-labile enterotoxin, are powerful mucosal adjuvants that augment S-IgA and systemic IgG responses to co-administered antigens (Holmgren et al.1993, Levine and Dougan 1998). Mutants that show reduced toxicity, but still sustain sufficient adjuvant activity, have been engineered from the wild-type forms (Douce et al.1995, Douce et al.1997, Fontana et al. 1995). Another kind of nasal adjuvant based on caprylic-capric glycerides dissolved in polysorbate 20 and water, Rhino Vax, is a non-toxic adjuvant and thus acceptable for human use (Gizurarson et al. 1996). It enhances transepithelial flux and has been

proven to induce immunogenicity to protein antigens: diphtheria and tetanus toxoids, after intranasal administration in humans (Aggerbeck et al. 1997). Recently, this adjuvant was also shown to enhance the systemic and mucosal responses after intranasal administration of pneumococcal conjugate vaccine, PncT, in mice and to protect mice against invasive pneumococcal infections (Jakobsen et al. 1999). Protection against colonisation and invasive disease has also been shown in mice after intranasal immunisation with pneumococcal PS conjugated to the cholera toxin B subunit (Seong et al. 1999). In addition to adjuvants mentioned above, novel strategies, including enclosure of antigens in biodegradable microspheres, proteosomes, or liposomes, or their expression in viral and bacterial vectors, and even in plants, are currently under research in modern vaccinology (reviewed by Mestecky et al. 1997 and Levine and Dougan 1998).

AIMS OF THE STUDY

The aim of the thesis was to characterise the circulating antibody-secreting cell (ASC) response and the mucosal antibody response induced by pneumococcal polysaccharide (PncPS) antigens

- in adults and toddlers immunised parenterally with pneumococcal vaccines
- in children with pneumococcal acute otitis media (AOM)

and to evaluate the relevance of the ASC response as a marker of the local IgA response.

To this end we measured

- the number of PncPS specific ASCs in the peripheral blood
- the PncPS specific antibodies in saliva and nasopharyngeal aspirates (NPA)

and compared the results of the two measurements. The overall immunogenicity of the vaccines was evaluated by measuring the serum antibody response to PncPS.

MATERIAL AND METHODS

This thesis consists of four separate studies: three vaccine studies (I-III) and one study on acute otitis media (IV). Each vaccine study was carried out with healthy volunteers, each of them participating in only one of the studies. The vaccines and the subjects for each of the vaccine studies are shown in Table 2. In two of them the study subjects were adult volunteers (I-II,) and in one the subjects were toddlers (III). The otitis media patients were children aged 9 to71 months.

1. Study subjects

1. 1. Vaccinees

The adult volunteers (n=40) participating in the studies were healthy students or laboratory personnel (aged from 18 to 56 years). None of them had received a pneumococcal vaccine earlier. During the study they were vaccinated intramuscularly in the deltoid muscle (dose 0.5 ml). In the first study, eight volunteers were vaccinated with the PncPS vaccine, and ten volunteers with the PncOMPC vaccine (I). In the second study, 22 volunteers were vaccinated with PncD or PncT vaccine in a randomised, double-blind trial (II) (Table 2).

A group of 40 healthy children, mean age 24 months (range 23 to 25 months), were enrolled in the third study and vaccinated intramuscularly in the deltoid muscle, each receiving one of the four vaccine formulas in a randomised, double-blind trial (Table 2). No other vaccines were given at the same time. All the children had previously received the immunisations recommended by the Finnish national immunisation programme, i.e., DTP (diphtheria-tetanus-pertussis vaccine) at the ages of 3, 4, and 5 months, Hib conjugate vaccine at the age of 4, 6, and 14 to18 months, and the inactivated polio vaccine at the age of 6 and 12 months.

1. 2. Patients with acute otitis media

Streptococcus pneumoniae was cultured in the MEF of 30 children with AOM enrolled in a study on pathogenesis of AOM at the Department of Otolaryngology at the Helsinki University Hospital during the winter of 1991-1992 (Virolainen et al. 1994). Samples for this analysis were available from 17 of them. Children, aged 9 months to 5 years (median 4 years), were defined as having AOM on the basis of pneumatic otoscopic findings suggesting middle ear fluid (MEF) behind an inflamed tympanic membrane, and at least one of the following symptoms of acute infection: otalgia, tugging at or rubbing of the ear, rectal/axillary temperature at least 38.0°C, irritability, restless sleep, acute gastrointestinal symptoms (vomiting or diarrhoea), or other simultaneous respiratory infection. Patients with secretory otitis media, tympanostomy tubes, spontaneous perforation of the tympanic membrane, or antibiotic treatment within one week before enrolment were excluded.

2. Vaccines

The pneumococcal polysaccharide vaccine PncPS (Pneumovax^{R}, Merck Sharp & Dohme, West Point, PA), is a mixture of capsular polysaccharides of 23 different types (25 µg/dose of each). The pneumococcal conjugate vaccine PncOMPC (Merck Research Laboratories) is a mixture of four pneumococcal capsular polysaccharides, each separately conjugated to the meningococcal outer membrane protein complex of group B *N. meningitidis*. The polysaccharide/protein ratio in the vaccine varies from 0.11 to 0.17 depending on the serotype. The pneumococcal conjugate vaccine, PncD, (Pasteur-Mérieux-Connaught, USA) is a mixture of four pneumococcal polysaccharides conjugated to diphtheria toxoid. The PncT vaccine (Pasteur-Mérieux-Connaught, France) is a mixture of four conjugates consisting of the pneumococcal polysaccharides, each separately conjugated to tetanus protein. Two different dosages of the PncD and PncT conjugates were used: each dose of the vaccines contained either 3µg (PncD03 and PncT03) or 10µg (PncD10 and PncT10) of each polysaccharide. The PS/protein ratios for each of the PS varied from 2.0 to 3.1 in PncD and from 1.4 to 2.2 in PncT, respectively. No adjuvants were used in any of the vaccines.

Table 2. Vaccines used in the studies

Study vaccines	Study	PS/dose	serotypes	carrier protein	The number of vaccinees	
PncPS[1)]	I	25µg	23 serotypes[2)]	none	8	adults
PncOMPC	I	1µg	6B, 14, 19F, 23F	the meningococcal outer membrane protein complex of *N. meningitidis*	10	adults
PncD	II	10µg	6B, 14, 19F, 23F	diphtheria toxoid	12	adults
PncT	II	10µg	6B, 14, 19F, 23F	tetanus protein	10	adults
PncD	III	3µg	6B, 14, 19F, 23F	diphtheria toxoid	10	toddlers
PncT	III	3µg	6B, 14, 19F, 23F	tetanus protein	10	toddlers
PncD	III	10µg	6B, 14, 19F, 23F	diphtheria toxoid	10	toddlers
PncT	III	10µg	6B, 14, 19F, 23F	tetanus protein	10	toddlers

1) Pneumovax^{R}

2) 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F

3. Specimen collection

3. 1. Vaccine studies

Blood samples from the vaccinees were collected for enumeration of antibody- and immunoglobulin-secreting cells (ASC and ISC) and for antibody measurement before and after immunisation (Table 3). ASC/ISC analysis was performed on 10 to 20 ml of

heparinized blood within an hour from sampling. Serum samples (5 ml of serum) were stored at -20° C until analyzed. Unstimulated saliva samples (1 ml) were collected from the vaccinees with a plastic pipette and stored at -70°C until analyzed. Frozen samples were thawed only once, when analyzed. Nasopharyngeal samples were collected by swabbing (Transpocult, Orion Diagnostica, Espoo, Finland) on days 0 and 28 and transported to the bacteriological laboratory to test for carriage of *Streptococcus pneumoniae*.

Table 3. Schedule for sampling

		days after immunisation		
Vaccine	Study	blood for Elispot	serum for EIA	saliva for EIA
PncPS/PncOMPC	I	0, 5, 7, 9, 14, 28	0, 28	0, 5, 7, 9, 14, 28
PncD/PncT; adults	II	0, 7, 28	0, 28	0, 7, 28
PncD/PncT; toddlers	III	7	0, 7, 28	0, 28

3. 2. Acute otitis media study

Serum and nasopharyngeal aspirate (NPA) samples from the AOM patients were obtained at the initial visit in the acute phase, and at the control visit 2 weeks later. The NPA sample was collected by insertion of a suction catheter through a nostril as described by Virolainen et al. (1995a). MEF samples were obtained at the initial visit only. A whole-blood sample for ASC analysis was taken on day 7 after the initial visit in order to asses the peak number of ASC.

The nasopharyngeal swabs and MEF and NPA samples were cultured and the isolated bacteria identified by standard methods (Balows et al. 1991). Pneumococcal strains were serotyped/grouped by counterimmunoelectrophoresis (CIE) or latex agglutination (groups 7 and 14) (Leinonen et al. 1980) with type-specific antisera from Statens Seruminstitut, Copenhagen, Denmark.

4. Serological assays

4. 1. Antigens for the assays

Pneumococcal capsular polysaccharide antigens (serotypes 6B, 9, 14, 19F, 23F) were from the American Type Culture Collection, Rockville, MD, USA. Pneumococcal C-polysaccharide (CPS) was obtained from Lederle-Praxis, West Henrietta, NY, USA. Diphtheria toxoid and tetanus toxoid were produced by the Vaccine Laboratory at the National Public Health Institute, Helsinki, Finland. In the AOM study (IV), ASCs and antibody concentrations were measured against the same type/group as cultured in the MEF and against one heterologous control serotype (either 6B, 14, 19F or 23F).

4. 2. Antibody-/Immunoglobulin-secreting cells

Specific antibody-secreting cells (ASCs) to pneumococcal polysaccharide and to both diphtheria and tetanus toxoids, as well as the number of total immunoglobulin-secreting cells (ISCs), were measured with an enzyme-linked immunospot assay (ELISPOT) (Czerkinsky et al. 1983, Sedgwick and Holt 1983). The mononuclear cells were separated from heparinised blood with Ficoll-Paque density gradient centrifugation (Pharmacia, Uppsala, Sweden) and adjusted to a concentration of 2 x 10^6 cells/ml in RPMI culture medium supplemented with 10% heat-inactivated fetal calf serum, gentamicin (15 µg/mL), and L-glutamine (3 mg/ml). Microtiter plates were coated with pneumococcal polysaccharides as for EIA (Käyhty et al. 1995) and blocked with 1% bovine serum albumin (BSA) in PBS for 30 min at 37ºC. For enumeration of diphtheria- and tetanus-toxoid-specific cells, the plates were coated with diphtheria toxoid: 2.5 Lf/ml and tetanus toxoid: 0.5 Lf/ml.

Lymphocytes were allowed to secrete antibodies in the wells. Cells secreting IgA, IgG, and IgM were measured from the vaccinees (I-III) and cells secreting IgA, IgA_1, IgA_2, IgG, and IgM were measured from the AOM patients (IV). Monoclonal antibodies to human IgA (Oxoid M26012, Unipath Ltd, Hampshire, England), IgA_1 or IgA_2 (Nordic, MaHu/Iga_1/asc NI69-11 or MaHu/IgA_2/asc NI 512, Tilburg, the Netherlands), were added to the wells, and the plates were incubated overnight at room temperature before alkaline phosphatase-conjugated antisera to mouse IgG (Jackson H&L 315-055-045, West Grove, PA) was added. Alkaline phosphatase-conjugated porcine anti-IgG and anti-IgM antibodies (Orion) (I, IV) or goat anti-IgG and anti-IgM antibodies (Sigma; A3188, A3437) (II, III) were used. The substrate (5-bromo-4-chloro-3-indolyl-phosphate, Sigma) was applied in agarose. Spots were counted under low magnification.

Results were expressed as the number of IgA, IgA_1, IgA_2, IgG, and IgM ASC/10^6 cells. The peak number of ASCs, detected on day 7 or 9, was the chosen value for the ASC response. Five or more ASCs/10^6 cells on day 7 after immunisation was considered a response.

4.3. Measurement of antibodies

Type-specific pneumococcal capsular polysaccharide antibodies were measured by EIA in paired saliva (I-III), NPA (IV) and serum samples (I-IV) after neutralisation of CPS antibodies (Käyhty et al. 1995, Virolainen et al. 1995b, Virolainen et al. 1996, Åhman et al. 1996), which can be induced by the pneumococcal vaccines (Skov Sorensen and Henrichsen 1984). For the vaccine studies, the reference serum pool, 89-SF, (Quataert et al. 1995) was used, and the serum antibody concentrations were expressed as ug/ml. The saliva results in Study I and the NPA results in the AOM study (IV) were expressed as end-point titers read at an optical density (OD) of 0.3. Saliva and NPA samples were centrifuged at 15 000 rpm for 10 min at room temperature before analysis. The supernatant was used for pneumococcal enzyme immunoassays (I-IV) and measurement

of total IgA by a radial immunodiffusion technique (Mancini) (LC-Partigen IgA, Behringwerke, Marburg, Germany) (II-IV). For measuring IgA and secretory component (SC), monoclonal antibodies to human IgA (Oxoid M26012) and to human SC (Sigma 1-6635) were used, followed by alkaline phosphatase-conjugated antiserum to mouse IgG (Jackson H&L 315-055-045). Saliva and NPA samples were analyzed in triplicates with plates coated with PBS used as blank plates for each assay (Kauppi et al. 1995, Virolainen et al. 1995).

The specific IgA concentrations in saliva were divided by the concentration of total IgA in each sample and expressed as ng of specific IgA /ug of total IgA to standardise the degree of dilution in each sample (II-III). Samples with undetectable anti-Pnc polysaccharide IgA were assigned values that were one half log less than the detection limit for each pneumococcal serotype: 1 ng/ml for serotypes 6B, 14, 23F, and 2.5 ng/ml for serotype 19F. In Study I, the saliva results are given as EIA-units (OD-readings). A twofold rise (specific/total IgA) between day 0 and 28 was regarded as a response (I-III). The saliva results for specific IgG were given as ng/ml (II-III). Samples with undetectable anti-Pnc polysaccharide IgG were assigned the values (half log less than the detection limit) 2 ng/ml for serotype 6B and 3 ng/ml for serotypes 14, 19F, and 23F. The NPA results for specific IgA in the AOM patients were calculated by subtracting the background OD from the specific OD, and dividing by concentration of total IgA in the sample (IV). If the total IgA was below the detection limit (4.5 IU/ml), the value 2.25 IU/ml was used. A threefold rise between the acute and convalescent phase results (specific/total IgA) was regarded as a response. The NPA results for specific IgG and IgM were calculated as above but not compared to total IgA.

5. Statistical analyses

The results are given as geometric mean (GM) of ASCs or antibody concentrations (GMC).The comparison of the mean in the different vaccine groups (for each serotype) was done with ANOVA, by use of log-transformed data. If significant differences were found, pairwise comparisons between vaccine groups were performed by the Bonferoni test (adults), LSD-test (least significant difference; assuming equal variances) (III), or Tammhane's T2-test (assuming unequal variances) (III). The T-test was used when toddlers were compared to adults. The significance of the correlation between the number of ASCs and the antibody concentrations was estimated by Pearson's or Spearman's correlation (I-III). Proportions of children with antibodies or antibody responses to each of the serotypes were compared with McNemar’s test (III). The paired t-test with log transformed data was used when the ASC responses of the AOM patients were compared in the two age-groups (IV).

RESULTS

1. Antibody-secreting cell response

1.1. ASC response in the vaccinees

Altogether 40 adult volunteers and 40 toddlers were vaccinated with one of the study vaccines (Table 2). Few pneumococcal polysaccharide-specific ASCs could be detected in the peripheral blood on day 0 in adults (I, II). After immunisation, their number increased rapidly, so that the peak number of ASCs was seen on day 7 or 9 after immunisation (I). On day 7 after immunisation ASCs were seen in all vaccinees to each of the serotypes (I, II). Thereafter, the number of ASCs decreased, and on day 28 no ASCs could be detected (I). The peak number of ASCs varied by vaccine used. As a whole, the responses in adults were higher after PncT and PncD conjugates than after PncPS and PncOMPC conjugate ($p<0.001$, except: $p=0.034$ for serotype 14 and $p=0.004$ for serotype 19F in the comparison of PncPS with PncD, and $p=0.004$ for serotype 14 in the comparison of PncPS with PncT) (Fig 4).

In toddlers, the ASC response was studied only on day 7 after immunisation. Pneumococcal polysaccharide-specific IgA, IgG, and IgM ASCs could be detected in all the 40 vaccinees (III). This was true for all the serotypes (for serotype 23F only 29 samples were analyzed), with one exception (one child did not respond to serotype 19F). In toddlers the responses were lower than in adults after PncD and PncT ($p<0.001$, except for $p=0.015$ for serotype 14 in comparison with PncT responses), but comparable to those seen in adults after PncPS (Fig 4).

The ASC response consisted mostly of IgA- and IgG-secreting cells, whereas the number of IgM ASCs remained low (2-28 ASC/10^6 cells) (I-III). The dominant antibody class in the ASC response in most of the cases was IgA (Fig 4). In adults, this was true for all four serotypes after immunisation with PncPS and PncD and for two serotypes after PncOMPC (I, II). In response to PncT, however, the number of IgG ASCs exceeded the number of IgA ASCs (II). The peak number of IgA-ASCs (GM) was higher after vaccination with PncPS than after PncOMPC, but the number of IgG ASCs did not differ between PncPS and PncOMPC (I) (Fig 4). On the other hand, the number of IgA ASCs did not differ between PncD and PncT, but the number of IgG ASCs was 3.0 to 4.4-fold higher after immunisation with PncT than after PncD; the difference was statistically significant for serotypes 6B, 19F, and 23F ($p=0.02$). In toddlers, the ASC responses to all the serotypes were dominated by IgA (III) (Fig 4).

In the PncD and PncT vaccine groups we also measured ASC response to the carrier proteins, diphtheria toxoid or tetanus protein (II, III). The ASC responses were completely specific: no ASCs to diphtheria toxoid were detected in vaccinees immunised with PncT nor were any ASCs to tetanus protein found in vaccinees immunised with PncD. The ASC responses to the carrier proteins were clearly dominated by IgG in all the vaccinees (Fig 5).

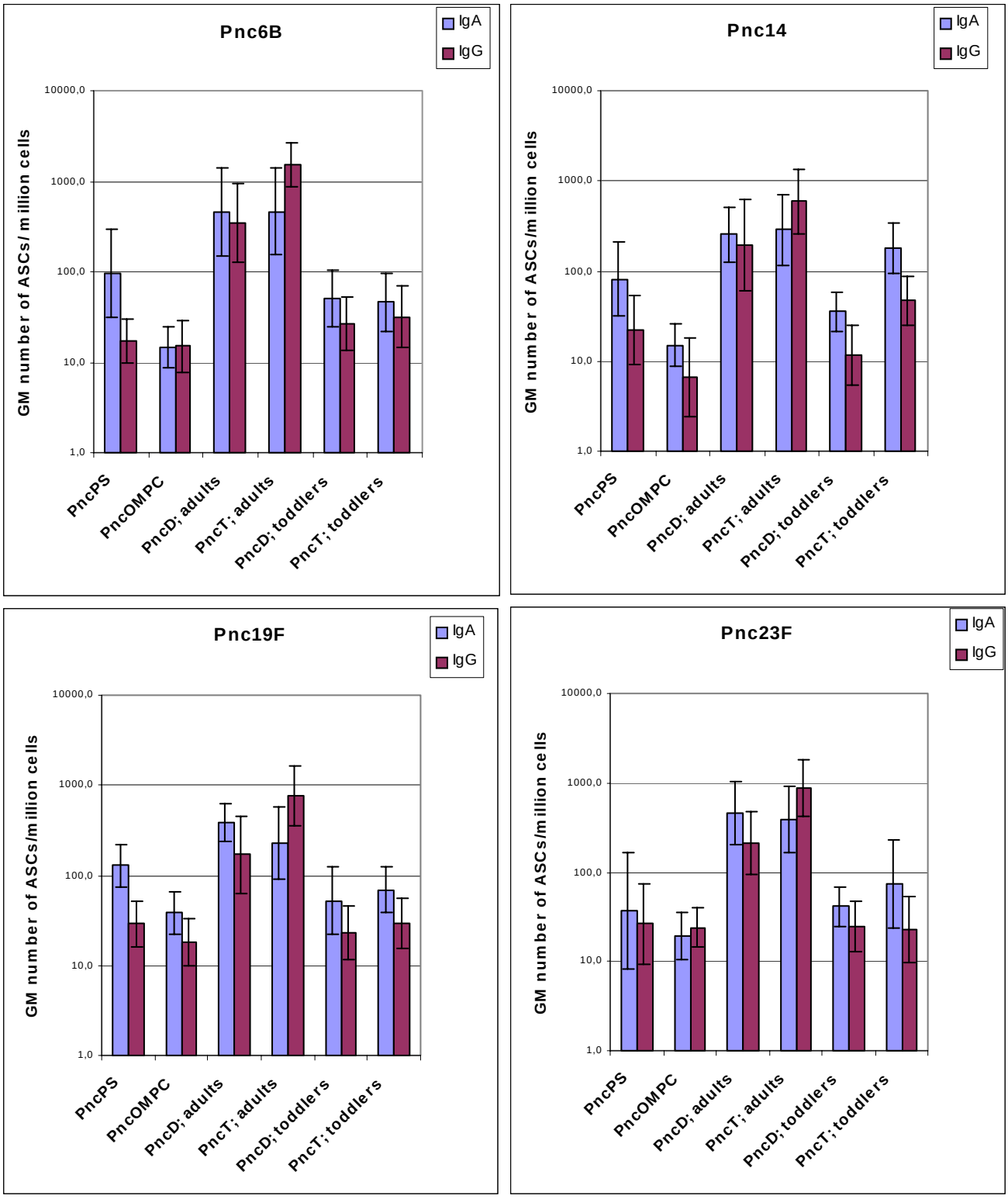

Figure 4.
The geometric mean number of IgA- and IgG-ASCs to pneumococcal serotypes 6B, 14, 19F, and 23F on day 7 after parenteral immunisation with the pneumococcal vaccines (with 95% confidence intervals shown).

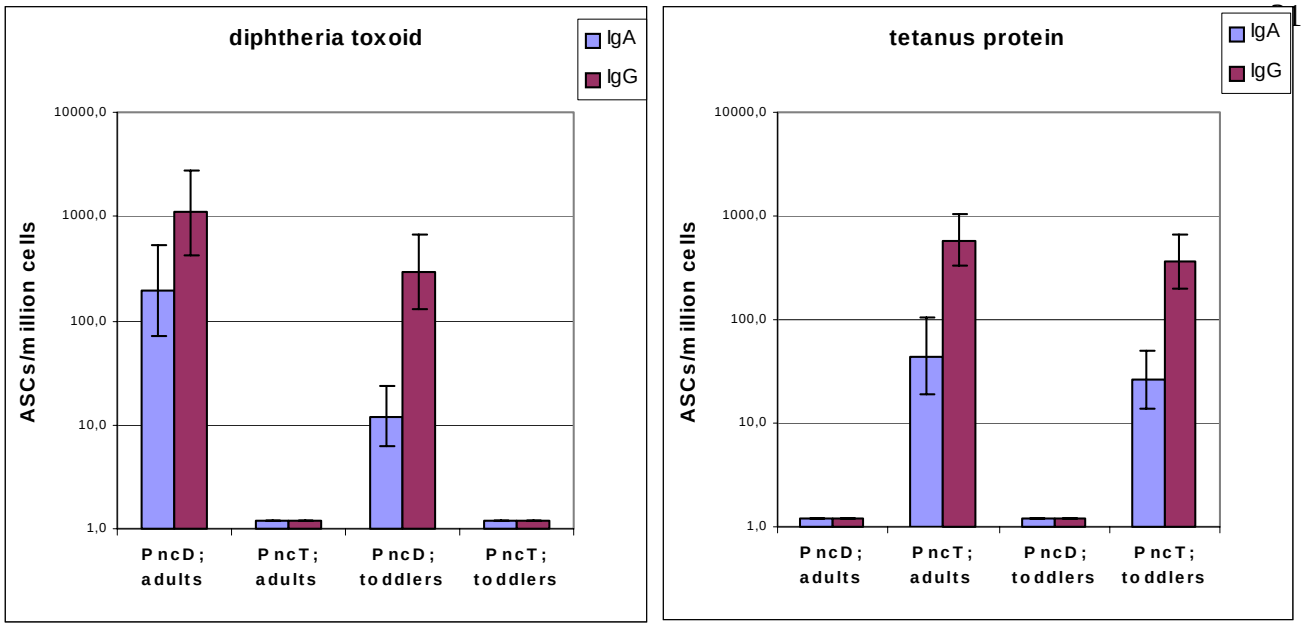

Figure 5.
The geometric mean number of ASCs to the carrier proteins (diphtheria or tetanus toxoid) on day 7 after immunization with the pneumococcal conjugate vaccines PncD and PncT in adults and toddlers (with 95% confidence intervals shown).

1. 2. ASC response in children with pneumococcal acute otitis media

ASCs to the capsular PS of the pneumococcal type isolated from the MEF could be detected in all of the 17 patients studied, varying from 7 cells to 1100 ASC/10^6 cells (all immunoglobulin classes combined). The geometric mean of the ASC response was clearly age-dependent; whereas the overall geometric mean (GM) was 32 ASC/10^6 cells, it was 63 ASC/10^6 cells in children older than 24 months and 18 ASC/10^6 cells in these vaccinees ($p = 0.03$). Seven (78%) of the nine older children, and three (38%) of the eight younger children had at least 15 ASC/10^6 cells. The ASC response was independent of gender.

Three children, all younger than 24 months, had their first AOM episode during the study (IV: Table). The causative agents were of serogroups 6 and 19 (two children). These children had only a low or no ASC response, but did not differ significantly from the other five children in this age-group (IV: Table). On the other hand, four older children had had at least 10 previous AOM episodes. Their infection was caused by group/type 9, 14 (two cases), or 19, and they all had an ASC response, although a weaker one than that of each of the other five children in this age-group.

In ten of the children in the study, the dominant antibody class was IgA. IgG-ASC were dominant in one child and IgM-ASC in another; both of these children had had over 10 AOM episodes in their lifetimes.

In order to confirm the specificity of the ASC response in the AOM patients, we enumerated the ASC response to a Pnc-type (6B, 14, 19F, or 23F) different from the

type cultured in MEF. Most of the ASCs in the control assays were IgA-producing cells (IV: Table), and IgG or IgM-ASC were detected in only few patients. The number of IgA-ASCs thus detected was less than 10 ASC/10^6 cells in all the children, supporting the specificity of the assay (Table in IV).

Table 4. Number of vaccinees with IgA antibodies in saliva before (pre) and after (post) vaccination and the number of those with a twofold or higher increase in salivary IgA concentration.

	number of positive		response		number of positive		response	
Pnc-type	pre	post	number	%	pre	post	number	%
	PncPS, n=8; adults				PncOMPC, n=10; adults			
6B	3	4	2	25	3	3	-	-
14	7	7	2	25	8	6	-	-
19F	6	7	2	25	8	7	1	10
23F	2	2	2	25	5	3	-	-
	PncD, n=12; adults				PncT, n=10; adults			
6B	2	4	5	42	2	3	2	20
14	7	10	5	42	6	8	3	30
19F	7	9	5	42	5	6	3	30
23F	4	6	6	50	7	7	2	20
	PncD, n=20; toddlers				PncT, n=19; toddlers			
6B	0	4	2	11	2	9	8	50
14	2	2	1	6	-	11	10	67
19F	3	12	7	37	4	14	11	69

2. Antibodies to pneumococcal polysaccharide in saliva (Tables 4 and 5)

Pneumococcal PS-specific IgA antibodies were already detected in the saliva of most of the adult vaccinees before immunisation, most frequently to serotypes 14 and 19F (I, II). In toddlers, PS-specific IgA antibodies were detected only occasionally before immunisation (III). The increases in the IgA concentrations in adults were modest, but could be seen on day 7 after immunisation (I, II). A total of eight IgA responses (>twofold increase in salivary IgA concentration) was seen in six of the eight vaccinees (25% of the measurements showed a response) who had received PncPS, but only one response was seen in the ten vaccinees who had received PncOMPC (I). The responses were seen with equal distribution among the four serotypes (I). The 22 adults who had received PncD or PncT showed a salivary response in 32% of the measurements (II). The specific IgA concentration on day 28 ranged from <3 ng/ml to 222 ng/ml. The toddlers receiving PncD or PncT showed a more than twofold increase in 35% of the measurements, most frequently to serotype 19F; the specific IgA concentrations in saliva ranging from <3 ng/ml to 52 ng/ml on day 28.

Salivary IgA antibody concentrations showed an excellent correlation with the secretory component (SC) concentrations, indicating that the IgA measured was secretory in nature. This was true both before and after (day 28) immunisation (r=0.93-0.97; I-III).

Table 5. Number of vaccinees with IgG antibodies in saliva before (pre) and after (post) vaccination

	number of positive			number of positive		
Pnc-type	pre	post	% post	pre	post	% post
	PncPS, n=8; adults			PncOMPC, n=10; adults		
6B	-	1	13	1	2	20
14	-	1	13	2	4	40
19F	-	2	25	3	4	40
23F	-	-	-	-	-	-
	PncD, n=12; adults			PncT, n=10; adults		
6B	-	7	58	-	8	80
14	-	3	25	-	4	40
19F	2	7	58	-	2	20
23F	-	7	58	-	6	60
	PncD, n=20; toddlers			PncT, n=19; toddlers		
6B	-	-	-	-	1	5
14	-	-	-	-	-	-
19F	-	1	5	-	1	5

IgG antibodies were rarely detected in saliva before vaccination. In adults, IgG antibodies could be detected on day 7, and more frequently on day 28. IgG responses were seen in one of the eight volunteers who had received PncPS, in three of the ten who had received PncOMPC, in seven of the twelve who received PncD and in eight of the ten who received PncT. In toddlers, IgG antibodies were not detected in any of the saliva samples before immunisation, and were found in only two samples even after immunisation (day 28).

3. Antibodies to pneumococcal polysaccharide in serum

Pneumococcal PS-specific IgG antibodies were already present in the serum of all the adult vaccinees before immunisation (I, II). The PncPS, the PncD, and the PncT vaccines induced serum antibody responses to all the four PncPS studied. The GM fold increase in IgG concentration varied from 3.5- to 6.0-fold after PncPS (I: Table 2) and from 10- to 22-fold after PncD/PncT (II: Table 3), depending on the serotype. Responses after PncOMPC were lower, less than 3-fold to each of the serotypes (I: Table 2). The IgM responses remained lower in response to each of the vaccines. The IgA concentrations were low before immunisation, below the detection limit in most of the vaccinees. The GM increase in IgA concentration varied from 1.0- to 2.5-fold after

PncOMPC (I: Table 2) and from 2.4- to 7.7-fold after the other vaccines (II: Table 3), depending on the serotype.

In toddlers, IgG antibodies were detected in only 25 to 40% of the vaccinees before immunisation, depending on the serotype. IgM antibodies were present in 65 to 83% of the 40 children. After immunisation, increased concentrations of IgM antibodies were detected in virtually all vaccinees, and IgG antibodies were detected in 75 to 95%, depending on serotype. Serotype-specific IgA antibodies were very rarely present in the serum of the toddlers before immunisation (serotype 19F-specific antibodies detected in three of the 40 vaccinees). After immunisation, IgA antibodies were detected in 63 to 68% of the vaccinees on day 7 after immunisation and in 38 to 45% of the vaccinees on day 28 (III: Table 1). Thus the GM IgA antibody concentration was higher on day 7 than on day 28 (III: Fig 4). This was true for each of the vaccines (data not shown) and for each pneumococcal serotype studied. The antibody concentrations in toddlers on day 28 ranged from 0.9 µg/ml to 6.6 µg/ml for IgM and from 0.5 µg/ml to 6.4 µg/ml for IgG (III: Fig 4). On day 7, the GM IgA concentration ranged from 0.2 µg/ml to 1.1 µg/ml, depending on vaccine and on the pneumococcal serotype measured.

4. Serotype-specific responses

Only a little variation in the ASC response was observed between the four serotypes in each of the vaccine groups (Fig 4), whereas serum antibody responses varied by pneumococcal serotypes. Thus, low serum antibody responses were often detected to serotype 6B, (especially after PncPS and PncOMPC). The highest responses, in general, were to serotypes 14 and 19F in adults and to serotype 19F in toddlers. In adults, the secretory IgA antibodies in saliva were mostly to serotypes 14 and 19F before immunisation, but the responses after vaccination occurred equally in all serotypes. In toddlers, IgA antibodies were rarely detected before immunisation, but could be seen most frequently to serotype 19F, although frequently also to other serotypes. Of the two IgG-positive saliva samples detected in children, one was specific for serotype 6B, the other for 19F. The latter was associated with an exceptionally high serum concentration of serotype 19F-specific IgG (223 µg /ml).

In children with AOM, the infection caused by serogroup 19 evoked the highest ASC response, but group 23 was also associated with high ASC responses (Table in IV). The ASC responses to other types were lower, but low responses were also detected in some children to groups 19 and 23. Thus the magnitude of the response showed no clear-cut correlation with the serotype causing the infection.

5. Nasopharyngeal carriage of the pneumococci

S. pneumoniae was identified by culture in the nasopharyngeal swabs from four adult vaccinees before immunisation (I, II). One of them was colonised with the vaccine serotype, 23F (I), and three with nonvaccine serogroups 3, 9, and 11 (II). None of the vaccinees was colonised with any pneumococcal serotype on day 28 after

immunisation. *S. pneumoniae* was identified by culture in the nasopharyngeal swabs from 8 children before immunisation (serogroups 6, 14, 15, 18, 19, and 23) and in 11 children (7 new acquisitions) on day 28 after immunisation (serogroups 6, 18, 19, 23) (III). The ASC and antibody concentrations and responses of these culture-positive vaccinees did not differ from those in the other vaccinees

The same serogroup as in MEF was cultured in the NPA sample of each of the 17 patients with pneumococcal AOM. Pneumococcal group 19 was the most common serogroup involved (7 patients). Group 23 was cultured in 4 patients, group 6 in 3 patients, and type 14 in 2. One patient had an AOM caused by group 9.

6. Relation of salivary and serum antibodies to number of antibody-secreting cells
In adults, the IgA response in saliva was associated with a high number of IgA-ASCs (35/38 of the salivary responses were related to an IgA ASC response of >100 IgA ASC/10^6 cells to the same serotype). On the other hand, a high ASC response was detected only rarely when no salivary IgA response was seen. In Study I, more than 100 IgA ASC/10^6 cells were detected in 7 of the 62 cases that showed no salivary IgA response to the same serotype. Furthermore, in most of these cases (5 of 7) antibody concentration was high before immunisation and a 1.2- to 1.9-fold increase in IgA concentration was still seen. In Study II, more than 100 IgA ASC/10^6 cells were frequently detected even when no salivary IgA response was demonstrated, but very rarely (6 cases) when more than 1000 IgA ASC/10^6 cells were detected: also in Study II, high salivary antibody concentrations were often already detected before immunisation, but we did not attempt to correlate these cases with those with high ASC responses. In toddlers (III), IgA was rarely detected in saliva before immunisation. After immunisation, a positive correlation was demonstrated between the salivary IgA concentration (on day 28) and the number of IgA ASCs after PncD or PncT vaccination in toddlers ($r=0.57$, $p=0.01$; IV: Fig 3).

The ASC responses were also compared to the serum responses to see whether any correlation could be demonstrated. A significant correlation between the fold rise in serum IgA concentration and the number of IgA ASCs appeared in adults in Study II ($r=0.64$, $p<0.01$) (III; Fig 3), but in Study I no correlation (Fig 3, $r=0.4$). The correlation between the responses was stronger after immunisation with PncD ($r=0.75$, $p<0.01$) than after PncT ($r=0.56$, $p<0.05$). The fold rise in serum IgG concentration showed no correlation with number of IgG ASCs ($r=0.3$) (I, II). However, in Study II, the serotype-specific IgG concentrations in serum and in saliva correlated on day 28 ($r=0.56$, $p<0.01$) (II: Fig 4); ($r=0.62$ after PncD and $r=0.51$ after PncT), whereas no correlation appeared between IgA concentrations in serum and in saliva.

In toddlers, IgA antibodies were rarely detected in serum before immunisation. After immunisation, a positive correlation existed between the serum IgA antibody concentration and number of IgA ASCs ($r=0.70$, $p=0.01$). The IgA concentrations in

serum and saliva did not correlate. A weak positive correlation was found between the fold increase in the serum IgG and the number of IgG ASCs ($r=0.60$, $p=0.01$).

Among the AOM patients, all three (Patients # 1, 2, 3) who had more than 100 IgA-ASC/10^6 cells also had an IgA response in NPA and serum (IV: Table). Each of them also had a serum IgG and/or IgM response. An IgA response in NPA was detected in three additional patients (# 7, 8, 15), whose IgA ASC responses were low. Furthermore, IgA in acute or convalescent NPA could be detected in four other children (# 6, 10, 13, 14) with an IgA ASC response. No IgG responses were detected in NPA.

DISCUSSION

1. Study design

This study consists of four separate studies on pneumococcal PS responses: three phase I vaccine studies and one study on children with pneumococcal AOM. Each study was planned separately, and initially, we did not attempt to compare different vaccines. Instead we were interested in studying the mucosal response to pneumococcal conjugate vaccines, mainly based on the earlier knowledge that nasopharyngeal carriage of Hib was dramatically reduced after Hib-conjugate vaccination (Takala et al.1991, Murphy et.al. 1993, Mohle-Botani et al. 1993, Takala et al.1993). AOM was chosen to represent a local, nonsystemic pneumococcal infection in order to study the mucosal response also in a natural infection.

One of the aims in these studies was to consider how well the measurement of the ASCs in the peripheral blood (measured with the ELISPOT method), reflects the mucosal antibody response, measured as salivary antibodies (with EIA). Measuring ASCs is an indirect way to estimate the mucosal response, since the cells committed to mucosal sites transiently appear in the peripheral blood when trafficking via the circulation before homing to their destination. The present study does not provide direct data on the final distribution of the circulating ASCs into different tissues. Such data could be obtained by analysing the homing receptors expressed on the surface of the ASCs (McDermott and Bienenstock 1979, Butcher et al. 1996). However, only a few mucosal homing receptors are known: those directing the cells to the vascular endothelium of the gut lamina propria. Receptors directing the cells to respiratory mucosa are still poorly characterised, and suggested to differ both from the homing receptors of the peripheral lymph nodes (for serum antibody production), or of the gut mucosa (Abitorabi et al. 1996). Thus, optimal tools to study adhesion molecules of the ASCs committed to the respiratory mucosa do not exist. In this thesis, the ASC results were compared with the salivary antibody results, and the results demonstrated that an IgA response in saliva is related to a high IgA-ASC response.

2. Methodological aspects

In each of the studies, the number of circulating ASCs were counted in the peripheral blood. In addition, mucosal antibodies were measured in secretions, i.e., in saliva in the vaccine studies, and in the NPA in the AOM study. Finally, serum antibodies were also detected in order to estimate the overall immunogenicity of the vaccines in each study group. For the reasons described above, methods varied slightly between different studies. Since Study II began, all the assays in our laboratory have been conducted with the same methods and same reagents. They are therefore comparable, including Studies II and III. However, the saliva antibody results from the first vaccine study (I) cannot be directly compared to those in Studies II and III, because at the time Study I was carried out, the method was still in the process of being improved. Thus, in Study I the salivary IgA results are not expressed in relation to total IgA in saliva, which has been widely accepted as a means to compensate for the diurnal and individual variation in the

protein concentration of secretory samples. In other studies, the salivary IgA results are given as specific IgA/total IgA (ng/µg). The method for measurement of the ASCs has remained essentially the same during all these studies. The antisera in the ASC measurement to detect IgG and IgM differ in Studies I and IV from those used in Studies II and III. However, the new antisera were first tested, and the ASC results did not differ from those obtained with the antisera formerly used.

The kinetics of the ASC response after pneumococcal vaccination was analyzed in Study I. Serotype-specific ASCs were not detected on day 0. Consistent with several earlier vaccine studies (Kantele et al.1986 and 1990, Tarkowski et al. 1990, Nieminen et al. 1996), the peak number of ASCs was detected on day 7, thereafter the number of ASCs decreased, and on day 14 only a few ASCs could be detected. On day 28 or 30 after immunisation, no specific ASCs existed. Occasionally the number of ASCs on day 9 exceeded the number detected on day 7. However, we adopted day 7 as the peak day to represent the ASC response in our later studies. The IgM ASC peak is regularly already seen a few days before the IgA and IgG peak, typically on day 5 (Heilmann et al.1987, Kantele 1990), as also demonstrated in Study I. Thus, the study design in II and III did not allow us to detect the IgM ASC peak. Neither were the days when serum samples were collected optimal to detect this isotype. Therefore, IgM is not discussed further in this thesis.

A major problem with saliva samples is the instability of the antibodies; enzymes in the saliva with degradative activity affect the antibodies in the sample. Therefore special precautions must be taken in transport and storage. This degradation can be prevented by addition of enzyme inhibitors and glycerol, and by storing the samples at -70°C (Butler et al. 1990). In this study, the samples were frozen immediately in dry ice, stored at -70°C, and thawed only once. Preservatives were not added to the samples, to avoid additional dilution of the samples, already containing only very low antibody concentrations. In addition to those sensitivity problems, specificity problems also existed for salivary EIA, as nonspecific binding was frequently seen. Background plates were used to control this binding.

IgG in saliva is suggested to be mostly derived from serum. Consistent with this, a positive correlation was noted between the IgG response in serum and in saliva, a finding to be discussed later in more detail. However, a considerable number of measurements in saliva remained negative for IgG, whatever the IgG concentration in serum. This could be due to saliva samples too diluted for EIA to detect the IgG antibodies, resulting from individual and diurnal variation in the salivary protein concentration (Sörensen et al. 1987) or from degradation of the immunoglobulins by the proteases in saliva. The first hypothesis is supported by our results, since most of the saliva results that remained negative for IgG were derived from a small number of vaccinees, and these vaccinees lacked salivary antibodies to three or four serotypes at the same time. On the other hand, the total IgA concentration in these saliva samples with no specific IgG antibodies was no lower than in the remaining samples, indicating

that these samples were not more diluted. The explanation for this could be that IgG in saliva may be more prone to unspecific degradation by enzymes in saliva than is the dimeric S-IgA.

The fold increases in serum antibodies after immunisation are also sometimes difficult to interpret. Comparison of individual responses is disturbed by widely varying antibody concentrations even before immunisation. On the other hand, measurement of ASCs detects the response at cell level; no specific ASCs are detected before immunisation and the number of cells appearing in the blood after immunisation represents the response: indicating the magnitude of the response as the number of activated cells. Detecting the number of ASCs in the peripheral blood is therefore a useful alternative complementing more traditional serological methods for evaluation of the antibody response On the other hand, ELISPOT is labourious to perform and requires fresh blood samples for the analysis. It is therefore not useful in phase II vaccine studies, or in efficacy trials, where large populations are vaccinated, but can be adapted in phase I vaccine studies to add information on immunogenicity of the vaccines.

3. Immune responses

3. 1. Responses of vaccinees

Parenterally administered pneumococcal vaccines, the polysaccharide vaccine PncPS, and the conjugate vaccines PncOMPC, PncD, and PncT were all able to induce mucosal and serum antibody responses. An ASC response was seen in all vaccinees. In adults, the responses were higher after the PncD and PncT conjugates than after the PncPS or PncOMPC. It was initially surprising that in Study I, the ASC and serum antibody responses in adults were actually lower after the PncOMPC than after PncPS, because the conjugate was expected to have enhanced immunological properties. This was most likely a direct consequence of the dose of the PS antigen which was radically different between the two vaccines: 1 µg/ml of each polysaccharide in the PncOMPC vaccine and 25 µg/ml in PncPS. Some studies with pneumococcal vaccines have also suggested that in adults, conjugate vaccines might not be significantly better immunogens than PS vaccines (Powers et al. 1996, Eby 1995). However, our later study (II) in adults showed that the ASC response and the humoral antibody responses were significantly higher after PncD and PncT conjugates than those seen after PncPS vaccine, indicating that the immunogenicity of a PS vaccine can be improved by conjugating the PS to a protein carrier also in adults. This is most likely due to the T-cell help provided that seems to be beneficial also in adults responding to PS antigens. This finding supports results from earlier studies showing a significant difference between PS administered as such and when conjugated to carrier proteins (Fattom et al. 1990).

Differences were also seen in the magnitude of the responses between the two conjugate vaccines PncD and PncT; both the total number of serotype-specific ASCs and the serum antibody response were higher after immunisation with PncT than after PncD, whereas salivary antibodies were seen more often after PncD than PncT. The structure

of PncD and PncT vaccines is quite similar, since they both represent a PS-protein-conjugate vaccine. However, each of the conjugate vaccines still has its own characteristics. The polysaccharides in the vaccines can vary in length as well as in the terminal structure of the saccharide chains. Furthermore, different carrier proteins also have their own characteristics, and the PS/protein ratio and the coupling of the PS to the protein differs in each conjugate. These structural characteristics of the PS-protein conjugates have been shown to have an influence on the amount and the quality of serum antibody induced by the vaccine (Anderson et al. 1989, Seppälä et al. 1989, Verheul et al. 1989, van den Wijgert et al. 1991), even though the determinants of the immunogenicity are not yet understood in detail. It seems likely that the characteristics of the conjugate vaccines may also have an effect on the induction, amount, and quality of the mucosal antibody responses.

The PS antigens are able to induce only poor, if any, antibody responses in young children. The response to many of the pneumococcal serotypes improves by the age of 2 years, but for some of the serotypes, like 6B, it remains poor much longer, even until the age of 6 (Mäkelä et al. 1983, Leinonen et al. 1986). However, antibody responses to PS are seen in young children, because the PS is coupled to a protein carrier to provide T-cell help. The serum IgG concentrations detected in toddlers after a single dose of PncD or PncT (III) were comparable in magnitude to those detected in infants after primary immunisation series (three injections at the ages of 2, 4, and 6 months of age) (Åhman et al. 1998 and 1999). The booster response in infants was not dependent on the magnitude of the primary response, indicating that memory function was evoked by the primary immunisation, even if marked responses were not seen. The ASC responses seen in toddlers after PncD and PncT, on the other hand, were comparable to those we reported in adults after the PncPS, a vaccine that is widely used in adult populations to prevent invasive infections, although it is not able to induce memory. However, the ability of the conjugate vaccines to generate immunological memory is believed to be an important factor in protection against invasive infections in children (Eskola et al. 1990). The memory function is likely to play an important role in protection at the mucosal surfaces, as well. However, these studies did not evaluate memory function in the mucosal immune system.

Although the ASC responses to PncD and PncT were comparable in toddlers to those in adults after PncPS, the response in toddlers remained lower than in adults after PncD and PncT. However, salivary IgA antibody responses were seen in toddlers as frequently as in adults after the same conjugates. Thus the salivary IgA responses were seen in children as frequently as in adults despite the lower IgA ASC responses in children. This might suggest that a higher proportion of the IgA ASCs detected in the peripheral blood is committed to the mucosal sites in children than in adults. Moreover, only very low serum IgA concentrations were detected in children, which is consistent with our knowledge of the late maturation of the serum IgA production in children. Furthermore, serum IgG concentrations also remained distinctly lower in toddlers than in adults. Together, these findings suggest that mucosal IgA responses induced in

adults and toddlers are comparable, although the overall ASC response in adults is higher. These findings support those of earlier studies suggesting that the mucosal compartment of the immune system matures and starts to function earlier in life than the systemic immune system (Pichichero et al.1981 and 1983).

3. 2. Responses in AOM

The AOM study (IV) is the first to report a pneumococcal PS-specific ASC response induced by pneumococcal acute otitis media. The responses measured were specific to the causative pneumococcal polysaccharide type/group cultured in the MEF, since at the same time the number of ASCs. measured to other pneumococcal types remained low. ASCs could be detected in all the children studied, and their number in three of the children was surprisingly high: 260 to 1100 ASC/10^6 cells. These values exceeded the average response detected in the toddlers vaccinated, although more than 1 000 ASC/10^6 cells were occasionally detected also in children vaccinated with PncD or PncT. The response in these three children with AOM was high also when compared to other infections. The number of pathogen-specific ASCs in urinary tract infection has been less than 100 ASC/10^6 cells in most adult patients and even less in children with pyelonephritis (Kantele et al. 1994 and 1995). The rest of the ASC responses in AOM children, however, were lower than the average response in vaccinated children. The induction of immune responses with purified or synthetic antigen preparations is likely to differ from the situation after contact with live bacteria. The PS antigen lacks T-dependent properties, but when it is presented on bacteria the number of antigenic epitopes is higher, and PS antigens conceivably are introduced together with T-dependent antigens. Furthermore, other factors enhancing or suppressing the response can be involved, e.g., an ongoing viral infection or mixed bacterial infections that can induce inflammation and release of various cytokines and thus affect the response in a way difficult to predict.

The ASC response in children with AOM was clearly age-dependent. High ASC responses were mostly detected in children 24 months of age or older. This supports the concept that T-independent polysaccharide antigens are poor immunogens in infants and young children and that the immunogenicity of the PS antigens is improved by the age of 2 years. The mucosal IgA-response, however, was induced earlier. None of the younger children (less than 24 months) had serum antibody responses or an IgG-ASC response, but six of them had an IgA-ASC response and one a local IgA response in NPA. Measurable IgA in NPA was further detected in three of the younger children. This again supports earlier findings that a mucosal IgA response may be induced even in the first year of life, also to PS antigens that are then unable to mount systemic responses (Pichichero et al. 1981 and 1983)

3. 3. Serotype-specific responses

In children with AOM, the highest ASC responses were seen to 19F, the serotype most commonly causing pneumococcal AOM. Furthermore, this high ASC response was related to serum and to salivary responses to the same serotype. In vaccinees, the ASC

responses detected were closely similar to all four serotypes in each vaccine group, whereas each serum antibody response varied by pneumococcal serotype. The low serum responses were generally seen after PncPS and PncOMPC and mostly to serotype 6B, a poor immunogen in several studies (Leinonen et al. 1986, Mäkelä et al. 1989). After PncD and PncT, the responses to serotype 6B were clearly improved, a finding also supporting the improved immunogenicity of the conjugate vaccines as compared to that of PS vaccines. The highest responses, in general, for both serum IgG and salivary IgA, were to serotype 14 and 19F in adults and to serotype 19F in toddlers. This is in accordance with earlier findings suggesting that the structure of each PS has a profound influence on the response, both on magnitude and distribution of the immunoglobulin classes in the response (van den Dobbelsteen et al. 1992, Korkeila et al. Vaccine, in press).

4. Isotype distribution of ASC response

After parenteral immunisation with protein vaccines considered to be T-cell-dependent antigens, the ASC response consists almost exclusively of IgG-producing cells (Lue et al. 1994, Trollmo et al. 1994). This was seen in the present study in response to the carrier proteins of the conjugates. Clearly IgG-dominant ASC responses were seen in adults and toddlers to the protein components of the vaccines. Several studies have shown that the ASC response to PncPS and other polysaccharide antigens, in contrast to that of protein antigens, is dominated by IgA-producing cells (Heilman et al. 1986, Heilman et al. 1987, Heilman et al. 1988, Lue et al.1988, Lue et al. 1990, Tarkowski et al. 1990). This was also the case in the present study. The IgA/IgG ratio of the ASC response to a PS that is conjugated to a protein carrier, on the other hand, is altered towards a pattern typical of T-cell-dependent antigens (Tarkowski et al. 1990). This was seen in adults (II) after pneumococcal conjugate vaccines, since both isotypes were observed in essentially equal numbers; this is in contrast to the response we saw to pure PS or protein antigens. However, variation in respect to the dominant isotype was observed between each serotype and different conjugates. In toddlers, on the other hand, the responses to PS components of the conjugate vaccines were clearly dominated by IgA-secreting cells.

The ASC response in children with AOM was always dominated by IgA-ASCs as well, in accordance with results from earlier studies showing an IgA-dominant ASC response after other mucosal infections such as gastroenteritis and urinary tract infection (Kantele et al. 1988 and 1994). The IgA ASC responses detected in AOM children were within the same range of magnitude as those seen in vaccinated toddlers. IgG ASC responses after AOM, on the other hand, were detected in only few children, less frequently than in the vaccinated toddlers. Thus, higher IgG ASC responses were evident in vaccinated toddlers than in children suffering from pneumococcal AOM infection.

As mentioned earlier, the ASC response in adults was higher after PncD and PncT conjugates, than after PncPS. This difference was more apparent in regard to the IgG

responses, whereas the numbers of IgA ASCs were more comparable to each other, indicating that improvement in response to a conjugate vaccine, as compared to a PS vaccine, mostly concerns the systemic IgG responses. The difference in response between toddlers and adults after the conjugates was also more evident when the numbers of IgG ASCs were compared, and less difference was observed between the numbers of IgA ASCs. This supports the suggestion that although the ability to produce systemic IgG responses matures with age (and thus better IgG responses are seen in adults than in children), mucosal IgA responses comparable to those seen in adults can be induced even at an early age.

In adults some differences were also observed between responses to the two conjugate vaccines PncD and PncT, not only in the magnitude of responses, but also in the quality of responses. The number of IgG ASCs was significantly higher after PncT, whereas equal numbers of IgA ASCs were detected after PncD and PncT. However, after both conjugates, the number of IgA ASCs was still higher than in response to pure PS vaccine. Although the ASC response was not dominated by IgA- or IgG-secreting cells as clearly as in the response to PS or protein antigens, a clear difference in the IgA/IgG ratio of the ASC response was obvious between the two conjugate vaccines; IgA cells dominating in response to PncD, and IgG cells in response to PncT. Furthermore, S-IgA responses were seen in the saliva more frequently after immunisation with PncD than with PncT. This occurred despite the equal numbers of IgA ASCs seen after the two vaccines. Surprisingly, the IgG response in saliva was also higher (although not significantly) after immunisation with PncD, despite the higher serum IgG concentration after PncT. Together, these findings suggest that the PncD vaccine is able to induce better mucosal antibody responses (both IgA and IgG), whereas the PncT seems to be more efficient in inducing systemic IgG responses. In children, however, such differences between the two conjugates were not apparent. On the contrary, the responses were very similar and, as mentioned earlier, the ASC response was always clearly dominated by IgA-secreting cells.

5. Correlation between antibody concentrations and ASC responses

The excellent correlation between serotype-specific IgA and serotype-specific antibodies associated with secretory component in saliva (Studies I-III) indicates that the IgA measured in saliva was secretory in nature. The lack of correlation between serotype-specific IgA in serum and in saliva further supports the fact that the IgA detected in saliva was locally produced.

In adults, the over twofold increase in specific IgA antibodies in saliva was associated with an IgA ASC response of more than 100 ASC/10^6 cells, and in toddlers the fold increase in IgA concentrations in saliva correlated with the number of IgA ASCs. These results suggest that a high number of IgA-ASCs detected with ELISPOT is an indicator of the mucosal IgA response after parenteral pneumococcal vaccination.

The IgG detected in saliva is mostly suggested to be derived from serum, high serum IgG concentrations leading to leakage of the antibodies into mucosal secretions (Brandtzaeg 1971, Korsud et al. 1980, Grönblad 1981). Study II showed that the IgG concentrations in saliva and serum were positively correlated, in accordance with the concept of the salivary IgG being from the same origin as is the serum IgG. In toddlers, salivary IgG was hardly ever detected, which can well be explained by the relatively low serum PS-specific IgG concentrations detected in toddlers as compared to those in adults.

On the other hand, in adults, lower salivary IgG concentrations were induced after PncT than after PncD, despite the higher serum IgG concentrations after PncT, and furthermore, in Study I, a salivary IgG response was seen more frequently after PncOMPC than after PncPS, despite higher serum IgG concentrations after PncPS than after PncOMPC. These findings suggest that salivary IgG may not be solely a result of leakage through the mucosal surfaces because of high serum IgG concentration, but that some of the salivary IgG may be produced locally. Thus, not only secretory IgA, but also IgG could be induced in secretions somewhat independently of the IgG at systemic sites. Another explanation could be a higher avidity of salivary IgG induced by PncOMPC than by PncPS; thus, lower IgG concentrations would be detected in the antibody assay after PncOMPC. The avidity could be expected to be increased after conjugated PS as compared to plain PS, as has actually been shown for serum IgG (Granoff et al. 1995, Anttila et al. 1998). However, it does not explain the differences between PncD and PncT in adults. One possible explanation is that the permeability of IgG molecules at mucosal surfaces differs individually or even diurnally in each individual.

One of the main aims of this study was to see how well the number of ASCs would correlate with the antibody responses, and this was determined also in respect to serum antibodies. In adults, no significant correlation could be shown between the number of IgG ASCs and serum IgG concentration. However, the study carried out in toddlers (III), showed a weak positive correlation. This may have been due to the low preimmunisation concentrations of pneumococcal IgG seen in toddlers. In adult volunteers widely varying concentrations of pneumococcal polysaccharide-specific IgG antibodies are often detected even before immunisation, which makes analysis of the correlation between IgG concentration and the number of IgG ASCs impossible. In toddlers the IgG concentration, as well as the fold increase in IgG concentration, represents more clearly a new induction of the IgG antibodies and can thus be compared to the number of IgG ASCs induced by the vaccine.

6. Characteristics of responses with respect to protection

In this study we have attempted to evaluate the mucosal immune responses, and therefore have focussed on IgA. An interesting finding was that the pneumococcal conjugate vaccines PncD and PncT were able to induce a salivary IgA antibody response in toddlers as frequently as was reported for adults in Study II. On the other

hand, it has been suggested that the reduction in Hib carriage seen after Hib immunisations is due to high IgG antibody concentrations induced by the vaccines, leading to leakage of the IgG antibodies to the mucosal surfaces (Robbins et al. 1995 and 1996, Kauppi et al. 1993). However, the serum IgG concentrations in toddlers in this study remained distinctly lower than in adults. Nevertheless, the serum IgG concentrations in toddlers were comparable to those reported in infants after a primary immunisation series (of three injections) of PncD and PncT (Åhman et al. 1998 and 1999), the same vaccines that are reported to reduce nasopharyngeal carriage of pneumococcus in infants (Dagan et al. 1996b).

The immune-mediated mechanisms behind the reduced carriage after Hib and pneumococcal conjugate vaccination still remain contradictory. Both IgA- and IgG-mediated mucosal immunity have been suggested to prevent colonization. In animal studies, intranasal administration of human anti-Hib antibodies in an infant rat model prevented colonization (Kauppi et al. 1993), suggesting that local antibodies play an important role. However, IgA and IgG were both effective, indicating that both isotypes prevent colonizations if sufficiently high concentrations are achieved in the nasopharynx. Furthermore, a high dose of IgG given intraperitoneally also can prevent colonization (Kauppi et al. 1993), suggesting that high systemic IgG concentrations would, indeed, leak into mucosal secretions. Moreover, studies with *S. pneumoniae* show that intralitter spread of colonization from one infant rat to another was prevented by anti-capsular antibodies given subcutaneously (Malley et al. 1998).

Human studies on passive immuno-prophylaxis have also suggested that systemic, pneumococcal PS-specific IgG may protect from pneumococcal AOM in high-risk children, even without stimulation of specific local immunity (Schurin et al. 1993). On the other hand, the presence of pathogen-specific anti-capsular S-IgA in the middle-ear fluid even at the onset of acute otitis media seems to be beneficial for the resolution of the disease (Sloyer et al. 1974, Sloyer et al. 1976, Karjalainen et al.1990). Together, these findings suggest that capsular polysaccharide specific antibodies of both isotypes, IgA and IgG, are beneficial in defence against colonization and acute otitis media; the exact role for the two isotypes still remaining somewhat obscure. Nevertheless, the improved serum IgG responses to PS antigens achieved with the conjugate vaccines in the first years of life may well explain the reduced colonization (Robbins et al. 1995 and 1996). However, these studies could not demonstrate IgG responses in salivary secretions in toddlers. Studies looking more specifically at this issue are still needed to clarify the role of IgA and IgG in mucosal protection.

SUMMARY AND CONCLUSIONS

Results of these studies demonstrated mucosal as well as systemic antibody responses to parenterally administered pneumococcal vaccines PncPS, PncD, and PncT. In these studies, the mucosal responses were estimated by measurement of salivary antibodies and by measurement of circulating ASCs in the peripheral blood. An ASC response of more than 100 IgA-ASC/10^6 cells was associated with an increase in specific IgA antibodies in saliva. This good correlation suggests that the ELISPOT method may be an useful alternative, complementing more traditional serological methods for evaluation of the mucosal antibody responses to pneumococcal vaccines.

In adults the pneumococcal conjugate vaccines PncD and PncT were able to induce higher responses than did a polysaccharide vaccine. The characteristics of the response varied by vaccine; the ASC response to PncPS was dominated by IgA-secreting cells, whereas the response to conjugate vaccines involved comparable numbers of IgA- and IgG-secreting cells. However, differences in the response were also seen between different conjugates. In toddlers, the ASC responses to PncD and PncT were comparable to those seen in adults after pneumococcal PS vaccination. Salivary IgA responses (over a twofold increase in IgA concentration) were detected in vaccinated toddlers frequently, at the same incidence as in adult studies with the same vaccines. Furthermore, pneumococcal polysaccharide-specific responses were induced in children with AOM; both mucosal and systemic responses were detected. Local immune responses were, however, seen independently of the systemic responses.

Capsular polysaccharide-specific antibodies, either IgA or IgG, are beneficial in defence against mucosal colonization and infection, the exact role for the two isotypes still remaining somewhat obscure. The high serum IgG concentrations of PS-specific antibodies that can be achieved with the conjugate vaccines have been suggested to explain the reduced colonization with encapsulated bacteria in infants and in young children. The results of these studies showed that the pneumococcal conjugate vaccines are able to induce high numbers of IgA-secreting cells and secretory IgA in the upper respiratory mucosa, which may also be important in mucosal protection. How the mucosal IgA response correlates with protection still requires further investigation. Studies looking more specifically at this issue are needed to clarify the exact role of IgA and IgG in mucosal protection.

FUTURE CONSIDERATIONS

The aim of this study was to estimate the mucosal immune response induced by parenteral pneumococcal vaccines. How the response correlates with protection still remains unanswered. However, pneumococcal conjugate vaccines have already been shown to reduce nasopharyngeal carriage of the pneumococcal serotypes included in the vaccines (Dagan et al. 1996, Dagan et al. 1997), indicating mucosal protection. Some preliminary data also suggest protection against AOM (Black et al. 1999), but specific proof of the efficacy of the conjugate vaccines in preventing pneumococcal AOM still remains to be achieved. The Finnish Otitis Media Vaccine Trial (FinOM) was initiated in 1995, the target of which is to establish PncCRM vaccine's protection against culture-confirmed pneumococcal AOM. Results from this study are expected this year.

The exact role of mucosal S-IgA and of serum IgG in reduction of nasopharyngeal carriage and AOM also needs to be elucidated. Experimental studies have suggested a role for both S-IgA and IgG in preventing colonization and AOM. Human studies on passive immuno-prophylaxis have suggested that systemic PS-specific IgG may protect from pneumococcal AOM (Schurin et al. 1993). On the other hand, the presence of pathogen-specific anti-capsular S-IgA in the middle-ear fluid has been shown to be beneficial for resolution of AOM (Sloyer et al. 1974, Sloyer et al. 1976, Karjalainen et al.1990). The FinOM trial is a prospective study that includes extensive serological analysis, both of mucosal and systemic antibody responses. After the results from these studies are available, we can expect to have better estimates of serological correlations with protection against AOM.

Because mucosal protection can be expected to be at least partially S-IgA-mediated, the mucosal administration route of antigens is being intensively studied and can be expected to provide new strategies against microbes invading the body through mucosal surfaces. Furthermore, pneumococcal proteins: PspA, PsaA, and pneumolysin, and their ability to induce antibody-mediated protection, is currently a field of interest. Both mucosal and systemic protection are being evaluated, and mucosal administration of these proteins has been intensively studied. Animal studies on mucosal vaccination have also suggested that mucosal administration can sufficiently induce biologically active serum IgG responses (Flanagan et al. 1997, Seong et al. 1999, Jakobsen et al. 1999). If the same can be accomplished in human beings, systemic infections that do not involve invasion through mucosal surfaces (e.g., tetanus) could also be prevented by mucosal vaccination; some preliminary data on mucosal immunogenicity in humans already exists (Aggerbeck et al. 1997). Significant effort is being invested in research on mucosal vaccines, and the feasibility of vaccination via mucosal routes should be appraised in few years. An optimistic view for the future would be a single-dose vaccine given to small infants via the mucosal route.

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