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Anu Nurkka

SALIVARY ANTIBODIES TO CAPSULAR POLYSACCHARIDES INDUCED BY POLYSACCHARIDE-PROTEIN CONJUGATE VACCINES IN INFANTS

National Public Health Institute,
Department of Vaccines,
Helsinki, Finland
2004

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Helsinki, Finland

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ACADEMIC DISSERTATION

To be publicly discussed with permission of the Faculty of Biosciences,
Department of Biological and Environmental Sciences, University of Helsinki,
in the auditorium no 2041, Viikinkaari 5, on the 26th of November, 2004, at 12 noon.

Helsinki 2004

Publications of the National Public Health Institute
KTL A14 / 2004
ISBN 951-740-463-8
ISSN 0359-3584
ISBN 951-740-464-6 (pdf)
ISSN 1458-6290 (pdf)
Hakapaino Oy

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Cover Saliva sampling

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CONTENTS

ORIGINAL PUBLICATIONS **4**
ABBREVIATIONS **5**
INTRODUCTION **7**
REVIEW OF THE LITERATURE **8**
1 Mucosal immunity **8**
1.1 Mucosal surface 8
1.2 Innate mucosal immunity 9
1.3 Adaptive mucosal immunity 10
1.3.1 Common mucosal immune system 10
1.3.2 Mucosal immune response 11
1.3.3 From a naïve B cell to an immunoglobulin A producing cell 14
1.3.4 Immunoglobulin A 15
1.3.5 Immunoglobulin G and other immunoglobulins at mucosa 18
1.4 Regulation of mucosal immune response 19
1.5 Mucosal memory 21
2 How to study mucosal immunity? **21**
2.1 Saliva samples 23
3 How to induce mucosal immunity? **24**
3.1 Natural immunity 24
3.2 Parenteral vaccines 25
3.2.1 Protein vaccines 25
3.2.2 Polysaccharide vaccines 26
3.2.3 Conjugate vaccines 27
3.3 Mucosal vaccines 28
3.4 Maternal immunisation 30
4 Pneumococcus and pneumococcal vaccines **30**
4.1 Streptococcus pneumoniae 30
4.2 Pneumococcal polysaccharide vaccines 32
4.3 Pneumococcal conjugate vaccines 33
4.4 Other pneumococcal vaccination strategies 34
5 Meningococcus and meningococcal vaccines **36**
5.1 Neisseria meningitidis 36

5.2 Meningococcal polysaccharide vaccines 37
5.3 Meningococcal conjugate vaccines 38
5.4 Other meningococcal vaccination strategies 38
AIMS OF THE STUDY 40
MATERIALS AND METHODS 41
1 Vaccines 41
1.1 Pneumococcal vaccines 41
1.2 Meningococcal vaccines 43
1.3 Other vaccines 43
2 Study subjects 44
3 The ethical issues 44
4 Samples 46
4.1 Saliva samples 46
4.2 Serum samples 48
5 Serological methods 48
5.1 Enzyme immunoassay (EIA) for measurement of pneumococcal antibodies in saliva 48
5.2 EIA for measurement of meningococcal antibodies in saliva 50
5.3 EIA for measurement of pneumococcal and meningococcal antibodies in serum 51
5.4 Single radial immunodiffusion 51
6 Statistical methods 52
RESULTS 53
1 Methodology for collection and storage of saliva samples 53
1.1 Effects of collection method on saliva samples 53
1.1.1 Volume of the saliva sample 53
1.1.2. Anti-pneumococcal IgA concentrations in saliva samples 54
1.2 Effect of storage method on anti-pneumococcal IgA concentrations 54
1.3 Co-effects of collection and storage methods on anti-pneumococcal IgA in saliva samples 55
2 Salivary anti-pneumococcal antibodies induced by conjugate vaccines 55
2.1 Anti-pneumococcal IgG 56
2.2 Anti-pneumococcal IgA 59
2.2.1 Anti-pneumococcal IgA in relation to total IgA 59
2.2.2 Anti-pneumococcal IgA subclasses, IgA1 and IgA2 62
2.3 Persistence of salivary antibodies after pneumococcal vaccination in infancy 62

2.4 The effect of a polysaccharide booster after a primary series with conjugate vaccines 63
3 Natural salivary anti-pneumococcal antibodies 64
4 Salivary anti-meningococcal antibodies induced by a MenA/C polysaccharide vaccine in previously primed and unprimed children 65
4.1 Anti-MenA IgG and IgA antibodies 65
4.2 Anti-MenC IgG antibodies 66
4.3 Anti-MenC IgA antibodies 67
4.4 Correlation of anti-Men IgA and sIg 68
4.5 Origin of anti-Men IgG 69
DISCUSSION 70
1 Study design 70
2 Methodology 70
2.1 Saliva samples 70
2.2 Collection and storage methods for saliva samples 71
2.3 EIA for detection of salivary antibodies 73
3 Salivary anti-pneumococcal antibodies induced by conjugate vaccines 74
3.1 Salivary anti-pneumococcal IgG 75
3.2 Salivary anti-pneumococcal IgA 75
3.3 Salivary anti-pneumococcal IgA subclasses, IgA1 and IgA2 76
3.4 Persistence of salivary antibodies after pneumococcal vaccination in infancy 77
3.5 The effect of a polysaccharide booster after a primary series with conjugate vaccines 77
4 Natural salivary anti-pneumococcal antibodies 78
5 Salivary anti-meningococcal antibodies induced by vaccines 79
6 Mucosal immunological memory 80
7 The significance of salivary antibodies 81
CONCLUSIONS 83
SUMMARY 84
FUTURE CONSIDERATIONS 85
ACKNOWLEDGEMENTS 87
REFERENCES 89

ORIGINAL PUBLICATIONS

The thesis is based on following original publications, which are referred to in the text by their Roman numerals. The original publications have been reprinted with the permission of the copyright holders.

I. Nurkka A, Obiero J, Käyhty H, Scott J.A.G. Effects of sample collection and storage methods on antipneumococcal immunoglobulin A in saliva. Clin Diagn Lab Immunol 2003;10(3):357-361.

II. Nurkka A, Åhman H, Korkeila M, Jäntti V, Käyhty H, Eskola J. Serum and salivary anti-capsular antibodies in infants and children immunized with the heptavalent pneumococcal conjugate vaccine. Pediatr Infect Dis J 2001;20(1):25-33.

III. Nurkka A, Åhman H, Yaich M, Eskola J, Käyhty H. Serum and salivary anti-capsular antibodies in infants and children vaccinated with octavalent pneumococcal conjugate vaccines, PncD and PncT. Vaccine 2001;20(1-2):194-201.

IV. Nurkka A, Lahdenkari M, Palmu A, Käyhty H, and the FinOM Study Group. Salivary antibodies induced by the seven-valent PncCRM conjugate vaccine in the Finnish Otitis Media Vaccine Trial. Vaccine 2004;23(3):298-304.

V. Nurkka A, Lahdenkari M, Palmu A.A.I, Käyhty H, and the FinOM Study Group. Salivary antibodies induced by the seven-valent PncOMPC conjugate vaccine in the Finnish Otitis Media Vaccine Trial. Manuscript.

VI. Nurkka A, MacLennan J, Jäntti V, Obaro S, Greenwood B, Käyhty H. Salivary antibody response to vaccination with meningococcal A/C polysaccharide vaccine in previously vaccinated and unvaccinated Gambian children. Vaccine 2000;19(4-5):547-556.

In addition, some unpublished data are presented.

ABBREVIATIONS

AOM	acute otitis media
APC	antigen presenting cell
ASC	antibody secreting cell
BALT	bronchus-associated lymphoid tissue
CMIS	common mucosal immune system
CI	confidence interval
CbpA	choline binding protein A
CPS	cell wall polysaccharide
CRM	non-toxic mutant diphtheria toxin, CRM_{197}
CT	exotoxin of *Vibrio cholerae*
DC	dendritic cell
D	diphtheria toxoid
EIA	enzyme immunoassay
ELISPOT	enzyme-linked immunospot assay
FAE	follicle-associated epithelium
FCS	foetal calf serum
FinOM	Finnish Otitis Media
GALT	gut-associated lymphoid tissue
GMC	geometric mean concentration
HBV	hepatitis B vaccine
Hib	*Haemophilus influenzae* type b
HIV	human immunodeficiency virus
Ig	immunoglobulin
IgA	immunoglobulin class A
IgG	immunoglobulin class G
IgM	immunoglobulin class M
KTL	Kansanterveyslaitos (National Public Health Institute, Finland)
LP	lamina propria
LT	heat-labile enterotoxin of *Escherichia coli*

MALT	mucosa-associated lymphoid tissue
MCV	meningococcal conjugate vaccine
Men	*Neisseria meningitidis*, meningococcus
MenA	meningococcal serogroup A
MenC	meningococcal serogroup C
MHC	major histocompatibility complex
MPV	meningococcal polysaccharide vaccine
NALT	nasal-associated lymphoid tissue
OD	optical density
OMPC	outer membrane protein complex
PBS	phosphate buffered saline
PCV	pneumococcal conjugate vaccine
pIgR	polymeric immunoglobulin receptor
Pnc	*Streptococcus pneumoniae*, pneumococcus
PncCRM	pneumococcal capsular polysaccharide-CRM conjugate vaccine
PncD	pneumococcal capsular polysaccharide-D conjugate vaccine
PncOMPC	pneumococcal capsular polysaccharide-OMPC conjugate vaccine
PncT	pneumococcal capsular polysaccharide-T conjugate vaccine
PP	Peyer's patch
PPV	pneumococcal polysaccharide vaccine
PS	polysaccharide
PsaA	pneumococcal surface adhesin A
PspA	pneumococcal surface protein A
SC	secretory component
sIg	secretory immunoglobulin
T	tetanus toxoid
TD	T cell dependent
TI	T cell independent

INTRODUCTION

Most of the pathogens invade the human body through mucosal membranes. Thus, mucosal surfaces provide the front line of defence by taking advantage of both innate and adaptive immune mechanisms. The innate immunity blocks invaders non-specifically, but the adaptive immune system consists of pathogen specific immune mechanisms. Further, the adaptive immune system has an ability to memorise, and during the consecutive encounter with the antigen it can act faster and with higher magnitude than at the first time. The local mucosal immune systems at various mucosal surfaces are known to communicate with help of circulating lymphocytes migrating from one site to another and, thus constitute the Common Mucosal Immune System (CMIS). According to the concept of CMIS antibody responses can be induced on a mucosal membrane even if the encounter with an antigen has taken place at a remote mucosal site.

Streptococcus pneumoniae (pneumococcus) and *Neisseria meningitidis* (meningococcus) are encapsulated pathogens, which are both able to colonise the human nasopharynx and, further, to cause a disease, e.g. pneumonia, meningitis or sepsis. Pneumococcus is also the leading bacterial cause of a local infection, acute otitis media. Vaccines against pneumococcal and meningococcal diseases have been developed, tested, and used for decades. The first licensed vaccines were based on capsular polysaccharides. Later, polysaccharides have been conjugated to carrier proteins in order to improve the immunogenicity of these vaccines in infants by turning the immune response from T cell independent to a T cell dependent type.

The encounter of microbes at mucosal surfaces may lead to the production of specific salivary antibodies. In animal models mucosal antibodies have also prevented the attachment of bacteria to the mucosal surface, an event necessary for the infection to proceed. The pneumococcal and meningococcal conjugate vaccines have been found to induce systemic immunity and immunological memory, but there are only few studies about the conjugate vaccines and mucosal immunity.

This thesis consists of a series of studies characterising the ability of parenterally administered pneumococcal and meningococcal conjugate vaccines to induce salivary antibodies in infants. We also conducted a study to find the best methods for collection and storage of saliva samples.

REVIEW OF THE LITERATURE

1 Mucosal immunity

Mucosal membranes are continuously exposed to a myriad of antigens, e.g. different food antigens, bacteria, viruses and different particles. Over 90% of pathogens invade the human body through mucosal membranes (Challacombe 1995), yet, a vast majority of them are eliminated by the local immune system. On the other hand, many antigens e.g. different nutritional components and bacteria functioning in symbiosis with the human body are essential for the well-being of man and have to be tolerated. Thus, the local immune system has to make right choices continuously between tolerance and immune response against different antigens. It has been estimated that events within the mucosal immune system cover more than two thirds of the activity of the entire human immune system (Russell *et al.* 2000).

The history of the concept of mucosal immunity starts in the beginning of the 20th century. In 1919 Russian-French serologist Alexandre Besredka presented the idea of local immunity (Besredka 1919). In 1965 Tomasi *et al*, confirmed the concept of mucosal immunity by finding secretory immunoglobulin A (sIgA) (Tomasi *et al.* 1965). Further, the idea of the common mucosal immune system (CMIS) indicating communication between different mucosal surfaces was introduced in 1970's (Mestecky *et al.* 1978). The knowledge on the local immune system has later on increased considerably.

1.1 Mucosal surface

Gastrointestinal, urogenital, and respiratory tracts, and ocular areas are all covered with mucosal membranes, which form together an area of approximately 400 m^2 in an adult human being (Brandtzaeg *et al.* 1998). The outer layer of mucosal membranes, epithelium, is formed of one layer of cells with different functions; undifferentiated epithelial cells, absorptive cells, Paneth cells, enteroendocrine cells, cup cells, tuft cells, intraepithelial lymphocytes, M (microfold) cells, and goblet cells. The structure of a mucosal membrane

differs depending on the part of the body, e.g. there are more absorptive cells in the small intestine than on the nasopharyngeal site.

Mucus forms a thick barrier on the mucosal epithelium. It mainly consists of well glycosylated high molecular weight glycoproteins called mucins, which are produced by goblet cells (Deplancke and Gaskins 2001). There are different mucins; at least six different genes encode the mucins of the airways (Lamblin *et al.* 2001). An important function of mucus is to trap unwanted microbes and other particles to be removed from the body, partly with the help of secretory immunoglobulin A (IgA) and mucociliary clearance (Corthesy and Spertini 1999). Further, mucus lubricates and insulates the mucosal epithelium.

Immediately beneath the mucosal epithelium is situated the lamina propria (LP). This is the major functional tissue of mucosa. The lamina propria contains many immunocompetent cells, including dendritic cells, macrophages, and lymphocytes.

1.2 Innate mucosal immunity

Mucosal membranes themselves form a physical barrier against pathogens. Antigens are also actively removed by ciliary and peristaltic movements, coughing, sneezing, and by the flow of saliva, mucus and urine. Further, pH ranges, and antibacterial substances like lysozyme, lactoferrin and peroxidases participate in the protection. In addition to mucins, goblet cells produce trefoil peptides. These small peptides interact with mucins to increase the viscosity of mucus, but also protect against different substances, e.g. bacterial toxins (Podolsky 1999). Human mucosal epithelial cells also make beta defensins, which help the local immune system to make difference between pathogenic and non-pathogenic bacteria based on the different signalling systems that harmless and harmful bacteria use (Chung and Dale 2004).

When invading the body, pathogens also need to cope with the resident microbial flora (Mayer 2003). Actually, there are estimated to be approximately 400 different species of microbes in the human gut (McCracken and Lorenz 2001). These microbes have different functions in the house-keeping of mucosal membranes, and they are also able to stimulate immune responses. Germ-free animals have defects in their mucosal immune system despite otherwise normal immune system organs (McCracken and Lorenz 2001).

In addition to physical and chemical barriers, mucosal surfaces have unspecific immunological prevention mechanisms against pathogens. Complement, polymorphonuclear leucocytes and macrophages take care of preventing infections to some extent at mucosal membranes (Jakobsen and Jonsdottir 2003).

1.3 Adaptive mucosal immunity

1.3.1 Common mucosal immune system

The adaptive immune system consists of two types of lymphocytes, B cells and T cells, providing antigen specific humoral and cell-mediated immune responses, respectively. The adaptive mucosal immune system is presumed to work quite independently from its systemic counterpart (McGhee *et al.* 1992; Brandtzaeg *et al.* 1998). It is based on the circulation and homing of mucosal lymphocytes, first described in rats by Gowans and Knight (Gowans and Knight 1964). Naïve B and T lymphocytes are continuously circulating through all secondary lymphoid tissues until they meet a specific antigen or die. After naïve lymphocytes are activated by an antigen at a mucosal site, they migrate to regional lymph nodes to mature. It has become evident that while naïve cells are able to migrate equally to all secondary lymphoid tissues, activated lymphocytes gain tissue-selective homing abilities and can, in addition, also invade tertiary lymphoid tissues, e.g. lamina propria and joints. This tissue-selective homing enables effective targeting of immune responses to sites where the antigen is most probably encountered. Tissue-selective trafficking of memory and effector lymphocytes is mediated by unique combinations of adhesion molecules and chemokines. This cycle of lymphocyte circulation and homing takes place also in humans (Kantele *et al.* 1986; Czerkinsky *et al.* 1987; Wenneras *et al.* 1994; Kantele *et al.* 1996).

It has been found that antigen stimulation at one mucosal site of the body can lead to an immune response also at remote mucosal sites (Figure 1). This interconnection of mucosal surfaces by circulating lymphocytes is called the common mucosal immune system (CMIS) (Mestecky 1987). Even if mucosal surfaces can communicate with each other via circulating lymphocytes, CMIS appears to be compartmentalised to some extent (Moldoveanu *et al.* 1995; Kantele *et al.* 1998). The CMIS consists of different mucosa-associated lymphoid tissues (MALT), e.g. genitourinary organs, inner ear, mammary, salivary and lacrimal glands

and gut-associated lymphoid tissue (GALT). In the airways MALT provide an important part of the immunity against respiratory pathogens (Brandtzaeg 2003b). The nasal-associated lymphoid tissue (NALT) functions in the area of the nasopharynx, nasal cavity and near the auditory tube (Mair *et al.* 1987). Previously, Waldeyer's ring in the human pharynx including palatine, lingual and nasopharyngeal tonsils (adenoids) was considered to be analogous to murine NALT. However, it has been recently proposed that young children have also NALT (Debertin *et al.* 2003). Laryngeal and tracheal areas are protected by larynx-associated lymphoid tissues (LALT) and lower respiratory tracts by bronchus-associated lymphoid tissue (BALT).

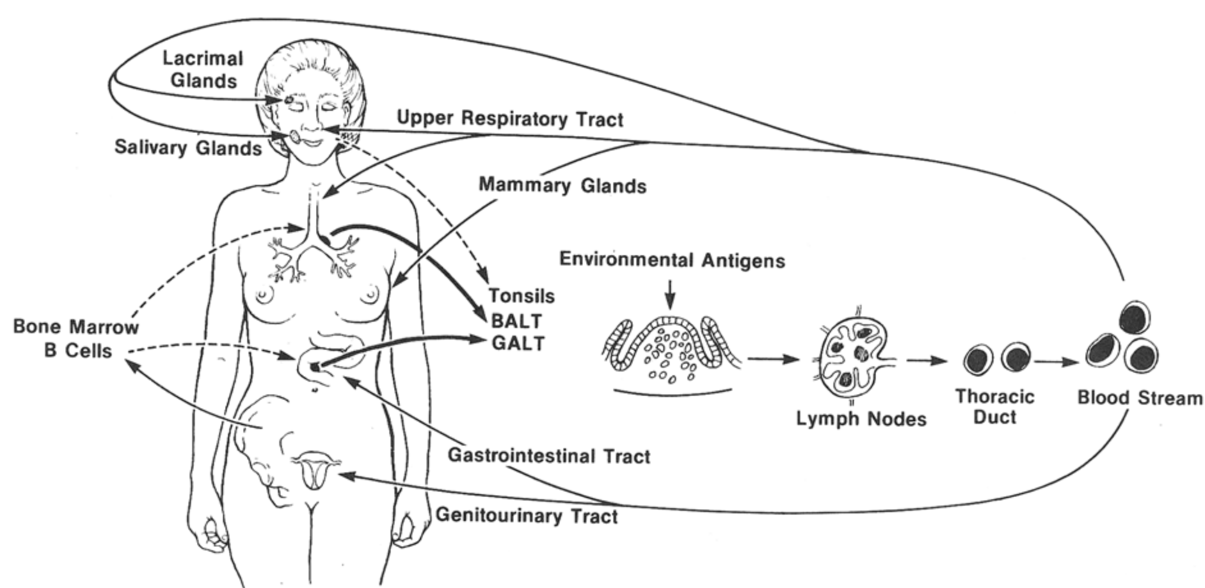

Figure 1. Hypothetical diagram of the common mucosal immune system (CMIS) in humans. Induction of immune response at one part of the system can induce immune response in another parts of the CMIS (Mestecky *et al.* 1994, with permission).

1.3.2 Mucosal immune response

GALT is the most thoroughly studied part of the mucosal immune system. It has three different types of inductive sites: Peyer's patches (PP), appendix and small solitary lymphoid nodules. Recently, isolated lymphoid follicles have also been suggested to be part of the murine GALT (Hamada *et al.* 2002). The structure of inductive sites in BALT is considered to be similar to GALT and thus suggested to function similarly (Tango *et al.* 2000). Also, NALT in mice is comparable to GALT with PP organisation (Wu *et al.* 1996).

Mucosal immune response begins when an antigen reaches the follicle-associated epithelium (FAE) of inductive sites e.g. at the dome like structures of PPs (Figure 2). Antigens are taken through the FAE either with help of microfold cells (M cells, synonymously used with follicle-associated epithelial cells) or dendritic cells (DCs). M cells are epithelial cells specialised in the uptake and transport of macromolecules and micro-organisms through the mucosa (Bouvet *et al.* 2002). They have short microvilli, small cytoplasmic vesicles and few lysosomes (Wolf and Bye 1984). It was thought for long that M cells are used only to transport antigens without their modification (Wolf and Bye 1984). More recent data suggested that M cells are able to process and present antigens (Allan *et al.* 1993). Some pathogens e.g. reovirus and salmonella use M cells as a route to invade the human body (Wolf *et al.* 1981; Weinstein *et al.* 1998).

Dendritic cells seem to have a dual role in the immune response. First, immature DCs are macrophage-like and function as vigorous phagocytes. After ingesting antigens they mature and start to act as effective antigen presenting cells (APCs). Further, DCs gather antigens from lumen by opening and closing tight junctions between epithelial cells and picking antigens with their dendrites (Rescigno *et al.* 2001). DCs are also suggested to collect bacteria, which have reached the lamina propria because of the leakiness of epithelium, tissue damage or invasion (Uhlig and Powrie 2003). There are at least three subpopulations of DCs at PP's: myeloid CD11b$^+$, lymphoid CD8α$^+$ and double negative DCs that lack expression of CD8α or CD11b (Iwasaki and Kelsall 2000). Lymphoid and double negative DCs can induce a Th1 type of cell-mediated immune response, while myeloid DCs are capable of inducing Th2 type of cells by producing IL-10, which leads to IgA response by B cells at effector sites. Myeloid DCs also produce TGF-β, and are suggested to induce oral tolerance. The role of DCs in keeping up the homeostasis of the mucosal immune system seems thus to be more important than previously known.

Inductive sites contain a variety of different cells needed in an immune response. The most important inductive sites, PPs, have organised area for both T and B cells including germinal centres. In fact, most of the immunoglobulin (Ig) producing lymphocytes are located at the intestinal mucosa (Brandtzaeg 1989). After transmission through the mucosal epithelium to inductive sites, antigens are first processed and presented to naïve T and B cells by APCs. Antigen presentation can take place locally, but DCs may carry antigens to be presented in

the lymph nodes. In the human gastrointestinal tract APCs include macrophages, a variety of DCs, and B cells (Brandtzaeg 2001). In human nasal mucosa instead closely related macrophages and DCs function as APCs (Jahnsen *et al.* 2004). After lymphocytes, including B cells, $CD4^+$ Th1/Th2 cells and CD8+ T cells, are primed at PPs they travel through lymph nodes and the thoracic duct to the blood stream, and finally home as effector cells in mucosal effector sites e.g. lamina propria (Figure 2).

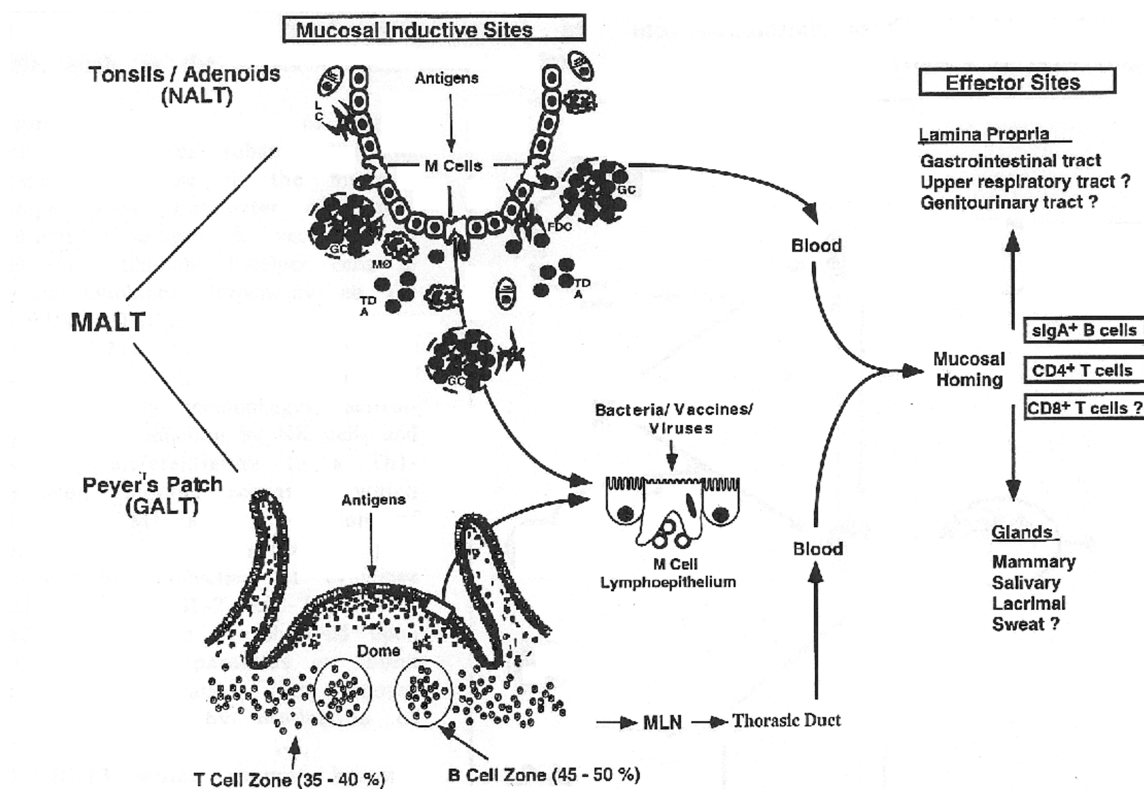

Figure 2. Induction of mucosal immunity. M cells are present in mucosal inductive sites in both the intestinal and upper respiratory tract, specifically in Peyer's patches and in the NALT, the tonsils and adenoids. M cells are thought to play an important role in antigen processing and possibly the induction of antigen-specific mucosal immunity in mucosal effector sites. Tissues followed by question marks are presumed sites since limited data are available on these tissues (van Ginkel *et al.* 2000, with permission).

The homing of mature B and T lymphocytes from circulation to their effector sites is a multi-step process, where the tissue-specific adhesion molecules have an important role. The extravasation of lymphocytes takes place through the endothelial cells of high endothelial venules (HEV) in the lymph nodes (Schoefl 1972). The extravasation appears to consist of four different steps: primary adhesion (including tethering and rolling), activation, arrest and

diapedesis (Picker 1994; Butcher and Picker 1996; Butcher *et al.* 1999). In the beginning of the process lymphocytes are rolling along the wall of a vessel allowing initial interactions. The speed of rolling is gradually decelerated providing contacts between the adhesion molecules on lymphocytes and on the endothelial cells. One of the central events in the homing of lymphocytes into tissues is regarded to be the binding of lymphocyte surface homing receptors (HR) to the ligands, addressins, on the endothelial cell walls. Three specific homing receptor – addressin pairs have been found: α4β7 is a counterpart to the mucosal addressin cellular adhesion molecule-1 (MAdCAM-1) on endothelial cells of the gastrointestinal tract (Berlin *et al.* 1993), L-selectin binds to PNAd in the peripheral lymph nodes (Berg *et al.* 1991a) and cutaneous lymphocyte antigen (CLA) to E-selectin in the skin tissue (Berg *et al.* 1991b). It has been suggested that primed lymphocytes tend to home back to their inductive sites as effector cells. Further, chemo attractant cytokines (chemokines) have a role in the homing of lymphocytes (Kunkel and Butcher 2003).

Lamina propria is the main effector site in GALT. LP contains mainly IgA-secreting plasma cells (Farstad *et al.* 2000) and T memory cells (Masopust *et al.* 2001a; Masopust *et al.* 2001b). LP plays also an important role in the regulation of mucosal immune responses (Bailey *et al.* 2001). Tissue-resident DCs in LP have a regulatory role to suppress immune response against commensal bacteria. When these DCs encounter non-pathogenic bacteria, they produce IL-2 and IL-10, which induce the differentiation of regulatory T cells. However, during inflammation macrophages attract circulating DCs from blood to phagocytose bacteria and to initiate an immune response (Granucci and Ricciardi-Castagnoli 2003).

1.3.3 From a naïve B cell to an immunoglobulin A producing cell

Several phases take place between the priming and maturation of a naïve B cell into an IgA producing plasma cell. The first step is taken at PPs, where naïve B cells with surface IgM are switched to IgA bearing cells with help of T cells (Kawanishi *et al.* 1983). However, IgA production can be also T cell independent (TI); a pathway to straight B cell differentiation to IgA producing plasma cells has been found (Macpherson *et al.* 2001). Recently, it has been suggested that 25% of murine IgA is produced T cell independently by B1 cells originated from the peritoneal cavity and representing a primitive system for recognition of commensal

bacteria by polyreactive antibodies. The remaining 75% of IgA is induced by a T cell dependent (TD) manner by B2 cells in germinal centres of MALT (Macpherson *et al.* 2001). However, a respective IgA production by peritoneal originated B1 cells unlikely takes place in humans (Boursier *et al.* 2002). The precursors of human B2 lymphocytes have been generated in bone marrow. At inductive sites the driving force for IgA switching of B cells is TGF- β (Cazac and Roes 2000) and the further maturation of $mIgA^+$ B cells is enhanced by IL-5, IL-6 and IL-10 (Salvi and Holgate 1999). The maturation of B cells takes place in draining mesenteric lymph nodes, where B cells further divide and differentiate, and it is finally completed after homing at mucosal effector sites (Lamm and Phillips-Quagliata 2002). During this process some B cells differentiate into memory cells (Tangye *et al.* 2003).

1.3.4 Immunoglobulin A

Immunoglobulin A (IgA) is the most abundant immunoglobulin (Ig) class of the immune system (Mestecky 1988). In serum, IgA covers only 15-20% of total Ig concentration, but at mucosal membranes IgA is the predominating Ig class (Hanson *et al.* 1985; van Egmond *et al.* 2001). Even if immunoglobulin G (IgG) predominates in serum, the daily production of IgA (66 mg/kg/d) exceeds the production of all other immunoglobulin classes combined (Monteiro and Van De Winkel 2003). IgA can be either monomeric (mIgA) or polymeric (pIgA). In adults over 90% of the IgA in serum is mIgA. Instead, in infants most of serum IgA can be pIgA (Weemaes *et al.* 2003). The proportion of pIgA decreases at the increase of total IgA concentration (Weemaes *et al.* 2003). In secretions instead, IgA is in general polymeric, most often dimeric.

There are two subclasses of immunoglobulin A, IgA1 and IgA2 (Figure 3). The major difference between these two subclasses is the absence of a 13-amino acid sequence in the hinge region of IgA2 (van Egmond *et al.* 2001). This part has many sites for O-glycosylation and is a target for IgA1 proteases induced e.g. by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenza*e type b (Kilian *et al.* 1996). Cleavage of IgA1 by IgA1 proteases can prevent e.g. the stimulation of a respiratory burst in neutrophils through Fcα receptors (Almogren *et al.* 2003). Further, IgA1 has been found to be T-shaped instead of Y, which is the common form of other Igs (Boehm *et al.* 1999). The distribution of IgA subclasses in different parts of the immune system varies notably (McGhee *et al.* 1993). In

serum, 90% of the IgA is produced in bone marrow and most of it represents subclass IgA1 (Delacroix *et al.* 1982; Kutteh *et al.* 1982). However, in secretions IgA is produced by plasma cells at effector sites. IgA1 predominates in the upper respiratory and in the upper gastrointestinal tracts. In the lower gastrointestinal tract the proportion of IgA2 is higher than of IgA1 (Brandtzaeg 1994). In saliva, IgA1 predominates normally. However, in infants subclass distribution of salivary IgA can vary more than in adults (Smith *et al.* 1989). The distribution of subclasses depends on the nature of the antigen: proteins induce IgA1 predominating response while the proportion of IgA2 producing cells is higher after immunisation with carbohydrates (Tarkowski *et al.* 1990; Simell *et al.* 2004). This has been seen both in adults and in infants.

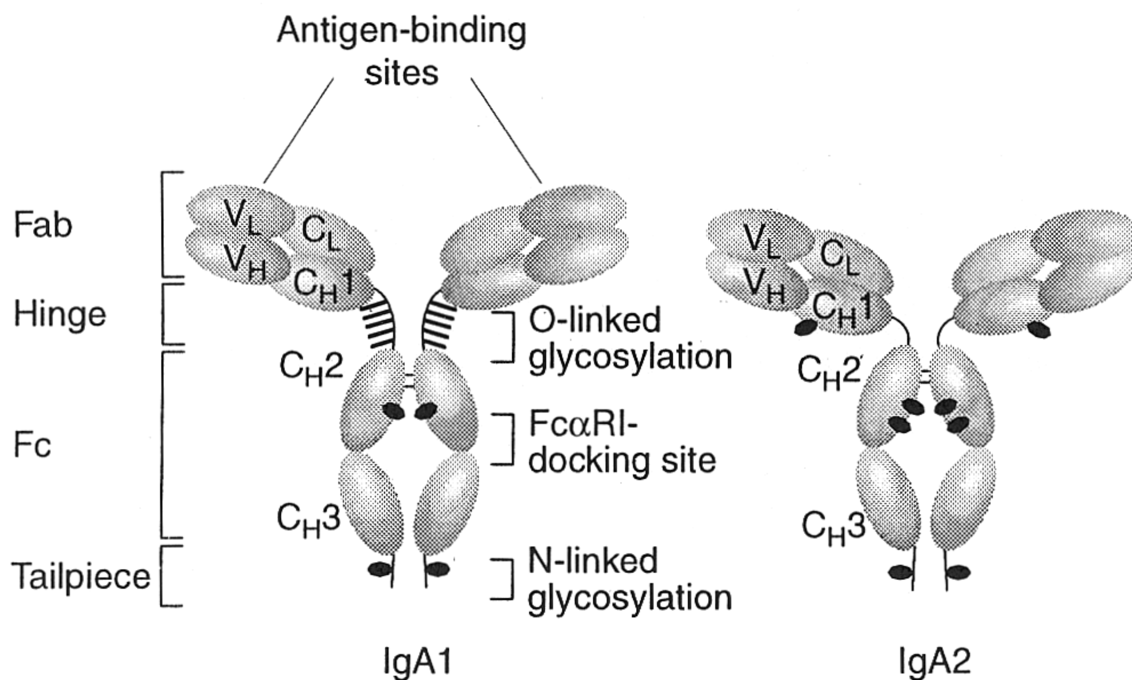

Figure 3. IgA isotypes. IgA is composed of two heavy and two light chains. Heavy chains consist of three constant regions (C_H1, C_H2, and C_H3), and one variable (V_H) region, whereas light chains are composed of one constant (C_L) and one variable (V_L) region. Positions of the FcαR1 docking site and the O- (**I**) and N- (●) linked glycosylation sites are marked (van Egmond *et al* 2001, with permission).

Most of the mucosal IgA is produced by plasma cells of lamina propria and secreted through epithelial cells to mucosal lumen by transcytosis (Figure 4). Secreted IgA is always polymeric, seen mostly as a dimeric form consisting of two monomeric IgA molecules linked together with a polypeptide called J (joining) chain. Mucosal epithelial cells have pIg

receptor (pIgR) on their basolateral surface, where pIgA can attach via J chain. Further, pIgA is actively transported through cells to an apical/luminal site of the cell through a vesicular route (Mantis *et al.* 2002). At the apical site of the cell, pIgA is cleaved from the pIgR. However, a part of pIgR, called secretory component (SC), is attached to the pIgA molecule forming together secretory IgA (sIgA). Thus, both plasma cells and secreting epithelial cells are essential for the assembly of sIgA.

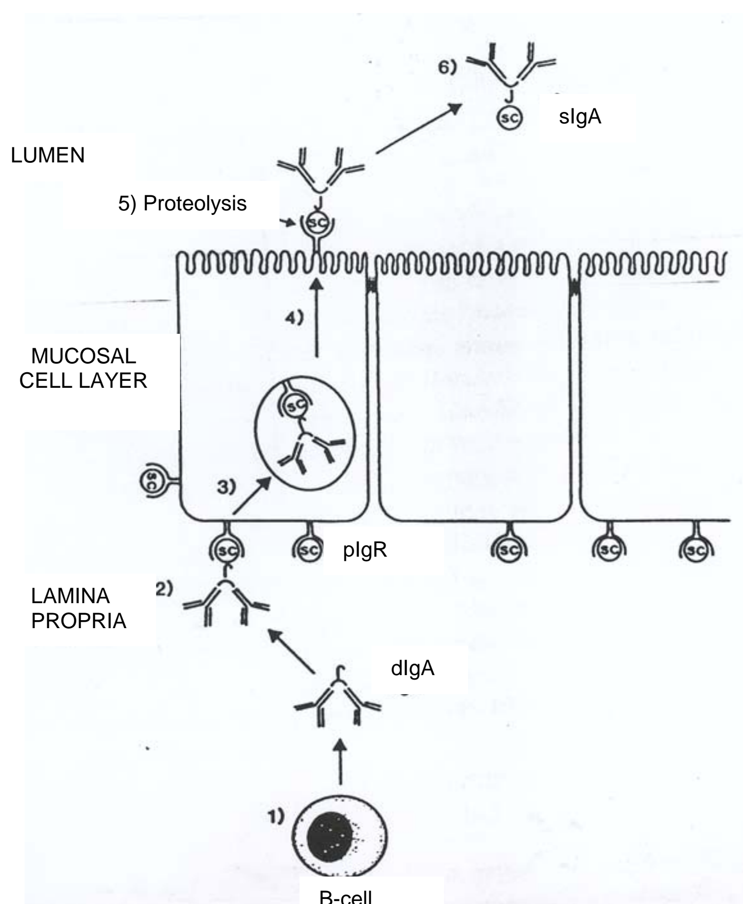

Figure 4. The transcytosis of dimeric IgA through epithelial cell layer to secretions. 1) The dimeric IgA (dIgA) is produced by the B-cells of the MALT. 2) dIgA binds IgA to polymeric Ig receptor (pIgR) via its J-chain. 3) The complex of dIgA and pIgR traverses the epithelial cell in an endocytic vesicle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

Secretory IgA participates in protection against pathogens in all mucosal secretions including saliva, tears, breast milk, and gastrointestinal fluids. SIgA is an effective isotype at mucosa, because of its stabile nature and resistance to many degrading enzymes. SC

prevents the proteolysis of sIgA and further SC protects the binding site for the Fcα receptor in sIgA, which improves sIgA's ability to activate phagocytic cells at the mucosal surface (Almogren *et al.* 2003). Further, the polymeric structure of sIgA enhances binding to antigens (Taylor and Dimmock 1985). SIgA prevents pathogens from entering the human body through mucosal epithelium and it neutralises viruses, enzymes and toxins (Russell *et al.* 1999). Further, sIgA functions synergistically with factors of the innate immune system, e.g. lactoperoxidase and lactoferrin (Marcotte and Lavoie 1998).

SIgA functions at all levels of the mucosal compartment. First, sIgA traps antigens in lumen and eliminates them together with mucus by a mechanism called immune exclusion (Stokes *et al.* 1975). The second level of function is the neutralisation of intracellular viruses (Mazanec *et al.* 1995; Bomsel *et al.* 1998). SIgA also acts at the third level, stromal side, eliminating antigens that have passed the epithelium by transporting them back through M cells to the luminal site using transcytosis (Robinson *et al.* 2001) or by FcαR-bearing phagocytes (Geissmann *et al.* 2001). Pathogens may also use the transcytosis route to invade human body (Phalipon and Corthesy 2003). *Streptococcus pneumoniae* can attach to human pIgR at nasopharyngeal epithelial cells by choline binding protein A (CbpA) and to use the reverse direction of the transcytosis route to invade the human body through nasopharynx (Brock *et al.* 2002). Serum IgA has anti-inflammatory effects, it can e.g. inhibit complement mediated IgG and IgM responses (Griffiss 1983; Kilian *et al.* 1988). Moreover, IgA in human amniotic fluid can protect a foetus by binding to natural maternal IgG autoantibodies (Quan *et al.* 1998). IgA is not able to activate complement by the classical pathway (Schaapherder *et al.* 1995), however, serum IgA can initiate complement activation by the alternative (Hiemstra *et al.* 1987; Janoff *et al.* 1999) or by the mannan-binding lectin pathway (Roos *et al.* 2001). Anti-Pnc PS specific IgA has also been found to activate neutrophil effector functions by binding to FcαR1 (van der Pol *et al.* 2000). Further, anti-Pnc PS specific sIgA in breast milk can initiate the killing of pneumococcus (Finn *et al.* 2002). However, the mechanism of this is not yet known.

1.3.5 Immunoglobulin G and other immunoglobulins at mucosa

All five Ig classes, IgA, IgG, IgM, IgE, and IgD are represented at mucosal membranes. IgG in mucosal secretions has traditionally been regarded as originating from serum by diffusion

(Brandtzaeg 1971). Serum derived IgG can enter the gut also through the hepatobiliary route in humans (Quan *et al.* 1996). In addition, IgG can be produced locally (Berneman *et al.* 1998; Bouvet and Fischetti 1999; Ogra 2000). A bi-directional transcytosis route can be used for transportation of IgG through the mucosal epithelium. IgG can attach to a major histocompatibility complex (MHC) class I-related Fc-receptor, FcRn, on human and mouse lung epithelium and can be transferred to the apical side of the cell (Spiekermann *et al.* 2002) to compensate functions of sIgA (Bouvet and Fischetti 1999). Actually, on the mucosal membranes of lungs IgG has been found more effective against viral infections than IgA (Palladino *et al.* 1995; Mbawuike *et al.* 1999). Also, in human adenoids B cells excreting IgG are more common than IgA producing cells (Boyaka *et al.* 2000; Zhang *et al.* 2002a). This suggests that both IgA and IgG are important in mucosal immunity.

It has been proposed that polyreactive sIgM is an important factor in primitive immune defence (Bouvet and Fischetti 1999). The sIgM molecule is pentameric and also has a binding site for pIgR and is transported through the epithelium in the same way as IgA (Brandtzaeg *et al.* 1999). However, because of non-covalent binding to SC, sIgM is not as resistant to proteolytic enzymes as sIgA (Bouvet and Fischetti 1999). In IgA deficient patients, higher concentrations of sIgM are produced to compensate the IgA deficiency (Brandtzaeg 1971).

Traces of IgE and IgD can be found in mucosal secretions. However, both IgD and IgE are fragile molecules and thus hard to detect in mucosal secretions. IgE has a role in protection against parasites and in allergy (Negrao-Correa 2001). The actual function of IgD in secretions is not known, but it is supposed to have a role in the maturation process of the mucosal immune system (Seidel *et al.* 2001).

1.4 Regulation of mucosal immune response

The successful regulation of mucosal immune response is a prerequisite for health. The bacteria of normal flora create homeostasis at mucosal membranes by promoting digestion, growth and differentiation of epithelial cells, producing vitamins, and further it is needed to establish both mucosal and systemic immunity (Mayer 2003). Mucosal sIgA and sIgM

antibodies have also a role in homeostasis; mucosal membranes have shown unusual leakiness in sIgA and sIgM deficient mice (Johansen *et al.* 1999).

T cell anergy, apoptosis, and active regulation are all used for the regulation of mucosal immune response (Bailey *et al.* 2001). When APCs present antigens to lymphocytes, two signals are needed for an immune response. First, antigens are presented in context with either class I or II MHC. In addition to this, a co-stimulatory signal is needed. A signal to the T cell through the CD28 molecule will lead to T cell activation and proliferation. Instead, a signal through CTLA-4 (CD152) is inhibitory (Nagler-Anderson 2001). Non-pathogenic bacteria are thought to block the NF-κB/IκB (nuclear factor kappaB/inhibitor kappaB) signalling pathway, which prevents the transcription of pro-inflammatory cytokines and further the immune response (Neish *et al.* 2000).

An important mechanism to regulate mucosal immune responses is oral tolerance. Oral tolerance can be induced by feeding an animal with a new protein antigen in increasing amounts (Challacombe and Tomasi 1980). When later challenged with the same antigen, the immune response decreases. The molecular mechanisms needed for immunological tolerance and the reason why harmless antigens can be tolerated, but an immune response against harmful antigens takes place, are not completely understood, yet. It has been suggested that mechanisms needed for tolerance take place at many stages of the immune response (Bailey *et al.* 2001). As previously mentioned, mature DCs are suggested to function in the regulation of oral tolerance. In lungs, DCs induce IL-10 production and further IL-10 producing regulatory type 1 (T_R1) T cells (Akbari *et al.* 2001). Instead, TGF-β transmits signals to generate T helper type 3 (T_H3) regulatory cells in the gut (Weiner 2001). The third important mucosal regulatory cells are $CD4^+CD25^+$ T cells (Sakaguchi *et al.* 1995). The regulation mechanism of these cells needs cell-to-cell contact and is thus different compared with T_R1 and T_H3 cells. $CD4^+CD25^+$ cells have been found to prevent autoimmune reactions. Neither M cells or PPs are required for the induction of oral tolerance (Alpan *et al.* 2001; Spahn *et al.* 2001). Tolerance can sometimes be broken and this is suggested to be a reason for chronic allergic and infectious diseases. Mucosal immunisation is suggested as prophylaxis against infectious diseases but also for treating allergy, autoimmune and immuno-pathological diseases (Holmgren *et al.* 2003).

1.5 Mucosal memory

When an antigen is encountered for the second time, the immune response occurs faster, and more antibodies with higher avidity are induced. This has been proven clearly within the systemic site of the immune system.

At mucosal sites, both B and T memory cells are available. sIgA producing B memory cells have been detected in the mucosal LP (Farstad *et al.* 2000). Mucosal intraepithelial T memory cells have been found to represent at least two subsets; CD8αβ TCRαβ and CD8αα TCRαβ. The first, CD8αβ positive ones, are conventional memory cells recognising foreign antigens, and CD8αα instead are self-reactive cells with diverse specifities (Cheroutre 2004). Natural rotavirus infection can induce $CD4^+$ memory T cell response in humans (Rott *et al.* 1997). The mucosal memory also takes place after oral immunisation with cholera toxin in mice and humans (Lycke and Holmgren 1986; Lycke *et al.* 1987), and intranasal immunisation induces effectively an immunological memory in mice (Asanuma *et al.* 1998). However, there have been controversial opinions about the existence of mucosal memory after intramuscular vaccination. In mice previous intramuscular vaccination with rotavirus induced an immune response with enhanced magnitude, but the response was not faster than before (Coffin and Offit 1998). Further, it has been speculated that protein antigens could induce mucosal memory, but polysaccharides would lack the effect (McGhee *et al.* 1993).

2 How to study mucosal immunity?

Mucosal immunity can be measured by investigating secretions from different mucosal areas. Saliva is the most easily accessible fluid, but also breast milk and colostrum are relatively simple to collect (Challacombe 1995). However, other mucosal secretions can also be used to study mucosal immunity, e.g. tears, nasopharyngeal washes, vaginal, intestinal, (Kantele *et al.* 1998) and bronchial lavages (Atis *et al.* 2001). Recently, adenoids and tonsils have been used to estimate mucosal immunity (Boyaka *et al.* 2000; Zhang *et al.* 2002a).

Detection of specific antibodies in serum or secretions is the traditional way to estimate both systemic and mucosal immune responses. Antibody concentrations in secretions can be analysed with different techniques. Previously, radio immuno assay (RIA) with

modifications and radial immunodiffusion were often used methods (Gleich and Dunnette 1977; Friedman 1982). Later, enzyme linked immunoassays (EIA), without radioactive agents, have been adapted as a more practical system. There are modifications of the basic EIA, in which an antigen is attached to the surface of a microtiter plate well to bind a specific antibody in a sample. One of them is an antibody capture assay, where a single class of human immunoglobulin is captured by an immobilised anti-immunoglobulin. The signal can be further amplified by the FITC/anti-FITC system (Vyse *et al.* 1999). Another modification of EIA is immuno-PCR, where an enzyme coupled conjugate is replaced by a specific antibody linked to a DNA molecule (McKie *et al.* 2002). Antibody concentrations can also be measured by a time-resolved fluorescence method based on a fluorescent antibody format (Hale *et al.* 2001). A new system, also founded on fluorescence, is a multiplexed bead assay Luminex (Seideman and Peritt 2002; Biagini *et al.* 2004). Luminex technology enables detection of antibodies to multiple different antigens at the same time. Yet, this method has been tested only with serum samples, but it has potential for analysing also saliva samples.

Mucosal immunity can be also studied by exploiting T and B cells. T cell responses have been analysed e.g. from palatine tonsils after immunisation (Davenport *et al.* 2003). The circulation of mucosal lymphocytes within the CMIS offers another approach to study mucosal immune response: the migrating cells can be caught from peripheral blood and investigated for antibody production. Specific ASC have been found in the circulation of humans after mucosal antigen encounter, e.g. after oral typhoid vaccination (Kantele *et al.* 1986), oral cholera vaccination (Czerkinsky *et al.* 1987; Czerkinsky *et al.* 1991; Quiding *et al.* 1991) and in patients with a mucosal infection (Kantele *et al.* 1994). During the last decade the ASC assay has been established as an important method for assessing the human mucosal immune response especially to oral vaccines. The most useful method for this is ELISPOT i.e. enzyme-linked immunospot assay (Czerkinsky *et al.* 1983; Sedgwick and Holt 1983). In that assay antigen-specific B cells homing into peripheral areas can be detected from blood by incubating samples with a specific antigen coated on a microtiter plate. If there are ASC secreting antibodies in a sample, antibodies bound to solid antigens can be detected as spots in the bottom of the well. It is noteworthy, that specific ASC appear in the circulation also after parenteral immunisation (Nieminen *et al.* 1996; Kantele *et al.* 1997; Kantele *et al.* 1998; Nieminen *et al.* 1998a; Nieminen *et al.* 1998b; Nieminen *et al.* 1999;

Kantele *et al.* 1999a; Kantele *et al.* 1999b). These cells are not regarded as homing into the mucosal but into the systemic immune system (Kantele 1996). In order to use the ASC assay as a measure of mucosal immune response, the expected site of homing of these cells needs to be considered. This can be performed by analysing the homing receptors on the surface of these cells (Kantele 1996).

2.1 Saliva samples

Saliva is secreted from parotid, submandibular, sublingual, and minor salivary glands, and fluid accesses the mouth also through gingival crevices (Challacombe and Shirlaw 1994). There is a nomenclature for saliva obtained from different glands (Malamud and Tabak 1993). Further, the mixture of secretions is named as whole saliva, and oral fluid as fluid obtained by placing absorptive collectors into the mouth. Here, the term saliva is referring to whole saliva.

Saliva is an important diagnostic specimen. It reflects the immunological, nutritional and even mental state of the human body (Mandel 1993). Saliva can be used for screening different hormones, therapeutic drugs, drugs of abuse, and antibodies of different isotypes (O'Neal *et al.* 2000; Gann *et al.* 2001; Shirtcliff *et al.* 2001; Tabak 2001). There are many advantages in the use of saliva to study mucosal immunity; it is excreted continuously and it can be collected non-invasively. Further, specially trained persons are not needed to collect saliva samples.

However, there are also disadvantages. Saliva samples can be either unstimulated or stimulated. Usually, unstimulated saliva best reflects the natural situation in the mouth. However, it is difficult to get a sample without any stimulation. Further, there can be diurnal and monthly variation in salivary antibody concentrations (Butler *et al.* 1990). In infants, breast-feeding can have an effect on the antibody concentration in the saliva. Thus, mothers are asked not to breast-feed children just before collecting saliva.

Saliva samples are often collected by suction with plastic pipettes, or simply by drooling. Collecting with a pipette can be enhanced by a mechanical suction device. There are also several commercial kits for saliva sampling e.g. OraSure, Omni-SAL, Oracol, Orapette, and

Salivette (Hodinka *et al.* 1998; Vyse *et al.* 2001; Judd *et al.* 2003). The use of method depends also if whole saliva (oral fluid) is preferred or if a sample consisting more of crevicular fluid is needed. Saliva can also be collected straight from individual salivary glands (Navazesh 1993).

Saliva contains many bacterial and other enzymes, e.g. IgA1-protease, which have been found to degrade antibody concentrations quickly. To hinder the decrease of antibodies in samples, the function of enzymes may be prevented either by freezing or by enzyme inhibitors. However, one study has shown that saliva samples can be stored even at +4°C for several days without degradation of Igs (Mortimer and Parry 1988). Others have found that antibody concentrations decrease in saliva samples rapidly when salivas are stored either at +4°C or –20°C (Butler *et al.* 1990) or during storage at –30°C for 3 months (Ng *et al.* 2003). Storage at –70°C with 50% glycerol could hinder the loss of antibodies during storage (Butler *et al.* 1990).

3 How to induce mucosal immunity?

3.1 Natural immunity

Human secretions contain natural polyreactive autoantibodies, which can react also with pathogens (Quan *et al.* 1997). These antibodies possibly provide protection at mucosal sites. However, natural encounters with bacteria e.g. carriage and acute otitis media can induce a specific mucosal immune response (Simell *et al.* 2001; Simell *et al.* 2002).

IgA can be detected in the saliva of an infant even on the first day of life (Seidel *et al.* 2001). Some studies suggest that adult levels of salivary antibodies are attained at the age of 6 to 8 years (Burgio *et al.* 1980). Others speculate that reaching adult Ig levels takes longer, and an increase in mucosal antibody concentrations continues even throughout the life (Grundbacher 1988). One study suggests that mucosal immunity to *Haemophilus influenzae* type b (Hib) develops earlier in life than systemic immunity (Pichichero *et al.* 1981).

Antibodies induced by natural contact with pathogens can be protective against mucosal infections. Both carriage of pneumococcus and pneumococcal AOM can induce specific

mucosal antibodies (Simell *et al.* 2001; Simell *et al.* 2002), that might have a role in defence against pneumococcal local infections like AOM (Simell 2003). Also, Hib infections induce mucosal antibodies (Pichichero *et al.* 1981; Gilsdorf and McDonnell 1991). Further, antibodies in breast milk can offer protection for an infant. For example, the risk of dying of diarrhoea is reduced 14-24 times in breast-fed infants compared to infants who have not been breast-fed (Brandtzaeg 2003a).

3.2 Parenteral vaccines

Parenteral vaccination leads to a systemic immune response. However, already in 1973 parenteral vaccines were shown to induce also mucosal antibody response (Ogra and Ogra 1973). There are different speculations about the mechanisms, how parenteral immunisation can induce mucosal response (Bouvet *et al.* 2002). It has been suggested that an intramuscular antigen migrates to draining peripheral lymph nodes, where it is internalised by APCs (Coffin *et al.* 1999). An mucosal immune response takes place, when these APCs reach mesenteric lymph nodes (MLNs) or PPs. An intramuscular soluble or phagocytosed antigen may also diffuse directly to MLNs or PPs, where it meets APCs and further induces a mucosal immune response. Instead, after transcutaneous or intradermal immunisation antigens can activate APCs of the skin and lead to a mucosal immune response (Kripke *et al.* 1990). Parenteral typhoid vaccination has been found to induce ASCs expressing homing receptor $\alpha4\beta7$ directing them to the gut. However, when an antigen is introduced parenterally only 58% of the specific ASCs carry $\alpha4\beta7$ on their surface, but after mucosal administration the respective number is 99% (Kantele *et al.* 1997). Certain adjuvants, CT, forskolin and an active form of vitamin D_3, have been found to enhance the mucosal immune response induced by intradermal diphtheria toxoid (DT) vaccine in mice (Enioutina *et al.* 2000). Vitamin D3 also improves the mucosal immune response following intramuscular vaccination in pigs (Van Der Stede *et al.* 2004).

3.2.1 Protein vaccines

Protein vaccines can consist either of living attenuated or inactivated pathogens, inactivated toxins or purified proteins. Proteins are T cell dependent (TD) antigens, which use T cell help to raise an immune response. TD antigens are presented to T cells through MHC

molecules by APCs. TD antigens are immunogenic already in infancy, and able to induce immunological memory, affinity maturation and isotype switching of antibodies.

The first study to indicate that intramuscular immunisation could induce a mucosal IgG response was conducted with polio vaccine (Ogra and Ogra 1973). Later parenterally administered vaccines e.g. cholera (Mascart-Lemone *et al.* 1988), *Salmonella typhi* (Kantele *et al.* 1991), tetanus (Smith *et al.* 1986), influenza (Brokstad *et al.* 1995), and measles (Bellanti *et al.* 2004) have been found to a induce mucosal antibody response.

3.2.2 Polysaccharide vaccines

Polysaccharide vaccines are based on purified capsular polysaccharides of bacteria. Polysaccharides are classified as T cell independent (TI) antigens, which do not require T cell help to induce an immune response. TI antigens can further be divided into TI-1 and TI-2 antigens. TI-1 antigens, e.g. lipopolysaccharides (LPS), function as mitogenic or polyclonal B cell activators and are completely independent from T cell help. TI-1 antigens can induce immune response early in life. TI-2 antigens, such as capsular polysaccharides, consist of highly repetitive epitopes and are able to activate only mature B cells. The mechanisms, how TI-2 antigens induce the immune response, are not entirely understood, yet. Anyway, TI-2 antigens can induce an immune response by cross-linking surface exposed immunoglobulin on B cells (Lesinski and Westerink 2001a).

TI-2 antigens are not presented to T cells through MHC II molecules. However, different T cell derived factors regulate TI-2 response (Baker 1992). How activation of T cells happens, is not known. One suggestion is that γδ T cells are involved in antigen presentation without MHC (Williams 1998). Another speculation is that DCs could present also polysaccharides along with lipids and glycolipids with the help of CD1 molecules on their surface (Porcelli and Modlin 1999). Natural killer (NK) cells and macrophages are also potential regulators of TI-2 responses (Snapper and Mond 1996) as well as CD5+ B cells (Neron and Lemieux 1997).

Immunity to TI-2 antigens in human develops slowly. One reason for the late maturation of immunity is that only B cells of adults express type 2 complement receptor (CR2) on their surface (Griffioen *et al.* 1992). CR2 is known to recognise PS combined with complement

component C3d and increase immunity to PSs. A second reason can be that CD5+ B cells, which are known to produce antibodies against PS, are rare in infants (Barrett *et al.* 1992). The responsiveness to polysaccharides has been found to increase along with CR2 and CD5+ B cells.

TI-2 antigens are not able to raise immunological memory. Neither, can they induce affinity maturation or class switching. Thus, after the second contact with the polysaccharide antigen the concentration of antibodies induced is not higher, and avidity and opsonophagocytic activity of antibodies are not increased.

Polysaccharide vaccines have been developed against Hib, *Neisseria meningitidis* (meningococcus), *Streptococcus pneumoniae* (pneumococcus), group B Streptococcus (GBS) and *Salmonella typhi*. Parenteral vaccination with both Hib (Pichichero and Insel 1983), meningococcus (Nieminen *et al.* 1996; Zhang *et al.* 2000; Zhang *et al.* 2001a), pneumococcus (Nieminen *et al.* 1998a; Nieminen *et al.* 1998b), and *Salmonella typhi* (Kantele *et al.* 1999a) polysaccharide vaccines have induced mucosal immune response.

3.2.3 Conjugate vaccines

Avery and Goebel found already in 1920's that saccharides can be turned immunogenic by binding them covalently to protein antigens (Avery and Goebel 1926). However, the first conjugate vaccine for use in humans was not licensed until 1987 against Hib (Anonymous 1988).

Conjugate vaccines consist of purified polysaccharides or/and oligosaccharides covalently linked to carrier proteins. Different conjugation techniques are available. However, every polysaccharide is conjugated separately to a carrier protein.

The linkage of polysaccharides with proteins is thought to induce internalisation of conjugated complexes by B cells and presentations of antigens through the MHC II route to T cells (Schneerson *et al.* 1980). The immunogenicity of a conjugate vaccine depends on the conjugation method, on the length of the polysaccharide, on the carrier molecule, and on the number of polysaccharides in the vaccine (Pawlowski *et al.* 2000). The concentration of a carrier protein is a critical factor for the immunity of the conjugate vaccine. The amount

needs to be high enough to turn the immune response T cell dependent. However, a too large quantity of carrier protein can cause competition between carrier and polysaccharide specific B cells (Fattom *et al.* 1999) and excess induction of carrier specific antibodies (Peeters *et al.* 1991). This can lead to an impaired immune response to the polysaccharides included in the vaccine (Dagan *et al.* 1998; Åhman *et al.* 1999). Parenteral conjugate vaccines have been found to induce mucosal immune response against Hib (Kauppi *et al* 1995), pneumococcus (Nieminen *et al.* 1998a; Nieminen *et al.* 1998b; Nieminen *et al.* 1999; Korkeila *et al.* 2000; Choo *et al.* 2000b), meningococcus (Borrow *et al.* 1999; Zhang *et al.* 2000; Zhang *et al.* 2001a; Zhang *et al.* 2002b), and *Salmonella typhi* (Singh *et al.* 1999).

3.3 Mucosal vaccines

Mucosal membranes provide the entry for most of the pathogens. Therefore, it would be important to have protection at the site where the infection naturally begins. Mucosal immunisation has several advantages. It could activate both B and T cells responses in the mucosal and systemic immune system and thus protect against both carriage and invasive disease. Mucosal memory may also be induced. Further, mucosal vaccines are easily administered and non-invasive. The immunisation without needles would be important especially in poor countries to prevent transmission of parenterally transmitted diseases such as hepatitis B and C, and human immunodeficiency virus (HIV). However, at the moment there is only one mucosal vaccine which is globally available for routine use, the oral poliovirus vaccine. Other mucosal vaccines are produced against typhoid fever, adenovirus, rotavirus, cholera, and cold-adapted influenza virus (Ogra *et al.* 2001).

A lot of problems need to be solved when developing mucosal vaccines. First, pure polysaccharides or proteins are not able to induce an immune response, thus proper adjuvants are essential for mucosal immunisation. Heat-labile enterotoxin of *Escherichia coli* (LT) and exotoxin of *Vibrio cholerae* (CT) and their detoxified variants have been used in many studies with animals (Rappuoli *et al.* 1999). Previous products have been too toxic to use in humans (Levine *et al.* 1983), but site-directed mutagenesis has enabled to generate non-toxic forms of these molecules (Pizza *et al.* 2001). The fully nontoxic mutant of LT, LTK63, has been tested in humans with a trivalent subunit influenza vaccine with good results (Peppoloni *et al.* 2003). Other potential adjuvants are: purified B subunit of CT

(Czerkinsky *et al.* 1991), oligodeoxynucleotides containing immunostimulatory CpG motifs (Gallichan *et al.* 2001), IL-12, (Lynch *et al.* 2003), IL-1 (Staats and Ennis 1999), IL-6 (Rincon *et al.* 1997) and IFN-γ (Proietti *et al.* 2002). With cytokines an immune response could also be directed towards Th1 or Th2 type.

The second problem is to solve how to transport antigens to inductive areas without degradation or inactivation. Liposomes (Childers *et al.* 1990), microcapsules (Lazzell *et al.* 1984), chitosan (Jabbal-Gill *et al.* 1998) and immunostimulating complexes (ISCOMs) (Thapar *et al.* 1991) are solutions, which have been tested, with variable results, to deliver vaccines to inductive sites.

The third problem is to get the antigens in contact with M cells to be transported through follicle-associated epithelium (Jakobsen and Jonsdottir 2003). Actually, targeting M cells to induce mucosal response is one of the strategies to induce mucosal immune response (Neutra *et al.* 1996). To overcome this problem, live bacterial and viral vectors have been used to transport antigens. Attenuated salmonella is an effective vehicle, because it invades the human body through M cells (Jepson and Clark 2001). Other potential bacterial vectors are *Mycobacterium bovis* BCG (Mederle *et al.* 2003), lactobasilli (Scheppler *et al.* 2002), streptococci (Lee 2003), *Yersiniae* (Sory *et al.* 1990), and *Shigella* (Vecino *et al.* 2002). Viral vectors studied are vaccinia (Ramirez *et al.* 2003), polio (Crotty *et al.* 1999), adeno (Xin *et al.* 2002), rhino (Dollenmaier *et al.* 2001) and influenza (Ferko *et al.* 2001) viruses. In the future plants may be used as a delivery system. Edible vaccines would be cheaper and easier to produce than current vaccines with industrial fermentation technique (Yuki and Kiyono 2003).

The possible development of tolerance to orally and nasally administered antigens also poses a problem. E.g. carrier induced suppression affects mucosal immunity when a conjugate vaccine is administered intranasally (Bergquist *et al.* 1997). This can be overcome by formulating an antigen. Live attenuated vaccines have been less tolerogenic than dead or subunits vaccines. Further, for example, live cold-adapted influenza vaccine protected better against the infections than inactivated influenza vaccine (Treanor *et al.* 1999).

Theoretically mucosal vaccines can be delivered by the rectal, vaginal, conjunctival, oral and nasal routes. However, the most practical and convenient routes for mucosal vaccination are

oral and intranasal. Further, the route to be used depends also on the target disease. For example, nasal vaccination has been found to induce better immune response in the upper respiratory tract than oral immunisation (Rudin *et al.* 1998). Also, aging has more impact on the immune responses induced by GALT than NALT (Boyaka *et al.* 2003).

3.4 Maternal immunisation

Maternal immunisation e.g. vaccination of women before or during pregnancy is routinely used against tetanus in developing countries. The passive transfer of IgG antibodies through placenta and/or sIgA and IgG via breast milk offers protection for the infant for the first months of life. Maternal vaccination has been studied also against Hib (Englund and Glezen 2003), Pnc (O'Dempsey *et al.* 1996a), Men (Shahid *et al.* 2002), influenza (Englund 2003), and respiratory syncytial virus (Munoz *et al.* 2003). It is a possible immunisation strategy to induce mucosal immune response (Shahid *et al.* 1995). In humans, meningococcal polysaccharide vaccine induced antibodies in breast milk (Shahid *et al.* 2002). Further, in pregnant chimpanzees both parenterally and mucosally administered HIV DNA vaccines have induced systemic and mucosal immune responses (Bagarazzi *et al.* 1999).

4 Pneumococcus and pneumococcal vaccines

4.1 Streptococcus pneumoniae

Streptococcus pneumoniae (pneumococcus, Pnc) is a Gram positive, facultatively anaerobic lancet-shaped coccus. Pneumococcus is covered with a polysaccharide capsule, which is an important virulence factor offering Pnc the ability to resist phagocytosis (Watson and Musher 1990). The classification of Pnc into 90 different serotypes is based on the polysaccharide capsule (Henrichsen 1995). Further, cross-reactive serotypes have been divided into serogroups. There are two nomenclatures for serotypes, Danish and American (Kaufman *et al.* 1960), the first one based on cross-reactions between polysaccharides, being widely accepted. Pnc strains can be further classified by multilocus sequence typing (MLST) (Feil *et al.* 2004).

Beneath the polysaccharide capsule pneumococcus has two more layers, cell wall and plasma membrane. The cell wall consists of a peptidoglycan backbone, where both capsular polysaccharides (PS), cell wall polysaccharides (CPS) and proteins e.g. Choline binding protein A (CbpA) are anchored (Gosink *et al.* 2000). The inflammatory reaction during pneumococcal disease is mainly caused by cell wall components e.g. cell wall polysaccharide (Tuomanen *et al.* 1985).

Several proteins participate in the pathogenesis of a pneumococcal infection. For adhesion to epithelial cells Pnc uses CbpA (Rosenow *et al.* 1997), pneumococcal surface adhesin A (PsaA) (Berry and Paton 1996b), and IgA1 protease (Weiser *et al.* 2003). The other important virulence factors of pneumococcus are pneumolysin (Ply) (Cockeran *et al.* 2002), pneumococcal surface protein A (PspA) (Crain *et al.* 1990), autolysin (LytA) (Berry *et al.* 1989), hyaluronidase (Kostyukova *et al.* 1995), zinc metalloproteinase (ZmpB) (Blue *et al.* 2003), and neuraminidase enzymes (NanA and NanB) (Berry *et al.* 1996a).

To adapt to ambient circumstances, pneumococcus can undergo spontaneous phase variation between opaque and transparent colony morphologies (Weiser *et al.* 1994). The opaque type is more virulent and contains more capsular polysaccharide and less teichoic acid than the transparent type (Kim and Weiser 1998). The transparent form has been associated with adherence and colonization of the nasopharynx (Cundell *et al.* 1995). In contrast, opaque type can more easily resist phagocytosis and cause an invasive disease (Kim *et al.* 1999).

Pneumococcus causes a wide variety of diseases, ranging from local acute otitis media (AOM) and sinusitis to severe life-threatening diseases, like pneumonia, meningitis and sepsis. Pneumococcus spreads from person to person by aerosols. Inhalation of droplets containing pneumococcus can lead to the colonisation of the nasopharyngeal epithelium and further to asymptomatic carriage. The carriage rate is highest at the age of two years (Dagan *et al.* 1996) after which it starts to decrease (Stenfors and Räisänen 1990). In a Finnish study, 87% of the children carried pneumococcus in their nasopharynx at least once before the age of two years (Syrjänen *et al.* 2001). In developing countries carriage rate is even higher (Gratten *et al.* 1986; Lloyd-Evans *et al.* 1996). Carriage rate also varies depending on the season, being higher in winter than in summer (Gray *et al.* 1982).

Pneumococcal carriage may lead to AOM or pneumonia and invasive disease. Pneumococcus is the most important bacterial cause of both AOM (Luotonen *et al.* 1981; Virolainen *et al.* 1994; Kilpi *et al.* 2001) and community-acquired pneumonia (Heiskanen-Kosma *et al.* 1998; Juven *et al.* 2000; Jokinen *et al.* 2001). Along with meningococcus, pneumococcus has been the leading cause of severe invasive disease since the introduction of Hib vaccines (Dawson *et al.* 1999). The risk groups for pneumococcal diseases are infants, elderly and immunocompromised people. Other factors predisposing to pneumococcal disease are smoking (Nuorti *et al.* 2000), parental smoking, lack of breast feeding, and crowded living conditions (Pukander *et al.* 1985; Ghaffar *et al.* 1999). Further, viral infections often prepare the way for pneumococcus to cause a disease and have been found in association with e.g. AOM (Heikkinen 2000). Also, the pathogenicity of different pneumococcal serotypes varies (Brueggemann *et al.* 2003). For example serotype 14 causes more often an invasive disease than serotype 3 (Hausdorff *et al.* 2000). The increasing resistance of pneumococcus to penicillin makes the treatment of pneumococcal disease more difficult than before (Jacobs 2003; Amsden 2004). Further, the growing incidence of HIV, which predisposes to pneumococcal disease, complicates the pneumococcal problem especially in developing countries (Obaro 2000). Thus, pneumococcal vaccines are needed to prevent the disease in the first place.

4.2 Pneumococcal polysaccharide vaccines

The era of pneumococcal vaccines started in the beginning of the 20th century, when the first pneumococcal vaccine study was conducted by using whole heat-killed pneumococci in South African gold miners (Wright *et al.* 1914). However, the first vaccine was not very successful because of the many side-effects (Austrian *et al.* 1976). The significance of immunity to capsular polysaccharides was found in the 1930's by Francis and Tillet (Austrian 1975). This invention lead to the preparation of the first pneumococcal polysaccharide vaccine (PPV) in 1945 (MacLeod *et al.* 1945). The discovery of penicillin in the 1940's decreased the enthusiasm to develop pneumococcal vaccines, because it was thought that pneumococcal diseases could easily be treated. In the 1960's the interest towards pneumococcal vaccines rose again, because the high morbidity of pneumococcal diseases continued (Smit *et al.* 1977). In 1977 the PPV containing 14 serotypes was licensed

in the United States. Later the vaccine was modified (Robbins *et al.* 1983) and since 1983 the 23-valent vaccine containing 25 µg of each serotype has been in use. These 23 serotypes included in the vaccine cover approximately 80-95% of the invasive pneumococcal diseases depending on the geographical area.

PPVs are safe, immunogenic and effective against invasive diseases and pneumonia in adults and older children (Austrian *et al.* 1976; Smit *et al.* 1977; Leinonen 1982; Leinonen *et al.* 1986; Sims *et al.* 1988; Shapiro *et al.* 1991). PPVs have also found to induce mucosal immune response (Nieminen *et al.* 1998a; Nieminen *et al.* 1998b). However, even if the parenteral 14-valent PPV can induce immune response in middle ear (Koskela 1986), it has limited efficacy against AOM (Karma *et al.* 1985). PPVs are not protective in infants under two years of age or in immunocompromised patients (Leinonen 1982). Further, there are controversial opinions on the effectiveness of PPV among the elderly and the immunocompromised (Örtqvist *et al.* 1998; Honkanen *et al.* 1999; Moore *et al.* 2000; Fedson 2003; French 2003). However, the 23-valent PPV is recommended in many industrialised countries as a prophylaxis in these risk groups.

4.3 Pneumococcal conjugate vaccines

The development of pneumococcal conjugate vaccines (PCV) was encouraged by the successful introduction of Hib conjugate vaccines. The number of serotypes included in the experimental PCVs has been varied from one (Schneerson *et al.* 1986), to 11 different polysaccharides (Wuorimaa *et al.* 2001a; Wuorimaa *et al.* 2001b; Nurkka *et al.* 2002; Puumalainen *et al.* 2002; Dagan *et al.* 2004). Several different carrier proteins have been tested, e.g. non-toxic mutant variant of diphtheria toxin (CRM197), protein D of *H. influenzae* (PD) (Gatchalian *et al.* 2001), outer-membrane complex of Men group B (OMPC), diphtheria and tetanus toxoids (D and T, respectively). Also, a mixture of D and T carriers have been studied (Wuorimaa and Käyhty 2002).

The PCVs studied have been safe and immunogenic in adults, toddlers and infants (Käyhty *et al.* 1995; Åhman *et al.* 1996; Mbelle *et al.* 1999; Shinefield *et al.* 1999; Miernyk *et al.* 2000; Choo *et al.* 2000a; Obaro *et al.* 2000; Wuorimaa *et al.* 2001a; Wuorimaa *et al.* 2001c; Zangwill *et al.* 2003). They are also protective against invasive disease, pneumonia, and to

some extent AOM (Black *et al.* 2000; Eskola *et al.* 2001; Klugman 2001; Black *et al.* 2002; Shinefield *et al.* 2002; Fireman *et al.* 2003; Kilpi *et al.* 2003; O'Brien *et al.* 2003). Further, pneumococcal conjugate vaccines reduce carriage (Dagan *et al.* 1997; Mbelle *et al.* 1999; Dagan *et al.* 2002), and induce immunological memory (Åhman *et al.* 1998; Eskola 2000; Kamboj *et al.* 2003), and affinity maturation leading to better avidity and opsonophagocytic activity of antibodies (Anttila *et al.* 1998; Vidarsson *et al.* 1998; Anttila *et al.* 1999a; Anttila *et al* 1999b Wuorimaa *et al.* 2001b; Puumalainen *et al.* 2003). Pneumococcal conjugate vaccines are also able to stimulate mucosal immunity (Nieminen *et al.* 1998a; Nieminen *et al.* 1998b; Nieminen *et al.* 1999; Korkeila *et al.* 2000; Choo *et al.* 2000b).

In 2000, the first PCV, PncCRM (Prevnar®, Wyeth-Ayerts Laboratories, Philadelphia, USA), was licensed in the Unites States, in 2001 (Prevenar®) in the European Union, and later in many other countries. At the moment, this 7-valent vaccine is the only pneumococcal conjugate vaccine available. However, serotypes 1 and 5, not included in this vaccine, are important serotypes in many developing countries (Sniadack *et al.* 1995; O'Dempsey *et al.* 1996b; Kanungo and Rajalakshmi 2001).

4.4 Other pneumococcal vaccination strategies

Maternal immunisation with PCVs results in specific antibodies in newborn infants and in breast milk (Shahid *et al.* 1995; Lehmann *et al.* 2003). Further, no tolerance or suppression of immunity induced by maternal vaccination were found (Lehmann *et al.* 2003). Also, immunisation of post partum women can generate anti-Pnc IgA in breast milk (Finn *et al.* 2002). These antibodies were able to initiate killing of pneumococcus. Anti-Pnc antibodies induced by vaccination of pregnant mice with PCV were protective in their offspring against pneumococcal infections by homologous PSs (Richter *et al.* 2004). The antibody concentrations persisted for several weeks and slowly degraded over time.

Pneumococcal proteins common to all 90 different serotypes are suggested to induce broader protection than polysaccharide-based vaccines. Another reason for the search of pneumococcal protein vaccines is that non-vaccine serotypes may replace vaccine serotypes (Mbelle *et al.* 1999; Dagan *et al.* 2002). There are several candidates for future protein vaccines e.g. pneumolysin, PsaA, PspA, PspC (Briles *et al.* 2000a), alpha-enolase, IgA1

proteinase, streptococcal lipoprotein rotamase A, putative proteinase maturation protein A (Adrian *et al.* 2004), PhpA (Zhang *et al.* 2001b), and protein of the Pht family (Adamou *et al.* 2001; Hamel *et al.* 2004). A combination of two or more different proteins could also be a solution (Briles *et al.* 2000b). One more approach is to conjugate pneumococcal polysaccharides and proteins. In mice serotype 9V conjugated to inactivated pneumolysin or autolysin could induce a protective immune response against invasive pneumococcal infection (Lee *et al.* 2001).

Mucosal vaccination has been considered as a protective strategy also against pneumococcus. The first study on mucosal vaccination against pneumococcus was carried in 1991 in guinea pigs (Yoshimura *et al.* 1991). The first results were promising, and later intranasal immunisation of mice with whole heat-killed encapsulated pneumococci has been found to protect against systemic infection (Hvalbye *et al.* 1999). Also, unencapsulated pneumococci administered with CT as an adjuvant protected mice against pneumococcal infection (Malley *et al.* 2001). Oral vaccination with PCV in enterocoated microencapsules have been tested in mice (Flanagan and Michael 1999). However, this strategy could not induce a proper mucosal IgA response. Instead, better results were achieved with intranasal immunisation with PCV (Jakobsen *et al.* 1999). IL-12 as an adjuvant further enhanced the immune response in mice (Lynch *et al.* 2003). Anyway, others have found oral vaccination with pneumococcal PSs in microspheres with cholera toxin B subunit adjuvant protective in mice (Seong *et al.* 1999). Also, PspA and PsaA administered orally were protective against pneumococcal infection in mice (Yamamoto *et al.* 1997; Seo *et al.* 2002), These proteins were also able to prevent pneumococcal carriage in mice (Briles *et al.* 2000c). The route of mucosal Pnc vaccination may have an impact on the immune response (van den Dobbelsteen *et al.* 1995).

A new vaccination strategy against pneumococcal infections are DNA vaccines. A DNA sequence encoding a peptide mimicking pneumococcal serotype 4 has resulted in specific antibodies in mice (Lesinski *et al.* 2001b). Also, DNA vaccines coding proteins PspA and PsaA have been immunogenic in an animal model (Miyaji *et al.* 2001).

5 Meningococcus and meningococcal vaccines

5.1 *Neisseria meningitidis*

Neisseria meningitidis (meningococcus, Men) is a Gram negative bacterium. Meningococcus has two cell membranes and between them a rigid peptidoglycan layer. The outer cell membrane contains amphiphilic lipo-oligosaccharide (LOS) molecules and it is further covered by a capsule consisting of polysaccharides. Meningococcus can be divided into serogroups, serotypes, subtypes and immunotypes according to capsular polysaccharides, PorA outer membrane proteins (OMP), PorB OMP and LOS, respectively. Meningococcus can further be classified by clonal families by using multilocus enzyme electrophoresis (Hart and Rogers 1993), but multi-locus sequence typing and pulse-field gel electrophoresis are nowadays used for the characterisation of different meningococcal strains (Morley and Pollard 2001).

Meningococcus can vary surface antigens quickly depending on the environmental and host factors. The most important virulence factor of meningococcus is the polysaccharide capsule (Vogel and Frosch 1999). The switching of capsule serogroup between similar meningococcal types promotes further the ability to escape host immunity (Swartley *et al.* 1997). Other factors involved in the pathogenesis of meningococcus are pili, OMPs with different functions and IgA1 protease (Griffiss 1995).

Humans are the only natural reservoir of meningococcus. It spreads by respiratory droplets from carriers and may lead to disease, either meningitis or bacteraemia. The meningococcal disease is always severe, the mortality rate in developed countries varies between 7 and 10% in acute meningococcal disease (Steven and Wood 1995). Survivors often suffer from neurological sequelae, e.g. hearing loss and mental retardation. Very rarely severe meningococcal infection can lead even to the loss of a limb. When compared to carriage rates, meningococcus seldom causes disease, and often a disease is caused by a recently acquired strain (Gold *et al.* 1978). Long term carriage does not necessarily protect against meningococcal disease (Ala'Aldeen *et al.* 2000). Infants carry meningococcus in their nasopharynx rarely. The carriage rate is approximately 2% in children under 4 years of age (Cartwright *et al.* 1987). However, it increases by age being highest among teenagers and

young adults. In Europe, the rate of overall carriage of meningococcus is approximately 10% (Cartwright *et al.* 1987; Caugant *et al.* 1994). Risk factors for the carriage of Men and further for meningococcal disease are age, contact with carriers, season (peaking in spring and autumn in industrialised countries) (Peltola 1983), low socio-economic status (Filice *et al.* 1984), over-crowding (Baker *et al.* 2000), and active or passive smoking (Stuart *et al.* 1989; Caugant *et al.* 1994). Also, influenza or other viral infections are known to predispose to meningococcal diseases (Voss and Lennon 1994).

From the 13 different meningococcal serogroups A, B and C cause 90% of the meningococcal diseases (Ala'Aldeen *et al.* 2000). Meningococcal diseases may occur as epidemics, especially serogroup A (MenA). In 1970's there were many MenA epidemics around the world, one of them in Finland (Peltola 1983). In the sub-Saharan Africa, "meningitis belt", a MenA epidemic occurs every 7 to 10 years (Hart and Cuevas 1997; Molesworth *et al.* 2002). In Europe two thirds of meningococcal diseases are caused by serogroups B and approximately one third is serogroup C based (Cartwright *et al.* 2001). Serogroup Y has been found to infect elderly people (> 65 years old) as well as W135, which causes disease also in infants (Cartwright *et al.* 2001).

5.2 Meningococcal polysaccharide vaccines

In the beginning of the 20th century serum therapy was used to prevent high mortality from meningococcal meningitis (Frasch 1995). Later whole cell and exotoxin vaccines were studied (Pollard and Levin 2000). However, the early studies were neither well controlled nor successful.

The immunogenicity of meningococcal polysaccharides of serogroups A and C (MenC) was first demonstrated by Gotschlich *et al* (Gotschlich *et al.* 1969). Afterwards, both MenA (Mäkelä *et al.* 1975; Peltola *et al.* 1977) and MenC (Artenstein *et al.* 1970; Gold and Artenstein 1971) polysaccharide vaccines (MPV) have been found safe and effective to prevent meningococcal disease. Also, serogroup W135 and Y MPVs are safe and immunogenic (Griffiss *et al.* 1981). The MPVs have been available in different mono- and polyvalent combinations (Saxena *et al.* 1985). The current tetravalent polysaccharide vaccine containing A, C, W135, and Y polysaccharides has been found safe and immunogenic (Armand *et al.* 1982; Peltola *et al.* 1985). It was licensed in 1981 and it is widely used as

prophylaxis for risk groups e.g. army recruits in many countries and in Africa in the area of the meningitis belt during epidemics. However, the immunogenicity is poor in infants, except for Men A (Peltola *et al.* 1977).

5.3 Meningococcal conjugate vaccines

The development of meningococcal conjugate vaccines (MCV) started in the end of 1970' (Riedo *et al.* 1995). The protein carriers used for studied MCVs have been CRM, D and T (Mäkelä and Käyhty 2002). Experimental MCVs containing serogroup C, A and C or A/C/W135/Y have been found safe and immunogenic (Fairley *et al.* 1996; English *et al.* 2000; MacLennan *et al.* 2000; Campbell *et al.* 2002; Rennels *et al.* 2002) and able to induce immunological memory (Borrow *et al.* 2000), which persists at least up to 5 years for MenC (MacLennan *et al.* 2001). Meningococcal conjugate vaccines can also induce mucosal immune response (Borrow *et al.* 1999; Zhang *et al.* 2001a; Zhang *et al.* 2002b). However, serotype replacement can take place also after using meningococcal conjugate vaccines (Perez-Trallero *et al.* 2002).

In 1999, the first meningococcal serogroup C conjugate vaccine (MenC-CRM) was licensed in the UK (Maiden and Spratt 1999) an it is now part of the national vaccine program. After the licensure it has been found highly effective against serogroup C disease in the UK population (Ramsay *et al.* 2001; Lakshman and Finn 2002). Later, two other meningococcal C conjugate vaccines have been licensed (http://www.who.int/vaccines/en/meningococcus.shtml), and other developed countries have been taken MCV into their routine vaccination schedule because of the increased incidence of the disease.

5.4 Other meningococcal vaccination strategies

Protein vaccines have been developed to overcome the problem with MenB cross-reactivity with human tissue (Finne *et al.* 1983) Outer membrane protein based vaccines have been investigated in many studies with variable results (Bjune *et al.* 1991; Sierra *et al.* 1991; de Moraes *et al.* 1992). They have proved more protective among adults and teenagers as compared to children. The PorA protein has been found immunogenic and able to induce protective immunity. However, the immunogenicity of different PorAs is variable (Luijkx *et al.* 2003). Other meningococcal outer protein vaccine candidates studied are OpcA, NspA,

and iron regulated proteins: TbpA and B, FbpA, and FetA (Morley and Pollard 2001). There are also new solutions for the vaccine development, one is to make an anti-idiotypic mimic of meningococcal B polysaccharide (Beninati *et al.* 2004). The another strategy is so called reverse vaccinology, where genes encoding possible vaccine candidates are systemically searched from the meningococcal genome (Vermont and van den Dobbelsteen 2003).

One suggested vaccination strategy is to immunise against the meningococcal transferrin receptor (Banerjee-Bhatnagar and Frasch 1990). Meningococci use host-derived iron compounds and this would prevent the uptake of iron in meningococcus.

Mucosal vaccines against meningococcus have also been developed. An intranasally administered MenC-CRM conjugate vaccine with LT adjuvant induced a more enhanced immune response in mice than a parenteral vaccine (Baudner *et al.* 2004). Intranasal vaccination with MenB derived outer membrane vesicles (OMV) have induced an immune response both in mice and humans (Haneberg *et al.* 1998a; Haneberg *et al.* 1998b; Dalseg *et al.* 1999). Further, intranasally administered OMVs did not induce mucosal tolerance (Bakke *et al.* 2001).

AIMS OF THE STUDY

The immunogenicity of parenterally administered pneumococcal and meningococcal conjugate vaccines has been investigated intensively. However, there are only few studies about their ability to induce mucosal antibody response. In order to broaden the knowledge about the mucosal immunity induced by conjugate vaccines we

- compared different collection and storage methods of saliva samples to find out the best way to preserve the original antibody concentration in a sample (I).
- measured anti-pneumococcal polysaccharide antibody concentrations induced by polysaccharide-protein conjugate vaccines in the saliva of children in two phase two studies (II-III) and two studies connected to a phase three study, FinOM Vaccine Trial (IV-V).
- measured anti-meningococcal polysaccharide antibody concentrations induced by meningococcal polysaccharide-protein conjugate vaccines in the saliva of children (VI).

MATERIALS AND METHODS

This thesis consists of a pilot study to choose collection and storage methods for saliva samples (I), of two immunogenicity studies (II-III) with PCVs, of two immunogenicity studies connected to an efficacy study (IV-V) in which two PCVs were studied parallel (Eskola *et al.* 2001; Kilpi *et al.* 2003), and of one immunogenicity study (VI) with MCV. Study subjects were healthy infants in immunogenicity and efficacy studies and adults in the methodological study. Saliva samples were collected in all of the studies and their antibody concentrations against pneumococcal polysaccharides and/or proteins or meningococcal polysaccharides were studied with an enzyme immunoassay (EIA).

1 Vaccines

1.1 Pneumococcal vaccines

PCVs contain a mixture of pneumococcal polysaccharides and/or oligosaccharides independently conjugated to a carrier protein. PPVs are composed of free polysaccharides. The vaccines used (II-IV) are listed in a Table 1.

Table 1. Pneumococcal vaccines

Study vaccine	Carrier protein	Serotypes	PS/**OS** content	Adjuvant	Manufacturer	Paper
PncCRM1	CRM_{197}, non-toxic mutant variation of diphtheria toxin	4, 6B, 9V, 14, 18C, 19F, 23F	4 µg (6B), 2 µg (4, 9V, 14, 19F, 23F), 2 µg (18C **OS**)	Aluminium phosphate	Wyeth Pharmaceuticals (West Henrietta, NY)	II
PncCRM2	CRM_{197}, non-toxic mutant variation of diphtheria toxin	4, 6B, 9V, 14, 18C, 19F, 23F	4 µg (6B), 2 µg (4, 9V, 14, 19F, 23F), 2 µg (18C **OS**)	Aluminium phosphate	Wyeth Pharmaceuticals (Pearl River, NY)	IV
PncD	Diphtheria toxoid	3, 4, 6B, 9V, 14, 18C, 19F, 23F	3 µg	-	Aventis Pasteur (Swiftwater, PA)	III
PncT	Tetanus toxoid	3, 4, 6B, 9V, 14, 18C, 19F, 23F	1 µg	-	Aventis Pasteur (Lyon, France)	III
PncOMPC	The outer membrane protein complex of *N. meningitidis*	4, 6B, 9V, 14, 18C, 19F, 23F	5 µg (6B), 3 µg (23F), 2 µg (9V, 18C), 1.5 µg (19F), 1 µg (4, 14)	Aluminium hydroxide	Merck & Co., Inc. (West Point, PA)	V
PPV (Pneumovax®)	None	23 serotypes[1]	25 µg	-	Merck & Co., Inc. (West Point, PA)	V
PPV (Pnu-Imune®)	None	23 serotypes[1]	25µg	-	Wyeth Pharmaceuticals (West Henrietta, NY)	II

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

1.2 Meningococcal vaccines

Meningococcal conjugate vaccine MenA/C (Biocine, Siena, Italy), contained meningococcal serogroup A and C polysaccharides (11 µg of each) individually conjugated to a carrier protein CRM_{197}, which is a non-toxic variant of diphtheria toxin (VI). Aluminium hydroxide was used as an adjuvant.

Meningococcal polysaccharide vaccines were Menpovax A plus C (Biocine) and Mengivac A&C (Aventis Pasteur), in the Table 3, MPV1 and MPV2, respectively (VI). Menpovax A plus C contained 50 µg of serogroup A and C polysaccharides and Mengivac A&C 10 µg of each.

1.3 Other vaccines

IV-V: There was a control group of children vaccinated with a hepatitis B vaccine (HBV, Merck & Co., Inc.). One dose of HBV contained 5 µg of hepatitis B surface antigen.

Children also received the normal childhood vaccinations. II-III: The children were immunised with combined diphtheria-tetanus-whole cell pertussis (DTwP) and *Haemophilus influenzae* type b (Hib) conjugate vaccine (Tetramune®, Wyeth) at 2, 4, 6, and 24 months, inactivated polio vaccine (IPV; Polio Novum, National Institute for Public Health and Environment, Bilthoven, Netherlands) at 7, 12 and 24 months, and measles-mumps-rubella (MMR; Virivac, Merck Sharp & Dohme, West Point, PA) at 16 months of age. IV-V: The vaccination schedule was similar to papers II-III, but IPV was Imovax®, Aventis Pasteur, and MMR was MMR®II, Merck & Co., Inc. Further, MMR was given at 18 months of age. VI: Children received routine vaccinations according to the EPI schedule (Twumasi *et al.* 1995), including oral polio, DTwP, hepatitis B, measles and yellow fever vaccines.

2 Study subjects

I: Healthy Kenyan adults among the staff of the Wellcome Trust/Kenya Medical Research Institute, Kilifi, Kenya.

II-III: Healthy Finnish infants from Joensuu and Kerava area.

IV-V: Healthy Finnish children in the Kangasala subcohort of the FinOM Vaccine Trial. This study also contained a control group of children who received hepatitis B vaccine (HBV) instead of the PCVs.

VI: Five years old children who had been recruited for the trial of a meningococcal conjugate vaccine in the Upper River Division of the Gambia in 1992 as infants (Twumasi *et al.* 1995). The study also contained a control group of healthy children who had not received any meningococcal vaccine previously. These children were matched for age and village with the study children.

3 The ethical issues

Before enrolment each adult participant (I) and a parent or legal guardian of a child (II-VI) had received information about the study and had given their informed consent. Protocols with possible amendments were approved by the Ethics Committee of the National Public Health Institute of Finland (KTL) (II-V) and by the relevant local health authorities before the initiation. Studies complied with the latest revision of the Declaration of Helsinki and followed the European guidelines for Good Clinical Practice.

Table 2. Vaccination and sampling schedules in pneumococcal vaccine studies (II-V)

Study Vaccine		Primary series (mo)	Booster (mo)	Saliva samples (mo)	N	Paper
Primary	Booster					
PncCRM1	PncCRM1	2, 4, 6	15	7, 16	30	II
PncCRM1	PPV (Pneumovax®)	2, 4, 6	15	7, 16	30	II
PncD	PncD	2, 4, 6	15	7, 16	25	III
PncT	PncT	2, 4, 6	15	7, 16	25	III
PncCRM2	PncCRM2	2, 4, 6	12	7, 13	57	IV
PncOMPC	PncOMPC	2, 4, 6	12	7, 13	49	V
PncOMPC	PPV (Pnu-Imune®)	2, 4, 6	12	7, 13	7	V

Table 3. Vaccination schedule in the meningococcal vaccine study (VI)

Study group	Meningococcal vaccinations			
	In infancy	At 2 years of age	At 4 or 5 years of age	N
1.	No	No	MPV2	64
2.	1-3 doses of MCV	MCV	MPV2	51
	1-3 doses of MCV	No	MPV2	76
3.	2 doses of MPV1	MPV1	MPV2	15
	2 doses of MPV1	No	MPV2	14
	No	MPV1	MPV2	25
4.	1-3 doses of MCV	MPV1	MPV2	47
5.	2 doses of MPV1	MCV	MPV2	12

4 Samples

4.1 Saliva samples

I: Study subjects were asked not to eat or drink for 2 hours before the study. Saliva samples were collected from every subject by four different methods, conducted in random order 15 minutes apart.

The following collection methods were used:

1. Unstimulated specimen: the subject was asked to “drool” into a clean 50 ml container.

2. Pastette: the investigator sucked the saliva from under the tongue and the para-gingival gutter using a disposable plastic pipette.

3. OraSure® (Epitope, Beaverton, OR): consists of a cotton pad on a short plastic stick, which is placed between the gums and the cheek and left there for 2 minutes. After removing it from the mouth, the stick is broken off and the pad is placed in a storage container with approximately 800 µl of proprietary buffer.

4. Oracol® (Malvern Medical Developments Ltd., Worcester, UK): is a cylindrical plastic sponge mounted on a short plastic stick. The teeth, tongue and gums are brushed by the subject using the Oracol sponge for 60 seconds. The sponge is then placed into an Oracol tube with 1 ml of buffer; 10% foetal calf serum (FCS; Gibco, Paisley, UK) in 0.17 M phosphate buffered saline (PBS) (DulbeccoA, Oxoid, Hampshire, UK), pH 7.3, with 10 µg/ml of C-polysaccharide (CPS; Statens Serum Institut, Copenhagen, Denmark). CPS was added into the buffer, because anti-pneumococcal antibodies were going to be measured.

OraSure and Oracol samples were centrifuged for 5 and 10 minutes, respectively, at 3000 rpm before the pad or sponge was removed.

Each specimen was divided into three equal aliquots and processed and stored in three ways immediately after collecting.

1. Snap-freezing in liquid nitrogen. The first aliquot was mixed with an equal volume of 80% glycerol in H_2O and dipped into liquid nitrogen for a couple of minutes.

2. Enzyme inhibition. The second aliquot was mixed 10:1 with an enzyme inhibitor cocktail consisting of pefabloc, leupeptin and aprotinin (Roche Diagnostics, Mannheim, Germany); 25, 0.5, and 0.25mg, respectively, in 5 mls of PBS with 50mg bovine serum albumin and 20mg EDTA. Samples with enzyme inhibitors were stored at +4°C.

3. Plain. The third aliquot was stored immediately at +4°C without any processing.

At the end of the working day (4 to 8 hours after handling) all samples were placed in a –70°C freezer and stored for approximately one week before the analyses.

II-III: Samples were collected at the study centers with a plastic pipette from the cheek area, frozen immediately and transported in dry ice to KTL.

IV-V: Mothers were advised not to breast feed infants during one hour before saliva sampling. Unstimulated saliva samples (up to 2 ml) were collected at study clinics with gentle aspiration using an electronic suction device. Samples were immediately frozen at –70ºC. However, in the paper IV samples, taken at the age of 4 to 5 years, samples were first frozen in dry ice and then moved within 4 hours to the -70ºC freezer.

VI: Samples were collected with OraSure (Owen Mumford, High Wycombe, UK) between gums and upper teeth and 0.9 ml of saliva was added immediately to 0.1 ml of a 10 times concentrate of bacterial enzyme inhibitors; aprotinin, leupeptin and pefabloc (Roche Diagnostics, Lewes, UK). The samples were immediately frozen in liquid nitrogen until they could be transferred to a -70ºC freezer. They were transported from the Gambia to Finland in dry ice.

In every study, saliva samples were stored at -70ºC if not otherwise stated (I). Before the analysis, samples were centrifuged at 13 000 or 15 000 rpm for 10 minutes and the supernatant was used for the assay. Saliva samples were thawed only once.

4.2 Serum samples

II-VI: Venous blood samples were collected at the same time with saliva samples. Samples were centrifuged and the serum was stored at –20ºC.

5 Serological methods

5.1 Enzyme immunoassay (EIA) for measurement of pneumococcal antibodies in saliva

I: Microtiter plates (Costar 3591, Cambridge, MA) were coated with 15 µg/ml of capsular polysaccharides 1, 5, 6B, and 14 (American Type Culture Collection, Manassas, VA) or with 5 µg/ml of pneumococcal surface adhesin A, PsaA, (Aventis Pasteur, Toronto, Canada) diluted in PBS and incubated over night at room temperature. Plates coated with only PBS were used as a background control to determine non-specific binding. PBS plates were treated in the same way as antigen plates during the assay.

All plates were blocked with 10% FCS-PBS for one hour at +37°C and then emptied without washing. The frozen saliva samples were centrifuged for 10 minutes at 13 000 rpm. Human serum 89-SF (Quataert *et al.* 1995) from the U.S. Food and Drug Administration, Bethesda, MD and an in house serum with high anti-PsaA concentration were used as reference sera for the capsular polysaccharide and PsaA assays, respectively. Saliva samples were diluted 1:10 (expect OraSure samples 1:16.7) in FCS-PBS containing 10µg/ml CPS to neutralize anti-CPS antibodies. Samples were incubated at room temperature for 30 minutes. The neutralization step was conducted also to the first dilutions of references. Samples were assayed at a single dilution in triplicate, reference sera at five serial three-fold dilutions in duplicate. Samples were aliquoted (50 µl/well) and incubated for 2 hours at room temperature with horizontal rotation (200 rpm). Mouse monoclonal anti-human IgA (HP6123, Centers for Disease Control and Prevention, Atlanta, GA) 1:2000 in FCS-PBS was incubated for 2 hours at room temperature, followed by rabbit polyclonal phosphatase conjugated anti-mouse IgG (315-055-045, Jackson's Immuno Research Laboratories, West Grove, PA) in FCS-PBS incubated over night at room temperature (50 µl/well). Substrate, p-nitrophenyl phosphate disodium (Sigma Immuno Chemicals, St. Louis, MO) in phosphate

buffer, pH 9.8, was incubated for one hour at +37°C. Between steps 1-3 plates were washed four times with PBS containing 0.05% Tween 20 (PBS-T) (Merck, Leics, UK); after overnight incubation plates were washed three times with PBS-T and two times with deionised water. Absorbances were measured at 405 nm and results expressed as optical densities (OD) after subtraction of the corresponding sample OD value in a background control plate coated only with PBS.

II-III: Anti-pneumococcal IgG, IgA and sIg to serotypes 4, 6B, 9V, 14, 18C, and 19F was analysed. EIA was conducted as above with a few modifications. Microtiter plates (MaxiSorp™, Nunc, Roskilde, Denmark) were coated with 2.5 to 10 µg/ml of the pneumococcal PSs (ATCC) in PBS. Plates were incubated for 5 hours at +37ºC, and stored at +4ºC for a maximum of four weeks. Serum pool 89-SF was applied as a reference for IgG and IgA assays and an in-house milk with high anti-pneumococcal antibody concentration for sIg assay. Plates were incubated for 2 hours at +37ºC after adding the samples and the reference. Alkaline phosphatase conjugated anti-human IgG (A 3188, Sigma, St. Louis, MO) and monoclonal anti-human IgA (M26012, Oxoid, Unipath, Bedford, UK) and anti-human sIg (I 6635, Sigma) were diluted in the FCS-PBS and incubated for 2 hours at +37ºC. In the IgG assay, the substrate (p-nitrophenyl phosphate disodium in the carbonate buffer) was added and the absorbances were measured after an hour incubation at +37ºC during the same day as the analysis had been started.

IV-V: The EIA assay to measure IgA, IgA1, IgA2, IgG and sIg antibodies for serotypes 6B, 14, 19F and 23F was a mixture of the assays above. Microtiter plates used were Costar and the coating was performed like in Papers II&III. Serum pool 89-SF was applied as a reference for IgG, IgA, IgA1, and IgA2 assays and an in-house milk with high anti-pneumococcal antibody concentration for sIg assay. The plates were incubated with horizontal rotation (200 rpm) for two hours at room temperature after adding the samples and standard. Monoclonal anti-human IgA, HP6123, (Centers for Disease Control and Prevention, CDC, Atlanta, GA), anti-IgA1, HP6116, (CDC), anti-IgA2, HP6109, (CDC) and anti-sIg (Sigma) antibodies diluted in FCS-PBS were used and incubated similarly as after aliquoting the samples. In the IgG EIA, polyclonal alkaline phosphatase conjugated anti-human IgG (cat. number A-3188, Sigma) antibodies were incubated for 2 hours at +37ºC without rotation.

In the EIA for saliva samples (II-V), the OD value of 0.05 (≥ 2 SD of the blank) or higher was regarded as positive (cut off-value). Results for IgA and IgG were calculated in nanograms per millilitre (ng/ml) of saliva. The lowest detected concentration was 5 ng/ml for IgA and IgG for all the serotypes. Samples with undetectable IgA and IgG were assigned values 1.7 ng/ml, half a log less than the lowest detected concentration. IgA1 and IgA2 results are given as EIA units (U), which were calculated from ODs by using 89-SF as a reference with a given calibration factor. The detection limit of IgA1 and IgA2 results for all the serotypes was 1.3 U and samples with undetectable IgA1 and IgA2 concentrations were given the value 0.65 U.

5.2 EIA for measurement of meningococcal antibodies in saliva

Salivary IgG, IgA, and sIg concentrations against MenA and MenC polysaccharides were measured by inhibition EIA (VI). MenA and MenC PSs (Connought Laboratories, Swiftwater, PA) were diluted (5 µg/ml) in PBS containing methylated human serum albumin (5 µg/ml) to improve binding onto the microtiter plates (Immulon, Dynatech laboratories, Chantilly, VA) (Arakere *et al.* 1994). Diluted polysaccharides (100 µl) were pipetted into the wells of the microtiter plates, which were incubated overnight at +22ºC.

On the next day, the plates were washed four times with PBS-T and blocked with 10% FCS-PBS for one hour at +37ºC. After blocking, the plates were emptied. Saliva samples were diluted 1:5 in FCS-PBS and divided into three aliquots. One third of the sample was neutralized with MenA PS and one third with MenC PS for one hour at +4ºC at a concentration of 30 µg PS/ml, titrated to be optimal for absorption of both anti-MenA and MenC antibodies. One third contained only the dilution buffer, FCS-PBS. From each tube 50 µl were pipetted in triplicates into the wells coated with either MenA or MenC PS and incubated for 2 hours at +37ºC. After incubation the plates were washed four times with PBS-T. For the IgA and sIg assays, monoclonal murine anti-human IgA (M 26012 Bionostics, Wyboston, UK) or anti-secretory component (I 6635 Sigma, St. Louis, MO) and for the IgG assay, peroxidase-conjugated rabbit antibodies to human IgG (P 0214 Dakopatts, Glostrup, Denmark) diluted in FCS-PBS were added and incubated for 2 hours at +37ºC. For the IgG assay, the substrate, 1.1 M natriumacetat, 0.6% tetramethylbenzidine and 30% hydrogen peroxide diluted into sterile aqua was added after the plates were washed three

times with PBS-T and once with aqua. The plates were incubated for 15 minutes at +22ºC in the dark and the reaction was stopped by the addition of 2 M sulphuric acid. Absorbances (wavelength 450 nm) were measured with a Multiscan MCC/340 (Labsystems, Finland).

For the IgA and sIg assays, alkaline phosphatase conjugated polyclonal rabbit anti-mouse IgG (315-055-045, Jackson Immuno Research Laboratories) diluted in FCS-PBS was added and incubated overnight at +22ºC. The plates were washed three times with PBS-T and once with aqua. The substrate, p-nitrophenyl phosphate disodium (Sigma) in carbonate buffer (pH 9.8) was added. The mixture was incubated for one hour at +37ºC and absorbances (wavelength 405 nm) were measured as described above.

All the results are given as optical density units (OD unit; 1000 times the optical density reading). The mean values of three neutralized and unneutralised wells were calculated and the value of the neutralized sample was subtracted from that of the unneutralised sample. The concentrations were calculated after subtraction. The limit of positivity was set at 30 OD units, which was higher than 2 SD of 30 determinations of one positive saliva sample on a same plate. In statistical calculations, values lower than 30 OD units were assigned as 10 OD units.

5.3 EIA for measurement of pneumococcal and meningococcal antibodies in serum

II-V: Anti-pneumococcal IgG in serum was measured at KTL as described (Käyhty *et al.* 1995; Åhman *et al.* 1996).

VI: Anti-meningococcal IgG in serum was measured at the CDC as described (Carlone *et al.* 1992).

5.4 Single radial immunodiffusion

For the determination of total IgA concentrations in saliva samples, anti-human IgA LC-Partigen® immunodiffusion plates (Behringwerke AG, Marburg, Germany) were used with Protein-Standard-Serum LC-V (Behringwerke) (II). Before the assays, samples were treated

with dithiothreitol (Sigma, Ontario, Canada) to convert IgA molecules from dimeric to monomeric. The results for total IgA concentrations were given as µg/ml. When calculating the ratio of anti-Pnc PS IgA and total IgA, samples with undetectable levels of both anti-Pnc PS IgA and total IgA were excluded. Samples with undetectable anti-Pnc PS IgA, but detectable total IgA were assigned a value 0.0113, which was the lowest value found.

6 Statistical methods

I: Differences in log-concentrations between groups were tested using Student's T test or linear regression. The analysis was conducted with the Stata program (Statacorp, College Station, TX). Although separate studies were performed to evaluate laboratory storage and specimen collection, the data from the two studies were combined for the analysis of each explanatory variable into a single regression model. To overcome the uneven distribution of this data and to control variation attributable to differences between subjects, a subject number was introduced into the model as a fixed effect. Interactions were tested using the Wald test with a combination of dummy variables representing the interaction terms.

II-VI: The results are given as geometric mean antibody concentrations (GMC) with 95% confidence intervals (CI). Non-parametric statistical methods were used, because of the non-normality of salivary antibody data. Antibody concentrations at different ages were compared using the Wilcoxon signed ranks test. Differences in antibody concentrations between vaccine groups were analysed with the Kruskal-Wallis and Mann-Whitney tests. The proportions of children with detectable antibodies in different groups were compared with the Yates-corrected chi square (χ^2) test or with Fisher's two-tailed exact test. Log-transformed serum and salivary IgG, and IgA and sIg concentrations in saliva were compared by Pearson's correlation analysis. Differences were considered statistically significant when the p-value was <0.05.

RESULTS

1 Methodology for collection and storage of saliva samples

The effects of different collection and storage methods on anti-pneumococcal polysaccharide and protein IgA concentrations in saliva samples were studied by collecting samples from 30 healthy Kenyan adults in random order with four different methods yielding 120 specimens (I). Each specimen was further processed with three different storage methods (see Materials and methods). Thus, the total number of specimens was 360. However, all the EIA analyses were not performed on all of the 360 specimens. First, we analysed the effects of different storage methods by measuring anti-pneumococcal antibody concentrations in 30 specimen; 7 collected with Oracol and OraSure devices and 8 collected by pastette and drooling method. The snap-frozen samples gave the highest antibody concentrations and to detect possible differences between the four collection methods, we decided to use primarily snap-frozen specimens. On one day EIA did not meet the quality criteria and we repeated the analysis by using specimens collected with the same method, but by using plain storage method instead of snap-freezing. Further, the volume of every specimen was not sufficient for all the antibody analyses.

1.1 Effects of collection method on saliva samples

1.1.1 Volume of the saliva sample

The final volume of the saliva sample obtained with OraSure and Oracol devices is dependent predominantly on the volume of the sample buffer. The volume achieved by collecting samples with plastic pipette was significantly lower than with OraSure, Oracol or by unstimulated drooling. The mean volumes with different methods were 1105 µl, 1580 µl, 673 µl, and 1405 µl, collected by OraSure, Oracol, Pastette, and unstimulated drooling, respectively. The order of the sampling did not have any effect on the volume; the rank order of volume between different methods remained the same when only the first samples were included in the analysis.

There were no differences in the attained volume of saliva samples between men and women; the mean volume of the samples collected from women was 1214 μl and the respective number from men was 1181 μl.

1.1.2. Anti-pneumococcal IgA concentrations in saliva samples

The OraSure collection method yielded higher anti-Pnc PS IgA concentrations than the other three methods, but after adjustment of subject, storage method and serotype, the ratios of the geometric mean concentrations (GMCs) as compared with that of the OraSure specimens were 0.93 ($p=0.44$; 95% confidence interval [CI], 0.78 to 1.22) for the Oracol specimens, 0.84 ($p=0.07$; 95% CI, 0.70 to 1.01) for the Pastette specimens, and 0.98 ($p=0.81$; 95% CI, 0.82 to 1.17) for the unstimulated drooling specimens. Thus, there were no statistical differences between collection methods when the anti-Pnc PS antibody concentrations were examined. However, the ratio of anti-PsaA IgA GMC in specimens taken with Oracol was only 0.52 ($p=0.001$; 95% CI, 0.36 to 0.76) compared with the OraSure specimens.

1.2 Effect of storage method on anti-pneumococcal IgA concentrations

In all studied polysaccharides, unadjusted anti-Pnc PS IgA concentrations were higher in specimens which had been snap-frozen in liquid nitrogen than in plain specimens or in specimens which had been treated with enzyme inhibitors. After adjustment of subject, collection method, and serotype the ratios of GMCs relative to that of snap-frozen samples were 0.68 ($p<0.0005$; 95% CI, 0.58 to 0.79) for plain samples and 0.71 ($p<0.0005$; 95% CI, 0.60 to 0.84) for enzyme-inhibited samples. Anti-PsaA concentrations were higher in the samples stored with enzyme inhibitors as compared with the snap-frozen and plain samples. The ratios of GMCs of anti-PsaA IgA relative to snap-frozen samples were 1.30 ($p=0.11$; 95% CI, 0.94 to 1.81) for the plain samples and 1.44 ($p=0.024$; 95% CI, 1.05 to 1.98) for enzyme-inhibited samples.

1.3 Co-effects of collection and storage methods on anti-pneumococcal IgA in saliva samples

We also found a significant interaction between the collection and storage methods in the anti-Pnc PS antibody concentrations detected. This is illustrated in Table 4 by using samples taken by the OraSure method and snap-frozen in the laboratory as a baseline. The table reveals that the storage method has less influence on antibody concentrations in the samples collected by proprietary methods than in the samples taken by pastette or unstimulated drooling methods.

Table 4. Ratios of concentrations of anti-Pnc PS IgA determined by various EIAs to that in snap-frozen OraSure specimens (baseline) relative to the methods of storage. (* = significant difference from the baseline)

Storage method	Ratio of anti-Pnc PS IgA concentration by indicated collection method to concentration in snap-frozen OraSure specimen			
	OraSure	Oracol	Pastette	Unstimulated drooling
Snap-freezing	1.00	1.06	1.01	1.34*
Enzyme inhibition	0.93	0.76	0.71	0.75
Plain	1.06	0.80	0.63*	0.60*

2 Salivary anti-pneumococcal antibodies induced by conjugate vaccines

Salivary anti-Pnc PS IgA and IgG concentrations were measured after three and four doses of pneumococcal conjugate vaccine (PCV) in different studies using PncT, PncD, PncCRM and PncOMPC (II-V). Antibody concentrations were determined against serotypes 6B, 14 and 19F in all studies, against serotypes 4, 9V, and 18C in PncT, PncD and PncCRM1 studies (II-III), and against serotype 23F in PncCRM2 and PncOMPC studies (IV-V). Anti-

Pnc PS IgA1 and IgA2 against serotypes 6B, 14, 19F, and 23F were measured in two studies (IV-V). In a subgroup of children, anti-Pnc PS IgA and IgG were measured also 3 to 4 years after completing the series of vaccinations (IV). In two studies antibody concentrations were determined also after pneumococcal polysaccharide vaccine (PPV) booster (II and V). A control group of children vaccinated with HBV was included in two studies (IV-V).

2.1 Anti-pneumococcal IgG

At the age of 7 months, after three doses of PCV, there were only few anti-Pnc PS IgG positive saliva samples (II-V) (Table 5). The percentage of positive samples varied between 0 and 14%, depending on the study vaccine and serotype. Similarly, antibody concentrations were low, GMCs varying between 1.7 and 2.3 ng/ml, respectively (Table 6). Salivary anti-Pnc IgG was most often found against serotypes 14 and 19F at the age of 7 months. None of the children had anti-9V IgG in saliva after three doses of PCV.

One month after the fourth dose, either at the age of 13 or 16 months, the percentage of anti-Pnc PS IgG positive samples varied between 0 and 44%, and GMCs ranged between 1.7 and 5.2 ng/ml depending on the study vaccine and serotype (Tables 5 and 6). After the booster dose, anti-Pnc IgG was most often induced against serotypes 6B, 14 and 19F. Further, anti-Pnc IgG concentrations were highest against these serotypes.

In the FinOM vaccine trial (IV-V), anti-Pnc PS IgG was detected as seldom in the HBV as in the PCV group after the primary series. At the age of 13 months, samples were significantly more often anti-Pnc PS IgG positive in the PncCRM2 group for serotypes 6B and 23F, and in the PncOMPC group for serotype 14 than in the HBV group.

Salivary anti-Pnc PS IgG concentrations reflected corresponding concentrations in serum. When serum anti-Pnc PS IgG concentration exceeded 10 µg/ml, 24-29% of the saliva samples were IgG positive, depending on the study. When anti-Pnc PS IgG concentration in serum varied between 10 and 3 µg/ml approximately 8% of the saliva samples contained anti-Pnc specific IgG. And when specific IgG concentrations in serum was less than 1 µg/ml only 2% of the salivas were positive for anti-Pnc PS IgG.

Table 5. Percentages of anti-Pnc IgG positive saliva samples taken at the age of 7 and 13 or 16 months from children vaccinated with different PCVs or with a hepatitis B vaccine. (- = not determined)

Study vaccine	N	Proportion (%) of anti-Pnc IgG positive samples													
		4		6B		9V		14		18C		19F		23F	
		7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo
PncT	10-24	0	20	0	30	0	0	14	14	0	0	8	13	-	-
PncD	3-21	0	0	0	13	0	0	14	22	5	11	5	44	-	-
PncCRM1	28-55	4	7	4	29	0	7	2	32	0	18	4	25	-	-
PncCRM1+ PPV booster	22-23	-	14	-	32	-	4	-	22	-	27	-	61	-	-
PncCRM2	52-57	-	-	7	21	-	-	4	12	-	-	14	12	4	13
PncOMPC	44-56	-	-	2	7	-	-	4	14	-	-	4	14	2	0
PncOMPC+ PPV booster	5-6	-	-	-	17	-	-	-	50	-	-	-	60	-	0
control (HBV)	54-55	-	-	0	0	-	-	4	2	-	-	9	4	2	2

Table 6. Anti-Pnc IgG concentrations in saliva samples taken at the age of 7 and 13 or 16 months from children vaccinated with different PCVs or with a hepatitis B vaccine; geometric mean concentrations (ng/ml) with 95% confidence intervals. (- = not determined)

Study vaccine	N	Anti-Pnc IgG concentrations (ng/ml)													
		4		6B		9V		14		18C		19F		23F	
		7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo
PncT	10-24	1.7 (1.7-1.7)	2.3 (1.5-3.6)	1.7 (1.7-1.7)	3.1 (1.7-5.7)	1.7 (1.7-1.7)	1.7 (1.7-1.7)	2.3 (1.7-3.2)	2.5 (1.5-4.3)	1.7 (1.7-1.7)	1.7 (1.7-1.7)	2.1 (1.6-2.8)	2.6 (1.4-4.7)	-	-
PncD	3-21	1.7 (1.7-1.7)	1.7 (1.7-1.7)	1.7 (1.7-1.7)	2.0 (1.4-2.9)	1.7 (1.7-1.7)	1.7 (1.7-1.7)	2.2 (1.7-2.9)	2.9 (1.5-5.7)	1.8 (1.6-2.0)	2.1 (1.4-3.1)	1.9 (1.5-2.4)	5.2 (2.2-12.3)	-	-
PncCRM1	28-55	1.8 (1.7-2.0)	1.9 (1.6-2.3)	1.8 (1.6-2.0)	3.2 (2.2-4.6)	1.7 (1.7-1.7)	2.0 (1.7-2.3)	1.8 (1.6-1.9)	3.6 (2.4-5.5)	1.7 (1.7-1.7)	2.3 (1.8-2.9)	2.0 (1.7-2.3)	3.3 (2.1-5.0)	-	-
PncCRM1+ PPV booster	22-23	-	2.4 (1.6-3.7)	-	3.8 (2.2-6.7)	-	1.9 (1.6-2.3)	-	3.1 (1.9-5.0)	-	3.0 (2.0-4.7)	-	10.3 (5.5-19.6)	-	-
PncCRM2	52-57	-	-	1.9 (1.7-2.1)	2.5 (2.0-3.1)	-	-	1.8 (1.6-2.1)	2.2 (1.8-2.8)	-	-	2.2 (1.8-2.5)	2.1 (1.8-2.4)	1.8 (1.7-1.9)	2.2 (1.8-2.7)
PncOMPC	44-56	-	-	1.7 (1.7-1.8)	1.9 (1.7-2.1)	-	-	1.9 (1.6-2.4)	2.3 (1.8-2.9)	-	-	1.8 (1.7-1.9)	2.3 (1.8-2.9)	1.7 (1.7-1.8)	1.7 (1.7-1.7)
PncOMPC+ PPV booster	5-6	-	-	-	2.1 (1.3-3.4)	-	-	-	4.6 (1.4-15.0)	-	-	-	7.8 (1.1-56.2)	-	1.7 (1.7-1.7)
control (HBV)	54-55	-	-	1.7 (1.7-1.7)	1.7 (1.7-1.7)	-	-	1.9 (1.6-2.1)	1.8 (1.6-1.9)	-	-	2.0 (1.7-2.2)	1.8 (1.6-1.9)	1.8 (1.6-1.9)	1.8 (1.6-1.9)

2.2 Anti-pneumococcal IgA

Both salivary anti-Pnc IgA concentrations and the proportion of positive samples were higher than the corresponding values for IgG (II-V). At the age of 7 months, after three doses of PCV, the proportion of anti-Pnc IgA positive samples ranged between 0 and 60%, respectively (Table 7). Anti-Pnc IgA concentrations varied between 1.7 and 8.0 ng/ml depending on the vaccine and serotype (Table 8). At the age of 7 months, anti-Pnc IgA was found most often against serotypes 4, 14, and 19F.

After four doses, at the age of 13 or 16 months, the proportion of anti-Pnc IgA positive samples ranged between 0 and 78%, and anti-Pnc IgA concentrations varied between 1.7 and 11.8 ng/ml depending on the vaccine and serotype (Tables 7 and 8). After the booster, anti-Pnc PS IgA was often detected against serotypes 4, 6B, 14, and 19F. The number of positive samples and antibody concentrations were low against serotypes 9V and 18C.

In the FinOM vaccine trial (IV-V), at the age of 7 months anti-Pnc PS IgA was detected more often in the samples of the children in the PCV than in the HBV group for serotypes 6B, 14, and 23F (PncCRM2) and for 19F (PncOMPC). At the age of 13 months, there was a significant difference in the anti-Pnc PS IgA detection rate only for serotype 14 between the PncCRM2 and the HBV groups.

Salivary IgA and sIg concentrations correlated well. In the FinOM study, the correlation coefficient was 0.72 among control children and 0.77 and 0.79 in the PncCRM and PncOMPC groups, respectively, when the 7 and 13 months and serotype specific results were combined.

2.2.1 Anti-pneumococcal IgA in relation to total IgA

In paper II we proportioned anti-Pnc PS specific IgA concentrations to total IgA. After the adjustment, increases in the anti-Pnc PS concentration between 7 and 16 months of age were not found as often as without relation to total IgA. The GMCs of total IgA concentrations were at the age of 7 months 8.9 µg/ml, and at the age of 16 months 15.5 µg/ml and 24.8 µg/ml in the PCV and PPV booster groups, respectively. Thus, the total IgA concentration increased relatively more than the anti-Pnc PS specific IgA level.

Table 7. Percentages of anti-Pnc IgA positive saliva samples taken at the age of 7 and 13 or 16 months from children vaccinated with different PCVs or with a hepatitis B vaccine. (- = not determined)

Study vaccine	N	Proportion (%) of anti-Pnc IgA positive samples													
		4		6B		9V		14		18C		19F		23F	
		7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo
PncT	21-24	13	17	0	4	0	9	4	9	0	8	17	21	-	-
PncD	23-24	14	24	5	13	5	0	19	30	0	8	13	30	-	-
PncCRM1	28-55	7	32	11	36	5	14	33	46	4	14	9	43	-	-
PncCRM1+ PPV booster	24-26	-	42	-	73	-	23	-	50	-	13	-	81	-	-
PncCRM2	55-57	-	-	51	65	-	-	60	56	-	-	51	76	28	44
PncOMPC	45-56	-	-	30	51	-	-	41	42	-	-	57	78	20	40
PncOMPC+ PPV booster	7	-	-	-	71	-	-	-	71	-	-	-	100	-	71
control (HBV)	55	-	-	16	47	-	-	25	29	-	-	31	64	5	31

Table 8. Anti-Pnc IgA concentrations in saliva samples taken at the age of 7 and 13 or 16 months from children vaccinated with different PCVs or with a hepatitis B vaccine; geometric mean concentrations (ng/ml) with 95% confidence intervals. (- = not determined

Study vaccine	N	Anti-Pnc IgA concentrations (ng/ml)													
		4		6B		9V		14		18C		19F		23F	
		7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo	7 mo	13/16 mo
PncT	21-24	2.1 (1.6-2.7)	2.2 (1.7-2.8)	1.7 (-)	1.8 (1.6-2.1)	1.7 (-)	2.2 (1.5-3.1)	1.9 (1.5-2.4)	2.4 (1.5-3-8)	1.7 (-)	2.0 (1.6-2.4)	2.1 (1.7-2.7)	2.4 (1.8-3.2)	-	-
PncD	23-24	2.2 (1.6-3.0)	2.4 (1.8-3.2)	1.9 (1.5-2.3)	2.1 (1.6-2.8)	1.9 (1.5-2.3)	1.7 (-)	2.3 (1.7-3.2)	3.5 (2.1-5.8)	1.7 (-)	1.9 (1.6-2.1)	2.1 (1.7-2.7)	3.5 (2.2-5.7)	-	-
PncCRM1	28-55	2.0 (1.7-2.4)	2.9 (2.1-4.0)	2.1 (1.7-2.6)	3.9 (2.3-6.5)	2.0 (1.7-2.5)	2.7 (1.7-4.2)	3.6 (2.6-5.0)	5.3 (3.2-8.9)	1.8 (1.6-2.1)	2.1 (1.7-2.7)	2.0 (1.7-2.2)	4.9 (2.9-8.2)	-	-
PncCRM1+ PPV booster	24-26	-	3.1 (2.3-4.2)	-	7.8 (5.0-12.2)	-	2.8 (1.9-4.2)	-	6.1 (3.4-10.8)	-	2.1 (1.7-2.6)	-	19.3 (11.3-32.8)	-	-
PncCRM2	55-57	-	-	5.6 (3.9-8.2)	10.8 (7.0-16.5)	-	-	8.0 (5.2-12.1)	6.2 (4.3-9.0)	-	-	5.0 (3.6-6.9)	11.8 (8.1-17.1)	3.0 (2.3-3.8)	5.1 (3.5-7.4)
PncOMPC	45-56	-	-	3.2 (2.4-4.1)	5.8 (3.8-8.9)	-	-	3.4 (2.7-4.4)	4.4 (2.9-6.5)	-	-	5.7 (4.2-7.9)	11.2 (7.5-16.7)	2.3 (1.9-2.7)	3.7 (2.7-5.1)
PncOMPC+ PPV booster	7	-	-	-	7.5 (2.8-19.9)	-	-	-	12.8 (2.9-56.0)	-	-	-	50.2 (21.9-115)	-	6.6 (2.2-19.5)
control (HBV)	55	-	-	2.5 (1.9-3.3)	4.5 (3.3-6.2)	-	-	2.8 (2.2-3.7)	3.0 (2.3-4.0)	-	-	3.1 (2.4-4.1)	8.0 (5.5-11.5)	1.9 (1.7-2.2)	3.0 (2.3-3.8)

2.2.2 Anti-pneumococcal IgA subclasses, IgA1 and IgA2

After the primary series of either PncCRM2 or PncOMPC (IV-V), salivary anti-Pnc PS IgA2 was detected more often than IgA1; 62 to 94% of the samples were positive for anti-Pnc PS IgA1 and 83 to 96% for IgA2, depending on the vaccine group and serotype. The GMCs for anti-Pnc PS IgA1 and IgA2 varied between 2.0 and 7.7 EIA units and 2.2 and 5.3 EIA units, respectively.

After the booster, at 13 months of age, the number of both anti-Pnc PS IgA1 and IgA2 positive samples ranged from 82 to 100%, depending on the vaccine group and serotype. The GMCs for anti-Pnc PS IgA1 were higher than for IgA2 varying between 3.1 and 18.8 EIA units, and 1.6 and 6.3 EIA units, respectively. The anti-Pnc PS IgA2 concentrations were at the same level both after the primary series and after the booster. Thus, rises in the IgA concentrations after the booster are supposed to be due to IgA1.

2.3 Persistence of salivary antibodies after pneumococcal vaccination in infancy

In the FinOM study saliva samples were collected also at the age of 4 to 5 years from 32 children in the PncCRM2 and 29 children in the HBV group, i.e. 3 to 4 years after completing the series of four doses of the PncCRM2 or HBV (IV) (Table 9).

The salivary anti-Pnc PS specific IgG concentrations reflected the IgG levels in serum. Both salivary and serum anti-Pnc PS IgG concentrations were at 4 to 5 years of age similar to that after the booster dose at 13 months of age (Åhman *et al.* 2002). The percentage of anti-Pnc PS IgG positive samples was 16% (5/32) for serotype 6B, 13% (4/32) for 14 and 19F, and 6% (2/32) for 23F. The GMCs were 1.8 ng/ml for serotype 23F and 2.4 ng/ml for 6B, 14, and 19F. In the HBV group, anti-Pnc PS IgG was still seldom detected at the age of 4 to 5 years; one child had anti-Pnc IgG against serotypes 19F and 23F (Table 9). The detection rate of anti-Pnc PS IgG did not differ statistically between PncCRM and HBV groups.

IgA concentrations increased with age, and almost every child had anti-Pnc PS IgA in saliva 3 to 4 years after the booster; 97% of the saliva samples were positive for 6B, 14 and 19F and 78% for 23F. The GMCs were also significantly higher than at 13 months of age ranging from 14.6 (23F) to 50.1 ng/ml (19F). At the age of 4 to 5 years, the detection rate of anti-Pnc PS IgA was similar in the PncCRM2 and in the HBV groups (Table 9).

Table 9. Anti-Pnc PS IgG and IgA in the PncCRM2 and HBV groups at the age of 4 to 5 years; the percentage of positive samples (% pos) and geometric mean concentrations (GMC) ng/ml with 95% confidence intervals.

Serotypes	IgG				IgA			
	PncCRM2 (n=32)		HBV (n=29)		PncCRM2 (n=32)		HBV (n=29)	
	% pos	GMC	% pos	GMC	% pos	GMC	% pos	GMC
6B	16	2.4 (1.8-3.3)	0	1.7 (1.7.-1.7)	97	28.2 (18.5-42.9)	90	19.1 (12.2-29.9)
14	13	2.4 (1.7-3.5)	0	1.7 (1.7-1.7)	97	20.4 (14.5-28.8)	93	29.2 (17.6-48.4)
19F	13	2.4 (1.7-3.3)	3	1.8 (1.6-2.1)	97	50.1 (34.7-72.4)	93	32.2 (20.0-51.7)
23F	6	1.8 (1.6-2.1)	3	1.8 (1.6-1.9)	78	14.6 (8.8-24.3)	66	10.3 (5.7-18.6)

2.4 The effect of a polysaccharide booster after a primary series with conjugate vaccines

Salivary antibody concentrations and the percentage of anti-Pnc Ig positive samples were higher after the PPV than after the PCV booster (II and V) (Tables 5-8). After four doses of PncCRM1, the anti-Pnc PS IgG concentrations varied between 1.9 (4) and 3.6 (14) ng/ml, and after three doses of PncCRM1 and a PPV booster (at 15 months of age) between 1.9 (9V) and 10.3 (19F) ng/ml (Table 6). However, the difference between the groups was significant only for serotype 19F (II). Also, in the saliva of infants who had received three doses of PncOMPC and the PPV booster (at 12 months of age), anti-Pnc PS IgG concentration was highest against 19F (V) (Table 6). However, because of the small number of subjects in the group of PPV boosted infants, no statistical analysis could be performed. After four doses of PncOMPC IgG concentrations ranged from 1.7 (23F) to 2.3 (14 and 19F)

ng/ml and after three doses of PncOMPC and the PPV booster from 1.7 (23F) to 7.8 (19F) ng/ml (Table 6).

Anti-Pnc PS IgA was detected more often after the PPV than the PCV booster against all other serotypes except 18C (II) (Table 7). However, the difference was statistically significant only for serotypes 6B and 19F. Antibody concentrations ranged from 2.1 (18C) to 5.3 (14) ng/ml, and from 2.1 (18C) to 19.3 (19F) ng/ml after the PncCRM1 and PPV booster, respectively (Table 8). In the FinOM Vaccine Trial, anti-Pnc PS IgA was detected more often against all analysed serotypes after the PPV booster than after the PncOMPC booster. After the PCV booster antibody concentrations ranged from 3.7 (23F) to 11.2 (19F) ng/ml and after the PPV from 6.6 (23F) to 50.2 (19F) ng/ml (Table 8).

3 Natural salivary anti-pneumococcal antibodies

In the FinOM Vaccine Trial, there was a control group of 55 children who did not receive any pneumococcal vaccinations during the study period (IV-V). Salivary antibody concentrations were determined at the age of 7 and 13 months, and in 29 children also at 4 to 5 years of age.

The anti-Pnc PS IgG concentrations remained at a low level during the whole study period (Tables 5, 6, and 9). The concentrations were close to the detection limit and varied between 1.7 and 2.0 ng/ml depending on the serotype and age. The percentage of positive samples ranged from 0 to 9%, respectively. None of the children had IgG against serotype 6B during the 4 to 5 years follow-up (Tables 5, 6, and 9).

The anti-Pnc PS IgA concentrations and detection rate increased by age despite the fact that these children had not received any pneumococcal vaccinations during their life (Tables 7, 8, and 9). Anti-Pnc PS IgA was found most often in unvaccinated children against serotype 19F and most rarely against serotype 23F. At the age of 7 months 5 (23F) to 31% (19F) of infants had anti-Pnc PS IgA in saliva. At the age of 13 months the corresponding numbers were 29 (14) and 64% (19F), and when children were 4 to 5 years old, 66% had IgA against serotype 23F, 90% against 6B, and 93% against 14 and 19F. The anti-Pnc PS IgA concentrations ranged from 1.9 to 3.1 ng/ml at the age of 7 months, from 3.0 to 8.0 ng/ml at the age of 13

months and from 10.3 to 32.2 ng/ml at 4 to 5 years of age (Tables 7, 8, and 9).

4 Salivary anti-meningococcal antibodies induced by a MenA/C polysaccharide vaccine in previously primed and unprimed children

Children in Paper VI were divided into 5 groups according to the meningococcal vaccination history. In group 1, children had not been primed with meningococcal vaccines. In group 2 children had been vaccinated previously with meningococcal conjugate vaccine (MCV) and in group 3 with meningococcal polysaccharide vaccine (MPV). Subjects in group 4 had been primed with MCV in infancy and with MPV at the age of 2 years. In group 5 children received the same vaccines as in group 4 but in reverse order. At the age of 4 to 5 years, all children received one dose of MPV.

4.1 Anti-MenA IgG and IgA antibodies

The number of anti-MenA IgG positive saliva samples increased significantly after the revaccination in every vaccine group (Table 10). In primed children GMCs of anti-MenA IgG ranged from 10.4 to 12.6 OD units before the booster vaccination and from 19.4 to 69.1 OD units after the vaccination depending on the group. Children vaccinated with MPV in infancy and with a MCV at two years of age (group 5) had a significantly higher anti-MenA IgG concentration in saliva after the revaccination than children who had received MCV in infancy and MPV at the age of two years (group 4) ($p<0.03$). In unprimed children the anti-MenA IgG concentration increased significantly after MPV immunisation; the GMC was 10.7 OD units before the vaccination, and 19.4 OD units after the vaccination. After the MPV, salivary anti-MenA IgG concentrations were still significantly higher in children in groups 2, 3, and 5 than in unprimed children ($p<0.003$).

Salivary anti-MenA IgA detection rate increased after revaccination with MPV regardless of the previous vaccination history (Table 11). In primed children the GMCs varied between 17.0 and 24.5 OD units before the vaccination, and between 67.3 and 149.9 OD units after the vaccination, depending on the vaccine group. In unprimed children the GMC of anti-

MenA IgA was 18.5 OD units before the vaccination, and 88.5 OD units after the vaccination. There were no significant differences in salivary anti-MenA IgA concentrations between the groups either before or after MPV booster immunisation.

4.2 Anti-MenC IgG antibodies

There were no significant differences in the salivary anti-MenC IgG concentrations between different vaccination groups before the MPV vaccination at 4-5 years of age; most of the children were negative for anti-MenC IgG (Figure 5). After vaccination there was a significant increase in the number of anti-MenC IgG positive saliva samples (Table 10) and in the GMC in all groups ($p<0.008$) (Figure 5). The salivary anti-MenC IgG concentrations reflected the serum IgG concentrations, which have been described earlier (MacLennan *et al.* 2001). Children who had previously received only MCV (group 2) and children who had received MPV vaccine in infancy and MCV at the age of two years (group 5) had significantly higher anti-MenC IgG concentrations after revaccination than the other previously vaccinated children ($p<0.001$ for all comparisons) and the control group ($p<0.006$). In the previously vaccinated children the anti-MenC IgG GMCs varied between 33.5 and 148.8 OD units and in the unprimed group GMC was 36.2 OD units.

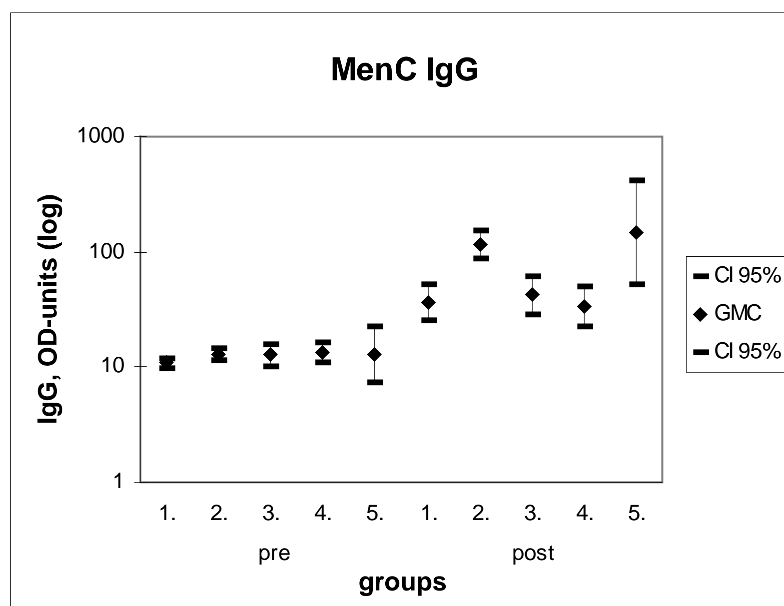

Figure 5. Anti-MenC PS IgG concentrations in different vaccine groups (see Table 3, Materials and methods) before and after vaccination with MPV at 4 or 5 years of age; geometric mean concentrations (GMC) and 95% confidence intervals (CI).

4.3 Anti-MenC IgA antibodies

Before revaccination children who had previously received 1 to 3 doses of MPV had significantly higher salivary anti-MenC IgA concentrations (GMC 22.5 vs. 11.0 OD units, p<0.001) than children who had been vaccinated with 1 to 4 doses of MCV (Figure 6). Further, the proportion of children with anti-MenC IgA positive saliva samples was lower among children who had previously received only MCV as compared with previously unprimed children (p<0.001). Before the vaccination, in previously unvaccinated children the anti-MenC IgA GMC was 17.2 OD units.

All the other children had significantly higher salivary anti-MenC IgA concentration after revaccination than before, expect children who had been previously vaccinated with 2 doses of MPV in infancy and with MCV at two years age (group 5). Anyway, the number of anti-MenC IgA positive samples increased in all groups (Table 11). After revaccination, anti-MenC IgA concentrations were significantly higher (p<0.001) in the group of children vaccinated previously only with MPV than in groups of children who had received previously only MCV (group 2) or MCV in infancy and MPV at the age of two years (group 4). Also, previously unvaccinated children had significantly higher anti-MenC IgA concentrations (54.8 OD units) after vaccination than children in group 4 (21.6 OD units) (p=0.02). In the other groups GMCs ranged between 34.7 and 85.0 OD units.

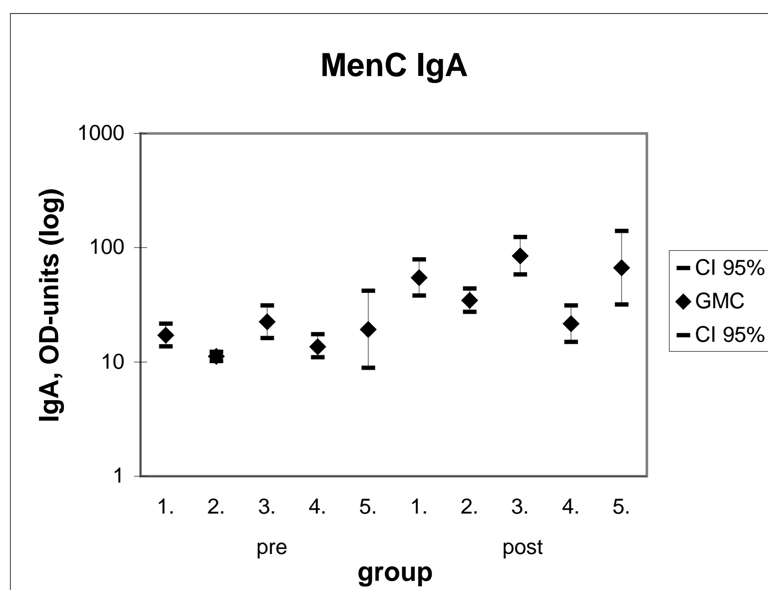

Figure 6. Anti-MenC PS IgA concentrations in different vaccine groups (see Table 3, Materials and methods) before and after vaccination with MPV at 4 or 5 years of age; geometric mean concentrations (GMC) and 95% confidence intervals (CI).

Table 10. The percentage of anti-MenA and MenC IgG positive saliva samples before and after vaccination with MPV at the age of 4 to 5 years.

Group[a]	N		MenA		MenC	
	pre	post	pre	post	pre	post
1.	64	64	3	30***	5	52***
2.	123	124	4	52***	11	78***
3.	54	54	7	61***	13	61***
4.	46	44	2	43***	15	52***
5.	12	11	8	82***	8	82***

[a] For groups, see Table 3. in Materials and methods
[b] Statistically significant difference between pre and post immunisation samples;
*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Table 11. The percentage of anti-MenA and MenC IgA positive saliva samples before and after vaccination with MPV at the age of 4 to 5 years.

Group[a]	N		MenA		MenC	
	pre	post	pre	post	pre	post
1.	64	64	28	78***b	28	63***
2.	126	125	25	78***	6	56***
3.	54	54	43	83***	37	81***
4.	47	45	40	78***	15	38*
5.	12	11	42	100**	25	82*

[a] For groups, see Table 3. in Materials and methods
[b] Statistically significant difference between pre and post immunisation samples;
*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

4.4 Correlation of anti-Men IgA and sIg

Both salivary anti-MenA and anti-MenC IgA and sIg concentrations were compared. There was significant correlation between IgA and sIg both before (r=0.89 and 0.94) and after (r=0.90 and 0.90) the revaccination with MPV for MenA and MenC, respectively.

4.5 Origin of anti-Men IgG

We also compared anti-MenA and C antibody concentrations in saliva and in serum. Salivary and serum IgG concentrations correlated both before (r=0.39 and 0.65) and after (r=0.51 and 0.75) the revaccination with MPV for MenA and MenC, respectively. Also, the proportion of MenA and MenC IgG positive saliva samples increased with the increasing serum IgG concentration.

DISCUSSION

1 Study design

This thesis consists of six studies (I-VI) carried out in 1994-2001 in Finland, the Gambia and Kenya. Five of them were conducted to investigate the ability of different pneumococcal vaccines (II-V) and one meningococcal conjugate vaccine (VI) to induce specific antibodies in the saliva of infants. We also ran a pilot study to test different collection and storage methods of saliva samples in relation to the anti-pneumococcal polysaccharide and protein antibody concentrations (I). The studies II, III, and VI were phase two immunogenicity studies, and IV and V were part of the FinOM Vaccine Trial, which was a phase three study. Mucosal immunity was only one arm of these studies, and saliva samples in the studies had already been collected before planning study I. Further, publications II and III included in this thesis contain detailed serum IgG results, which are out of the scope of this thesis and are not presented here.

2 Methodology

2.1 Saliva samples

Saliva samples were collected to measure mucosal immune response induced by pneumococcal and meningococcal vaccines. Saliva samples have advantages over other mucosal secretion samples: they are relatively easy to collect, can be taken non-invasively, and persons collecting the samples do not need special education. One of the major concerns in measuring salivary antibodies has been the presumed instability of antibodies in the saliva samples. In addition to this, immunoglobulin concentrations in saliva are low, which implies that the respective methods for detection of salivary antibodies have to be more sensitive compared with the serum assays. Further, saliva samples are often viscous and thus hard to handle. The volume attained from infants is frequently low, and therefore all the planned analyses cannot always be performed. This restricted the number of serotypes against which antibody concentrations were estimated also in our studies. Thus, we prioritised the most

often carried serotypes in our analyses.

Antibody levels in saliva have natural diurnal and monthly variation (Butler *et al.* 1990). Also, stimulus of the mouth can increase the flow of saliva and dilute it. Optimally, saliva samples should be unstimulated. Albumin and total IgA concentrations among others have been used as adjustment factors to equalise the dilution effect (Kugler *et al.* 1992; Kauppi *et al.* 1995). However, it has recently been found, that also concentrations of these proteins have within-subject variation (Rantonen and Meurman 2000). In study II, we indicated anti-Pnc PS IgA results also proportioned to total IgA. However, the total IgA concentrations increased relatively more than the specific IgA concentration, and increases in the specific IgA concentrations could not be detected as often as without adjustment. The immunodiffusion assay we used to measure total IgA concentration is not very accurate and this may lead to the distortion of results. Further, the increasing total IgA concentration may be due to increases in specific IgA levels to other antigens encountered. In studies IV and V we had a control group to compare the specific anti-Pnc PS IgA concentration in children with and without vaccination with PCV. We speculate that the difference in actual concentrations of specific IgA between control and vaccine groups is important. Thus, we decided to determine only the anti-PS IgA concentrations without relating them to total IgA in these studies.

In addition to antibodies, whole saliva consists of excretions of salivary glands, gingival crevicular fluid, mucosal products, viruses, bacteria, hormones, and traces of food. There are also plenty of different enzymes, for example IgA1 protease produced by e.g. pneumococcus, meningococcus and Hib (Kilian *et al.* 1996). Thus, antibodies in saliva samples are prone to degradation and to prevent this they need to be handled and stored properly. Therefore saliva samples are in general stored at –70°C and are commonly thawed only once.

2.2 Collection and storage methods for saliva samples

We found that there were differences between anti-Pnc PS and anti-protein IgA concentrations depending on the method of collection and storage. However, the sensitivity

of the anti-PsaA IgA EIA was low and thus, we based our conclusions about collection and storage of saliva samples on the anti-Pnc PS IgA concentrations.

We did not find any significant differences between the collection methods in anti-Pnc PS antibody concentrations, even if, Oracol has been better in terms of IgM yield in a previous study (Vyse *et al.* 2001). The storage method by contrast had less effect on the antibody concentration when samples had been collected with OraSure or Oracol methods than with drooling or plastic pipette. Proprietary methods OraSure and Oracol are also more practical than pastette and drooling systems. However, they are more expensive and have been designed for the investigation of crevicular fluid rather than whole saliva; and when samples are stored properly after collection by snap-freezing to –70°C, drooling appears to be the more advantageous method for sampling.

When different storage methods were compared, samples snap-frozen in liquid nitrogen contained 41 to 47% higher antibody concentration compared to samples stored with the other methods. Samples stored with enzyme-inhibitors or as plain were stored at +4°C for 4 to 8 hours before freezing at –70°C. The storage prior final freezing may have had an effect on the antibody concentrations. However, a previous study points out that saliva samples could be stored at +4°C without degradation of antibodies (Mortimer and Parry 1988). We have found before that in saliva samples, stored at –20°C with enzyme inhibitors, antibody concentration decrease quickly compared to the samples stored with glycerol or without additives at –20°C (unpublished data). Others have speculated that enzyme inhibitors in saliva samples may interfere with the EIA (Rosenqvist *et al.* 2001). Anyway, our studies are not consistent with this, because we have seen that when saliva samples are stored at –70°C, antibody concentrations can be maintained also in the samples stored with enzyme inhibitors (unpublished data). As a conclusion, the best way to preserve antibody concentrations in saliva samples is to freeze (most preferably to snap-freeze) them as soon as possible after collection and store samples at –70°C.

In the pneumococcal vaccine studies (II-V), saliva samples were collected with a plastic pipette or with gentle aspiration using an electronic suction device. After that, samples were immediately frozen either in dry ice or placed at –70°C in a freezer. In the meningococcal vaccine study (VI), saliva samples were collected with the Oracol method, stored with the

enzyme inhibitor cocktail, and snap-frozen also immediately after collection in liquid nitrogen and then stored at –70°C. Some of the saliva samples studied (II-III) had been stored even 1.5 years before analyses, and in studies IV-V even longer. This might have had an effect on the antibody concentration. Anyhow, we have seen that the anti-Pnc PS antibody concentration in saliva samples can be maintained at least for one year (unpublished data). With reference to the two previous paragraphs, the collection method does not have a major effect on the antibody concentration. But saliva samples should be frozen to –70°C as soon as possible after collection. This way of action was followed during studies II to VI.

2.3 EIA for detection of salivary antibodies

The EIA method to detect specific salivary antibodies is based on a serum assay (Käyhty *et al.* 1995). In the serum assay to measure anti-Pnc PS IgG, absorption of the samples with cell wall polysaccharide (CPS) has been used to prevent unspecificity caused by impurities in polysaccharide preparations (Koskela 1987). To further improve specificity, absorption with serotype 22F has been used in the serum EIA (Concepcion and Frasch 2001). The 22F absorption is becoming a standard procedure in the serum EIA. In our salivary EIA CPS neutralisation was a part of the protocol, but 22F absorption was not used in these studies. The problem of 22F EIA both in serum and saliva assays is however, that anti-Pnc PS IgG and IgA concentrations in the reference sera have been determined for EIA without 22F absorption and thus the concentrations may not represent the actual values attained by 22F EIA.

Saliva samples are much more heterogeneous in nature than serum samples, and in spite of CPS absorption, unspecific binding of antibodies on the microtiter plates has been a problem. This so called background binding was not similar in all samples; the degree of the binding appears to depend on the person. Consecutive samples of a person all seemed to have high background binding while all samples of another person had low background. We have used in all assays plates coated with PBS, which have been treated in the same way as plates coated with antigens. In the end, OD values on PBS plates have been subtracted from antigen plates. This system creates more reliable results, but the reduction of background OD values may sometimes reduce actual ODs too much and thus antibody concentrations may be lower than in reality. In some cases the PBS plate even gives higher OD values than the

antigen plate. When analysing anti-Men antibodies with MenA and MenC EIA we solved the background binding problem by absorbing samples with MenA or MenC polysaccharides; we had samples incubated with and without the corresponding polysaccharide, and in the end we subtracted the OD values of absorbed samples from the unabsorbed.

To further reduce unspecific background binding and to make the method more sensitive, we modified the salivary EIA for detecting pneumococcal antibodies during the time period the studies (II-V) were conducted. For example, in the IgA assay, after adding samples and monoclonal anti-human IgA antibodies, we changed the incubation at +37°C without rotation to incubation at room temperature with horizontal rotation. This resulted in the reduction of background binding and an increase in sensitivity. This can be due to better contacts between antibodies and antigens during incubation because of rotation. Because of the changes in the assay, IgA results of the studies II-III and IV-V are not directly comparable.

The IgA1 and IgA2 assays we used were differing in terms of their sensitivity; there were much more saliva samples positive for anti-Pnc PS IgA2 as compared with IgA1. Thus, the anti-Pnc PS IgA1 and IgA2 concentrations can not be compared to each other directly.

3 Salivary anti-pneumococcal antibodies induced by conjugate vaccines

We studied salivary antibodies against seven pneumococcal serotypes (included in the heptavalent vaccine currently on the market) induced by 4 different PCVs. We also investigated the effect of a pneumococcal polysaccharide vaccine as a booster compared with the conjugate. In the FinOM Vaccine Trial we were able to study the development of natural anti-pneumococcal antibodies in a group of children, who had not received any pneumococcal vaccines during the 4 to 5 year study period. The studies with the pneumococcal vaccines have been conducted separately and the method has been slightly modified between different studies. Thus, the results are not totally comparable to each other.

3.1 Salivary anti-pneumococcal IgG

Most of the salivary IgG have been suggested to transudate through capillaries from serum and especially through gingival crevices. In the present study anti-Pnc PS specific salivary and serum IgG correlated moderately. The low level of correlation most probably results from the small number of anti-Pnc PS IgG positive saliva samples. However, salivary IgG concentrations reflected corresponding IgG concentrations in serum. When anti-Pnc PS IgG concentration in serum exceeded 10 µg/ml, a quarter of the saliva samples were positive for IgG. In spite of that, there were IgG positive saliva samples with a low concentration in serum, which suggests that local production of IgG can take place. This has also been seen in previous studies (Berneman *et al.* 1998; Ogra 2000; Choo *et al.* 2000b).

At the age of 7 months, after three doses of a PCV, anti-Pnc PS IgG was detected seldom in saliva. After four doses the detection rate increased, being at highest 44% for serotype 19F in the PncD group. On the average the detection rate after the PCV booster was 15%. In general, anti-Pnc PS IgG was most often found against 6B, 14, and 19F, which are often carried serotypes in Finnish infants. Most seldom anti-Pnc PS IgG was found against serotype 9V.

Besides the serotype specific differences, we also found differences between the PCVs. Salivary anti-Pnc PS IgG was detected more often after PCV than HBV, but PncCRM and PncD tended to induce more specific salivary IgG than the other vaccines. Different conjugate vaccines have previously been found to vary in the ability to evoke systemic immune response (Käyhty *et al.* 1991). These disparities in the immunogenicity can be due to the characteristics of different conjugate vaccines due to the conjugation technique (Fattom *et al.* 1995). However, a possible explanation can be the different PS contents of the vaccines; e.g. PncT has 1 µg and PncD 3µg of each serotype.

3.2 Salivary anti-pneumococcal IgA

IgA is the predominating isotype on a mucosal surface. In concordance with this we detected salivary anti-Pnc PS IgA more often than IgG. Further, salivary anti-Pnc PS IgA

concentrations correlated with the anti-Pnc PS sIg concentrations, which indicates that salivary IgA against Pnc PSs is secretory in nature.

Anti-Pnc PS IgA was detected often already after three doses of a conjugate vaccine; even 60% of the infants had anti-Pnc PS IgA in their saliva in the PncCRM2 group (IV). At the age of 13 or 16 months, after 4 doses, 30% on the average had anti-Pnc PS IgA in their saliva. Salivary IgA was detected most often against serotypes 19F and 14 and least often against serotypes 9V and 18C. The serotype specific differences in the IgA concentrations are similar to IgG. In the FinOM study, anti-Pnc PS IgA was detected more often in the PCV groups than in the HBV group at the age of 7 months, but later the difference between the groups decreased. PncCRM and PncOMPC induced more often anti-Pnc PS IgA than the other PCVs. Further, PncD induced also more anti-Pnc PS IgA than PncT. This has been seen also previously in toddlers in studies with the four-valent PncT and PncD vaccines (Nieminen *et al.* 1999).

3.3 Salivary anti-pneumococcal IgA subclasses, IgA1 and IgA2

Human IgA is found in two subclasses IgA1 and IgA2. IgA1 is a predominant subclass both in serum and in saliva, but IgA2 is detected proportionally more often in saliva than in serum. The nature of the immunising antigen has an effect on the IgA1:IgA2 ratio; in adults polysaccharide antigens have been claimed to induce mainly IgA2 dominating response and protein antigens IgA1 dominating response (Tarkowski *et al.* 1990). In children the IgA1:IgA2 ratio is only slightly higher for a PS than a protein antigen (Simell *et al.* 2003).

In the FinOM Vaccine Trial we determined IgA subclass concentrations at 7 and 13 months of age. Due to methodological reasons we are not able to compare IgA1 and IgA2 concentrations directly. We found the rises in the IgA concentrations between 7 and 13 months of age to be due to IgA1. This is in accordance with previous studies with Hib and PCVs (Kauppi-Korkeila *et al.* 1998; Korkeila *et al.* 2000). Among many other bacteria, both pneumococcus and meningococcus produce proteases, which cleave IgA1 antibodies in the hinge region into Fab and Fc fragments (Kilian *et al.* 1996; Chintalacharuvu *et al.* 2003). Pneumococci are able to use Fab fragments to enhance the attachment on respiratory

epithelial cells (Weiser *et al.* 2003). It has been suggested that Fab fragments, by binding to the surface of the bacteria, protect against an inhibitory effect of the negatively charged capsule. Thus, in fact IgA2 antibodies would be more advantageous than IgA1 in defense against pneumococcus.

3.4 Persistence of salivary antibodies after pneumococcal vaccination in infancy

Three to four years after the series of four doses of a PCV, PncCRM2, the detection rate of salivary anti-Pnc PS IgG stayed approximately at the same level as one month after the booster. Still, it was higher than in the HBV group. Salivary IgG concentrations reflect the corresponding serum antibody concentrations (Åhman *et al.* 2002), and therefore it can be suggested that most of IgG in saliva has been transudated from serum.

On the contrary, salivary anti-Pnc PS IgA was detected more often 3 to 4 years than one month after the booster dose of PCV. Almost all children had anti-Pnc PS IgA in their saliva at the age of 4 to 5 years. However, anti-23F IgA was detected relatively less often than antibodies to 6B, 14, and 19F. At the age of 4 to 5 years, the detection rate of salivary anti-Pnc PS IgA was as high in the HBV as in the PCV group. Since completing the series of vaccinations 3 to 4 years earlier, the children have probably encountered pneumococci several times. Thus, it appears that pneumococcal contacts have induced the production of antibodies both in the vaccine and control groups.

3.5 The effect of a polysaccharide booster after a primary series with conjugate vaccines

In two studies (II and V) a 23-valent PPV was used as a booster at 12 or 15 months of age instead of a PCV. After the PPV booster, anti-Pnc PS IgG could be found in 60% of the infants. Also, antibody concentrations were higher than after a PCV booster. The same was true for anti-PncPS IgA concentrations after the PPV booster. However, there were serotype specific differences, e.g. anti-18C IgA was detected as often after four doses of PCV than after three PCV doses and a PPV booster.

A polysaccharide booster has been found to induce higher anti-Pnc PS IgG concentrations than a conjugate booster also in serum (Kilpi *et al.* 2003). The antibody concentrations and the detection rate of anti-Pnc PS IgG have been especially high for serotype 19F both in serum and in saliva. There are a couple of possible explanations for this phenomenon. High anti-19F antibody concentrations might either be due to the cross-reactive antibodies induced by 19A included in the PPV or by cross-reactive bacteria from the normal flora (Lee *et al.* 1984; Lee and Wang 1985). However, higher responses can also be induced by the higher PS content of the PS vaccine. The PPV contains 5 to 25 times more PS antigen depending on the vaccine and serotype than PCV.

Despite the high antibody concentrations, the use of a PS vaccine as a booster has been thought possible to trigger existing memory cells and further possibly to lead to depletion of memory cells and suppression of immunity (MacLennan *et al.* 2000). In contrast, a conjugate booster has been suggested to stimulate the generation of new high-affinity B memory cells resulting antibodies with better avidity (Anttila *et al.* 1999a). The clinical efficacy against AOM has been found the same after the PCV and PPV booster in healthy children (Kilpi *et al.* 2003). However, one or two doses of PCV and PPV booster did not reduce episodes in children suffering from recurrent AOMs (Veenhoven *et al.* 2003).

4 Natural salivary anti-pneumococcal antibodies

In the FinOM Vaccine Trial we had a group of children who did not receive any pneumococcal vaccines during the study period of 4 to 5 years. Anti-Pnc PS IgG was detected only seldom in the saliva of these children, and the detection rate of antibodies did not increase by age. None of the children had salivary anti-6B IgG during the study. These results are in concordance with the serum data; serum IgG concentrations stayed low until the age of 4 to 5 years in unvaccinated children. Thus, natural contacts with pneumococcus do not appear to augment a clear local IgG production.

Anti-Pnc PS IgA was detected often also in unvaccinated children and the detection rate increased with age. Previously, Simell *et al* have found that pneumococcal carriage and AOM can induce both anti-Pnc PS and protein antibodies in saliva of infants (Simell *et al.* 2001; Simell *et al.* 2002). Thus, it appears that an increase in antibody concentrations in

these children is a consequence of natural contacts with pneumococci.

In addition to this, anti-Pnc PS antibodies can be induced by cross-reactive bacteria of normal nasopharyngeal and enteric flora e.g. *Escherichiae coli*, *Klebsiellae*, GBS, and nongroupable streptococci (Tsui *et al.* 1982; Lee *et al.* 1984; Lee and Wang 1985; Reason and Zhou 2004). However, the role of naturally induced antibodies in protection is not clear.

5 Salivary anti-meningococcal antibodies induced by vaccines

Children in study VI had received MCV and/or MPV in infancy (Twumasi *et al.* 1995). At 4 to 5 years of age all the children were vaccinated with MPV in order to study immunological memory induced by meningococcal vaccines (MacLennan *et al.* 2001). To investigate mucosal immune response and memory induced by meningococcal vaccines, saliva samples were collected before and 9 to 14 days after the vaccination.

We found that the meningococcal vaccine was able to induce mucosal antibodies; there were significant IgG and IgA responses for both MenA and MenC. Both anti-MenA and MenC antibodies detected in saliva represented more often class IgA than IgG. Further, IgA found in saliva was secretory in nature.

Salivary antibody responses to the MPV were found in all vaccine groups, but there were differences in the anti-MenC antibody concentrations depending on the meningococcal vaccination history. Children who had been primed with one or more doses of MPV had significantly higher salivary anti-MenC IgA concentrations both before and after the revaccination with MPV than children who had been primed with MCV. In serum, the situation was vice versa: children who had been primed with MCV had higher IgG antibody concentrations than children who had received the MPV earlier (MacLennan *et al.* 2001). Also, bactericidal activity of the antibodies was lower in children primed with MPV. Salivary anti-MenC IgG response reflected these serum results indicating hyporesponsiveness to the polysaccharide vaccination after reimmunisation. A reason for discrepancies between salivary IgA and IgG responses can be that a conjugate vaccine is not able to reach the mucosal induction sites in a T dependent form. Also, conjugate vaccine contains shorter chain oligosaccharides compared to the polysaccharide vaccine, which are probably not able to activate specific B cells as efficiently as longer polysaccharides.

As to the MenA IgA concentrations, no differences were found between the vaccine groups either before or after revaccination. The anti-MenA IgG was detected more often in children who had been primed with meningococcal vaccines before than in the control group. There are two possible reasons for the differences between MenA and MenC responses. First, the MenA component of the vaccine was not able to induce immunological memory at the systemic site (Leach *et al.* 1997). Second, during the study there was a meningococcus A epidemic in the study area (MacLennan *et al.* 2001).

6 Mucosal immunological memory

When immunological memory has been induced, the next encounter with an antigen is expected to result in a faster response with higher concentrations of high avidity antibodies. In conjugate vaccine studies a polysaccharide vaccine has often been used as a challenge to mimic a contact with bacteria. Polysaccharide vaccines are T cell dependent antigens and therefore not regarded to induce immunological memory. Conjugate vaccines by contrast have been found to prime for systemic immunological memory. The results of the studies aiming to show the ability of conjugate vaccines to induce mucosal immunological memory have been controversial (Korkeila *et al.* 2000; Choo *et al.* 2000b).

We could not find any evidence of mucosal immunological memory induced by parenterally administered, either pneumococcal or meningococcal, conjugate vaccines. Children who had never been vaccinated with pneumococcal vaccines had at the age of 4 to 5 years anti-Pnc PS IgA in their saliva as often as children who had been immunised with a series of a PCV. Thus, it can be expected that if there were mucosal memory B cells, natural contacts with pneumococcus would have boosted higher antibody concentrations in children who had been primed with pneumococcal vaccines compared with the unvaccinated children. Further, children who received their first MenA/C vaccine at the age of 4 to 5 years, had even significantly higher anti-MenC IgA concentrations after vaccination than children who had been immunised with 1 to 3 doses of MCV in infancy and one dose of MPV at the age of two years. However, the serum and salivary anti-Men IgG data speak clearly for the systemic immunological memory (MacLennan *et al.* 2001).

Even if we could not find mucosal immunological memory after parenteral vaccination with

bacterial vaccines, others have found intramuscular rotavirus vaccination in mice to result in an enhanced specific mucosal immune response after challenge with a virus (Coffin and Offit 1998). However, the second response did not take place any earlier than the first one. The induction of mucosal memory may be dependent on the exposure to an antigen on a mucosal surface, which might explain the different responses to parenteral cholera vaccinations in different populations (Svennerholm *et al.* 1980). Also, because mucosal immunological memory has been induced by mucosal vaccinations, both oral and intranasal (Lycke *et al.* 1987; Asanuma *et al.* 1998), it has been speculated that the route of vaccination might have an impact on the emergence of mucosal immunological memory (Asanuma *et al.* 1998). Even if most of the children in our studies had been exposed naturally to Men and Pnc, they are not able to mount an immunological memory by themselves. This is suggested to be due to the fact that these bacteria are covered by a TI-antigen, polysaccharide capsule.

7 The significance of salivary antibodies

Both natural contacts with bacteria and parenterally administered vaccines can induce specific antibodies in the saliva of children. All the vaccines we studied were able to induce salivary antibodies, and also unvaccinated children had produced anti-Pnc and anti-Men PS specific IgA. Animal studies suggest that local antibodies have a role in defence; mucosal antibodies to PS antigens prevent acquisition of Hib or Pnc (Kauppi *et al.* 1993; Malley *et al.* 1998). However, salivary anti-PS antibodies have not been found to clearly have a protective effect against AOM or carriage (Simell 2003).

In the FinOM study we had an opportunity to relate salivary antibody concentrations to the vaccine efficacy results. We did not find any differences in the salivary anti-19F IgA concentrations between children vaccinated with PncCRM2 and HBV either at the age of 7 or 13 months. In accordance, the efficacy against type 19F AOM was low, 25% (95% CI –14 to 51%). However, protection was good against serotypes 6B, 14, and 23F varying between 59 and 84%, and we found that the IgA concentrations against these serotypes were significantly higher in the PncCRM2 than in the control group. According to this we could speculate that anti-Pnc PS IgA have a role in defence against AOM. Even so, in the PncOMPC group, there was a significant difference between the PCV and the HBV groups in the anti-Pnc PS IgA detection rate for 19F at the age of 7 months, and the efficacy against 19F AOM in this group was 37%. For other serotypes no differences between PCV and

HBV groups could be found, and the efficacy varied between 52% and 79% for 23 and 6B, respectively. However, the number of subjects was too small to allow statistical analyses, and to draw clear conclusions. The correlation of the anti-Pnc PS specific antibodies and the efficacy of the vaccine against AOM was not straightforward either on the systemic site (Jokinen *et al.* 2004).

It appears that both IgG and IgA have a role in defence, because bacteria have developed mechanisms to avoid the action of both of them. For example, pneumococci have the polysaccharide capsule to protect against IgG induced phagocytosis (Brown *et al.* 1983). Further, to avoid elimination by IgA it produces IgA1 protease (Kilian *et al.* 1996) and CbpA, which inactivates the antibody by binding to SC (Elm *et al.* 2004). We found that at the age of 7 months there was more often a difference between PCV vaccinated and unvaccinated children in the anti-Pnc PS IgA detection rate compared to IgG. Instead, at the age of 13 months, the situation was vice versa. Thus, it can be speculated that salivary IgA is more important than IgG early in life. Actually, the salivary IgA response has been found to mature early in life (Seidel *et al.* 2001). Later both salivary IgA and IgG can be suggested to have roles in mucosal defence.

Most of the pneumococcal and meningococcal vaccine studies have focused on antibody concentrations in serum, and the phagocytosis aided by anti-pneumococcal antibodies or serum bactericidal activity of anti-meningococcal antibodies have been thought to be the most important factors in defence against pneumococcal or meningococcal disease, respectively. However, these bacteria invade the human body through mucosal membranes and local antibodies most probably have a role in defence against them (Kauppi *et al.* 1993; Malley *et al.* 1998). However, how important salivary antibodies actually are, is not known. E.g. we do not know, which is actually more important, the salivary IgA or IgG concentration, and further, how high concentrations of antibodies are needed for protection. More profound knowledge on the function and protective capacity of the mucosal immune system would be helpful for the further development of efficient vaccines against upper respiratory tract pathogens, like pneumococcus and meningococcus.

CONCLUSIONS

The following conclusions can be drawn from the studies included in this thesis:

- The collection method of saliva does not have a significant effect on the anti-Pnc PS specific IgA concentrations.
- It is important to freeze saliva samples as soon as possible after collection to –70°C to prevent degradation of antibodies.
- Pneumococcal conjugate vaccines induce PS specific antibodies in saliva. However, there are differences between the vaccines in the ability to induce mucosal immune response and there are also serotype specific differences in the antibody concentrations and in the proportion of positive samples after a series of vaccinations.
- Salivary IgG is mainly derived from serum, but some local IgG production may take place.
- Salivary IgA is secretory in nature and thus locally produced.
- Increases in the anti-Pnc PS IgA concentrations appear to be mainly due to the IgA1.
- The pneumococcal conjugate vaccines in the present study were not able to induce mucosal immune memory; the anti-Pnc IgA concentrations increased by age also in the saliva of unvaccinated children.
- A booster immunisation with a pneumococcal polysaccharide vaccine induces stronger salivary antibody responses than a conjugate booster.
- Meningococcal conjugate and polysaccharide vaccines induce specific salivary IgG and IgA antibodies in infants. However, it appears that they are not able to induce mucosal immune memory.
- Responses to MenA and MenC differed from one another; meningococcal vaccination priming did not have an effect on the salivary anti-MenA IgA response, while children vaccinated with MPV previously had more anti-MenC IgA in saliva than children vaccinated only with MCV.
- Salivary anti-MenC IgG responses reflected the hyporesponsiveness to multiple MPV doses detected on the systemic site of immunity.

SUMMARY

Streptococcus pneumoniae and *Neisseria meningitidi*s come into contact with the human body at mucosal membranes. It is important to provide efficient protection at the portals of entry in order to prevent colonisation and ensuing local and invasive diseases. Salivary antibody concentration has been regarded to reflect antibody level at the nasopharynx. It is known that salivary antibody responses can be induced both by natural contact with bacteria and by vaccines. There are only few studies on conjugate vaccines and salivary immune response.

To improve methodology to investigate salivary antibodies, we studied different methods for collection and storage of saliva samples. We found that the collection method did not have an effect on the anti-pneumococcal antibody concentration in saliva samples. However, it is important to store them as soon as possible after collection at –70°C to preserve the antibody concentration.

We studied salivary antibody responses induced by four different pneumococcal conjugate vaccines and one meningococcal conjugate vaccine. All the studied conjugate vaccines evoked a salivary immune response in children. When polysaccharide vaccine was used as a booster the response was even stronger than with a conjugate booster. Still, we could not find evidence of mucosal immunological memory. Antibody concentrations increased by age also in unvaccinated children, suggesting natural encounters with bacteria.

In the future, it appears important to study the actual impact of mucosal immunity on immune defence against pneumococcal and meningococcal diseases. It has been suggested that protein vaccines and mucosal immunisation would be more potent in inducing systemic and mucosal immune responses. These and other potential approaches should be studied in order to develop more effective vaccines against both local and invasive infections.

FUTURE CONSIDERATIONS

Pneumococcal and meningococcal conjugate vaccines, and natural contacts with bacteria, have all been found to induce specific antibodies in saliva. Based on animal studies local antibodies have a role in defence. However, the actual importance of salivary anti-Pnc and anti-Men antibodies in humans is not completely clear. We do not know, which is more important, IgA or IgG or how high mucosal antibody concentrations are needed to prevent an acquisition or disease. In serum, surrogates of protection against pneumococcal disease have been sought for many years. Recently, serum anti-Pnc PS IgG concentration 0.35 μg/ml has been suggested as a surrogate of protection against invasive disease at population level (http://www.who.int/biologicals/Guidelines/Vaccines.htm). Five μg/ml in serum has been proposed to be a preventive level against pneumococcal carriage (Goldblatt *et al.* 2004).

Another issue is that antibodies in saliva have been thought to reflect antibody concentration in the nasopharynx where bacteria colonise the human body. Studies to compare how well anti-Pnc PS or Men antibodies in the nasopharynx and saliva correlate have not been conducted. Also, the quality of salivary antibodies has not been studied. We do not know, e.g. whether there is a difference between vaccine-induced and natural antibodies with respect to the avidity and functional activity. Neither is it known whether there are differences between the qualities of salivary antibodies after a polysaccharide booster compared with a conjugate booster.

Thus far the pneumococcal and meningococcal vaccines studied have been administered mainly parenterally. In animal models pneumococcal vaccines given through a mucosal route e.g. nasally have been protective against pneumococcal disease (Hvalbye *et al.* 1999; Jakobsen *et al.* 1999; Malley *et al.* 2001). Further, mucosally administered pneumococcal vaccines in mice have been found to induce better antibody responses both in serum and mucosa than parenterally given vaccines (Jakobsen and Jonsdottir 2003). Would that be the case also in humans, remains to be seen. In a study by Haneberg *et al* an immune response to MenB was not as strong after mucosal as after parenteral vaccination (Haneberg *et al.* 1998b), yet, the antibodies induced by mucosal vaccination were bactericidal.

To summarise, in the future more effort should be put to investigate the actual role of mucosal immunity against pneumococcal and meningococcal disease in humans, e.g. how antibodies function and the roles of both B and T cell immunity. Parenterally administered protein vaccines against pneumococcal disease are under development. The ability of these vaccines to induce mucosal immunity should be studied and the possible role of mucosal immunity as a surrogate of protection should be considered. However, according to the information available today, the best solution against pneumococcal disease might be a nasally administered protein or whole cell vaccine. Also a combination of nasal and parenteral administration could be considered. That would offer protection against different serotypes and further induce both systemic and mucosal immunities. Compared with the conjugate vaccines, the price of these vaccines might be lower, which would further facilitate the implementation of pneumococcal vaccines both in developed and developing countries.

ACKNOWLEDGEMENTS

This study was carried out between 1996 and 2004 at the Department of Vaccines, National Public Health Institute, Helsinki. I wish to thank all those who have contributed to this work and made this thesis possible.

I am grateful to the former Head of the Institute, Professor Jussi Huttunen and the present Director General, Professor Pekka Puska, for providing excellent working facilities.

I wish to thank the previous and current heads of the Department of Vaccines, Professor Juhani Eskola and Docent Terhi Kilpi, for pleasant collaboration.

My supervisor, Research Professor Helena Käyhty is warmly thanked for guiding me in the world of research. I appreciate her professional skills, good sense of humour and patience. It is very pleasant to work with her.

I thank all co-authors: Heidi Åhman, Juhani Eskola, Brian Greenwood, Virva Jäntti, Maija Korkeila, Mika Lahdenkari, Jenny MacLennan, Steven Obaro, Jack Obiero, Anthony Scott, and Mansour Yaich for collaboration and contribution in writing the articles included in this thesis. I am grateful to Anthony for giving me the possibility to work in the study group in Wellcome Trust/Kenya Medical Research Institute, Centre for Geographic Medicine Research – Coast, Kilifi, Kenya and for hospitality during my trip. Heidi is warmly thanked for friendship and encouragement to complete this thesis.

I appreciate statisticians Mika Lahdenkari and Virva Jäntti for help with statistical analyses, and Mika for patiently answering to all my questions concerning statistics. Jaason Haapakoski and Esa Ruokokoski are thanked for data management. Pia Korkeamäki and Jarkko Vieno are warmly thanked for help with my sometimes tricky problems with computers.

The staff at the library of the National Public Health Institute is acknowledged for kind and efficient service.

I am grateful to Professor Adam Finn who has kindly agreed to be the opponent in the public discussion. Germie van den Dobbelsteen, PhD, and Docent Anu Kantele, the reviewers of this book, are appreciated for careful reviewing of the thesis and useful comments. Suvi Rapola is thanked for the revision of the English language.

I want to thank all co-workers and friends at the Vaccine Department during these years. Especially, I want to thank persons at the Vaccine Immunology Laboratory and Clinical unit, with whom I have had been privileged to collaborate recently: Kari Auranen, Sirkku Blomberg, Nina Ekström, Sinikka Grönholm, Raili Haikala, Anne Holm, Emma Holmlund, Sinikka

Huotari, Teija Jaakkola, Ulla Johansson, Jukka Jokinen, Kaisa Jousimies, Hannele Lehtonen, Tuija Leino, Pamela Lindholm, Anna Lundqvist, Merit Melin, Pirjo Mäkelä, Hanna Nohynek, Päivi Paalanen, Sanna Piipponen, Taneli Puumalainen, Satu Rapola, Leena Saarinen, Tiina Salomäki, Pirjo-Riitta Saranpää, Birgit Simell, Anu Soininen, Ritva Syrjänen, Maija Toropainen, Camilla Virta, Kaija Vuontela, Arja Vuorela, and Rose-Marie Ölander. Specifically, I want to thank Hannele for teaching me in the beginning how to perform salivary assays, and Anna for efficient secratarial work.

I want to thank all volunteers, adults, children and their families for participating in these studies and the staff at the Child Health Centers.

Sharon Choo is acknowledged for offering me an opportunity to work at the Telethon Institute for Child Health Research, Perth, Australia. Peter Richmond and Deborah Lehmann are appreciated for pleasant collaboration. Willie Pomat and Ruth Thornton are warmly thanked for teamwork in the lab. I thank Ruth also for friendship and for organising outside lab activities. Deborah Lehmann and Michael Alpers are warmly thanked for their hospitality during my stay and for many dinner discussions with a glass of wine.

I am grateful to my fellow students: my friend Pirjo Matero for being my collaborator in numerous laboratory courses and my friend Leena Erkkilä for sharing experiences completing a thesis.

Finally, I thank all friends and relatives for happy events during these years. My sister Anne Korvenniemi and her family, Antti, Aino and Jussi are warmly thanked for all memorable moments we have had together. Hilarious kids, Aino and Jussi, have brought a lot of joy into my life. I also want to thank Anne for friendship and for sharing ups and downs in our lives. I am deeply grateful to my parents Marja-Leena and Jaakko Nurkka for taking good care of me and all the help they have given. I warmly thank my dear, Jani Poutiainen, for love and support during these years. Especially, I want to thank him for all the more or less deep conversations we have had and advice he has given. He knows how to bring a smile on my face.

Helsinki, October 2004

Anu Nurkka

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