ACTA UNIVERSITATIS AGRICULTURAE SUECIAE

VETERINARIA 48

ISCOMs as Delivery Systems for Mucosal Immunization

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SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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Akademisk avhandling som för vinnande av filosofie doktorsexamen kommer att offentligen försvaras i Ettans föreläsningssal, Klinikcentrum, Ultuna, Uppsala, fredagen den 28 maj 1999, kl 09.15.

Abstract

The overall objective of this thesis is to explore the potential of ISCOMs as mucosal adjuvant and delivery systems. The model consists of ISCOMs containing envelope proteins of respiratory syncytial virus (RSV) as vaccine antigen and targeting molecule, gp120 of HIV and ovalbum (OVA) as passenger vaccine antigens to be delivered to the mucosal immune system, and CTB as a model antigen and targeting molecule.

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Gp120 as a passenger antigen in RSV ISCOMs induced by i.n. potent mucosal IgA response in mice at various mucosal sites encompassing the remote genital tract, giving promise for the mucosal delivery concept with a targeting molecule and with passenger antigens, which is e.g. of interest for combating sexually transmitted diseases (STDs).

The dual adjuvant targeting system of ISCOMs bearing rCTB-OVA enhanced the mucosal antibody responses both to rCTB and the passenger. This ISCOM combination enhanced mucosal IgA response to OVA in the remote genital tract. ISCOMs complemented the rCTB-induced IgG1 response in mice by increasing the IgG2a level known to be promoted by IFN-γ, leading to a balanced Th1/Th2 immune response.

In conclusion, the present study provides direct evidences that ISCOMs are potent mucosal adjuvant and delivery systems, and promising alternative and complement to CTB, by increasing and modulating the immune response to CTB itself as well as to passenger antigens. The incorporation of a targeting antigen with combinations of adjuvants and passenger antigens in the same ISCOM, creates an attractive approach to modern vaccine design.

Key words: ISCOMs, RSV, mucosal immunity, IgA, gp120, OVA, CT, CTB.

Distribution:
Swedish University of Agricultural Sciences
Department of Veterinary Microbiology
S-751 23 Uppsala, Sweden

Uppsala 1999
ISSN 1401-6257
ISBN 91-576-5417-4

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 1999

Acta Universitatis Agriculturae Sueciae
Veterinaria 48

ISSN 1401-6257
ISBN 91-576-5417-4

Tryck: SLU Service/Repro, Uppsala 1999

To my parents
and my son

Abstract

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Appendix

Papers I-V

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I **Hu K.-F.**, Morein B., Ekström J., Merza M. Characterization of an ISCOM prepared with envelope proteins of human respiratory syncytial virus. *Manuscript.*

II **Hu K.-F.**, Elvander M., Merza M., Åkerblom L., Brandenburg A. and Morein B. (1998) The ISCOM is an efficient mucosal delivery system for RSV envelope antigens inducing high local and systemic antibody responses. *Clinical and Experimental Immunology. 113:235-243.*

III **Hu K.-F.**, Ekström J., Merza M., Lövgren-Bengtsson K. and Morein B. (1999) Induction of antibody responses in the common mucosal immune system by RSV ISCOMs. *Medical Microbiology and Immunology. In press.*

IV **Hu K.-F.**, Morein B. and Merza M. (1998) Using distilled water for the extraction of mucosal antibodies and the subsequent application in RSV neutralization test. *Journal of Immunoassay. 19:209-222.*

V Ekström J., **Hu K.-F.**, Lövgren-Bengtsson K. and Morein B. (1999) ISCOM and ISCOM-MATRIX enhance by intranasal route the IgA responses to OVA and rCTB in local and remote mucosal secretions. *Vaccine. In press.*

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Abbreviations

Ab	antibody
Ag	antigen
APC	antigen presenting cell
BALT	bronchus-associated lymphoid tissue
BRSV	bovine respiratory syncytial virus
BSA	bovine serum albumin
CPE	cytopathic effect
CRSV	caprine respiratory syncytial virus
CT	cholera toxin
CTL	cytotoxic T Lymphocyte
DTH	delayed-type hypersensitivity
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
FI-RSV	formalin-inactivated RSV
F protein	fusion glycoprotein
GALT	gut-associated lymphoid tissue
G protein	attachment glycoprotein
HRSV	human respiratory syncytial virus
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
i.m.	intramuscular
i.n.	intranasal
i.v.	intravenous
LP	lamina propria
LRI	lower respiratory tract illness
LT	Escherichia coli heat labile enterotoxin
MALT	mucosa-associated lymphoid tissue
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MoAb	monoclonal antibody
OVA	ovalbumin
PBS	phosphate-buffered saline
PP	Peyer's patches
rCTB	recombinant cholera toxin B subunit
s.c.	subcutaneous
sIgA	secretory IgA
Th1/Th2	T helper lymphocyte type 1 or 2
URT	upper respiratory tract
VN	virus neutralization

Introduction

Background

Great achievements have been made since a live vaccine against smallpox was first introduced on a broad scale for man by Jenner some 200 years ago. More lives have been saved, and more animal production has been safeguarded by vaccination than through all other medical activities. The worldwide eradication of human smallpox, and achieving the same goal for poliomyelitis and measles are cases in point. But these achievements are overshadowed by the continuous pandemic of AIDS, and the attempts to develop vaccines for many other old and new diseases have been unsuccessful.

Most vaccines today are given parenterally, providing poor or insufficient mucosal protection, because the majority of pathogens invade the host through mucosal surfaces. Live vaccines have been tried extensively, but this approach also has shortcomings: under- or over-attenuation or reversion to wild type, insufficient expression of foreign antigens or expression in an inappropriate form, induction of immune response to the vectors, uncontrolled spreading of infection, and tissue damage caused by delayed-type hypersensitivity (DTH) reaction. Killed and subunit vaccines are generally safe. However, most non-replicating antigens are relatively inefficient when administered mucosally, requiring multiple and large doses (milligram to gram) of immunogens, only yielding modest and short-lasting antibody responses.

Alternative strategies of antigen delivery include liposomes and biodegradable microspheres (Michale, Eldridge et al. 1994). Their preparation, however, requires large amounts of antigens, and the harsh conditions may result in the denaturation of antigens. The introduction of the powerful mucosal adjuvants, such as cholera toxin (CT) and the thermolabile enterotoxin of E coli (LT) made it possible for numerous antigens to be delivered through mucosal surfaces, inducing both local and systemic antibody- and cell-mediated immune responses. But CT and LT in their native forms are highly toxic, and the conflicting reports on the effectiveness of their B subunits as well as the induction of immune tolerance to antigens coupled with the B subunits, call for other alternatives to be explored.

One antigen delivery system is the immunostimulating complexes (ISCOMs). ISCOMs have been extensively studied in connection with a variety of antigens including antigens from parasites, bacteria, mycoplasmas and peptides. However, the experimental vaccines were mostly given by parenteral administration. Studies have also shown that ISCOMs have promise for mucosal application.

The ISCOMs are well suited for delivery of viral envelope proteins including respiratory syncytial virus (RSV), the most important cause of viral lower tract illness and death in infants; it is also a pathogen for calves. The virus gains entrance to the host through the respiratory mucosa. Thus immune protection against RSV requires a vaccine eliciting mucosal antibodies. The envelope proteins of RSV, like CT and LT and their B subunits, are all the natural targeting instruments for the corresponding microorganisms. It might be beneficial to incorporate these proteins into ISCOM particles, together with

passenger antigens to explore their targeting properties. Passenger antigens may by themselves have different immunological properties. Ovalbumin (OVA) is an inert or weak antigen while gp120 of HIV is a biologically active molecule. These antigens could be guided by mucosal targeting molecules in the ISCOMs to the mucosal immune system.

Respiratory syncytial virus

The first description of childhood pneumonia believed to be of virus etiology was published by Goodpasture in 1939 (Goodpasture EW 1939). Subsequently, Adams reported the occurrence of pneumonia in 32 infants with nine associated deaths, that had occurred in 1937 (Adams 1941). This outbreak was a nosocomial epidemic in a nursery for newborn, and the disease was characterized by cough, dyspnea, and cyanosis. The lungs from the deceased infants exhibited cytoplasmic inclusion bodies in the bronchial epithelial cells. Most likely, this report represented the first detailed description of respiratory syncytial virus (RSV). However, RSV was first isolated in 1956 from chimpanzees, when an epidemic of viral respiratory disease occurred in a colony in captivity (Morris, Blount et al. 1956). Soon after, it was isolated from children (Chanock, Roizman et al. 1957). The name, respiratory syncytial virus, reflects the characteristic effect the virus has on cell culture, in which it causes formation of multinucleated syncytia (Kingsbury, Bratt et al. 1978). Today, RSV remains the most important cause of lower respiratory illness (LRI) in infants (Hall 1994; Collins, McIntosh et al. 1996). Nearly 100,000 hospitalizations are attributable to RSV infection in the United States annually, resulting in costs approaching $300 million per year (Heilman 1990). Infants who are premature (Berkovich 1964; Cunningham, McMillan et al. 1991) or who have chronic lung disease (Groothuis, Gutierrez. et al. 1998) or congenital heart disease (MacDonald, Hall et al. 1982) are at particular risk for severe RSV disease. RSV can also cause life-threatening pulmonary disease in bone marrow transplant recipients (Fouillard, Mouthon et al. 1992) and in elderly people (Falsey, Treanor et al. 1992; Falsey, Cunningham et al. 1995; Dowell, Anderson et al. 1996; Mlinaric-Galinovic, Falsey et al. 1996; Falsey and Walsh 1998).

Epidemiology

By 2 years of age, almost all children will have been infected with RSV and approximately 50% will have been infected twice (Hemming, Prince et al. 1995). Reinfection can occur throughout life and is usually symptomatic. However, RSV infection does not generally cause lower respiratory tract infection in immunocompetent young adults and healthy older children (Hall, Geiman et al. 1976).

RSV epidemics occur yearly during winter and early spring in temperate climates and during the rainy season in some tropical climates (Spence and Barratt 1968; Mufson, Levine et al. 1973). Humans are the only known reservoir for human RSV (HRSV). Spread of this highly contagious virus via contaminated nasal secretions requires close contact with an infected individual or contaminated environmental surface (Hall and Douglas 1981).

Bovine respiratory syncytial virus (BRSV) infection has been reported most frequently in calves under six months of age (Kimman, Zimmer et al. 1988; Kimman and Westenbrink .1990). However, factors such as stress, gestation, lactation, previously existing infections, housing conditions, and antigenic

variations may affect virulence and severity of the disease (Baker, Wilson et al. 1992).

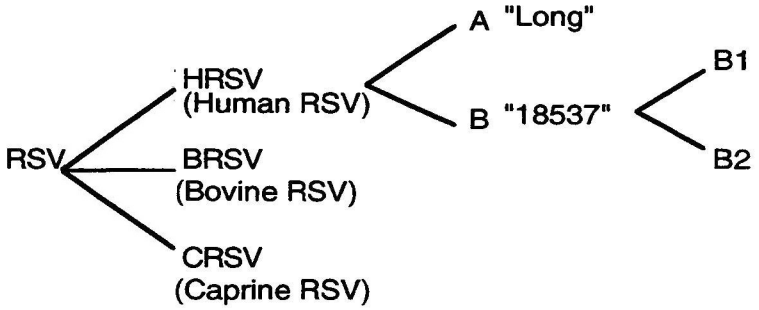
Classification and structure

The respiratory syncytial virus is a member of the genus *Pneumovirus* of the family *Paramyxoviridae*. It is a pleomorphic enveloped virus with a diameter of 300 to 350 nm, exhibiting both spherical and filamentous forms. RSV exhibits fusion activity but it does not have the hemagglutination, hemadsorption, hemolytic, or neuraminidase activities associated with some of the other paramyxoviruses (Richman, Pedreira et al. 1971). This virus has a genome comprised of single strand of negative-sense RNA, which (e.g. strain A2) is composed of 15,222 nucleotides, encoding ten proteins. They are three transmembrane surface proteins (F, G, and SH), two matrix proteins (M and M2), three nucleocapsid proteins (N, P, and L), and two nonstructural proteins (NS1 and NS2), which are present in infected cells only (Collins, McIntosh et al. 1996). The surface fusion (F) and attachment (G) glycoproteins are the only viral components that induce RSV-neutralizing antibody. The F protein, in combination with G and SH, is responsible for fusion of the viral envelope with host cell membranes and for the characteristic syncytium formation in cell culture, and its genome is highly conserved between RSV groups. The G protein mediates attachment to the host cell surface and is largely responsible for the antigenic diversity observed between RSV groups.

RSV major neutralizing epitope is on the F1 subunit of the fusion protein, probably in the region 221-236 of the amino acid sequence (Trudel, Nadon et al. 1987a; Trudel, Nadon et al. 1987b). Recently, three successive overlapping linear peptides that share the amino acid sequence 173STNKAVVSLS182 have been identified as another neutralizing epitope. The sequence of this novel neutralization site is conserved in all known HRSV and BRSV strains, and is located on the N-terminus of F1, adjacent to the hydrophobic, putative fusion-related region (Langedijk, Meloen et al. 1998).

G protein possesses fewer hydrophobic regions and is more difficult to incorporate into ISCOMs than the F protein. It is believed that unlike the F protein, the G protein is not involved in cytotoxic T cell response (CTL) (King, Stott et al. 1987; Pemberton, Cannon et al. 1987). The F and G proteins induce different cytokine profiles. The F protein generates T helper lymphocyte type 1 (Th1) cytokines, interleukin-2 (IL-2) and interferon gamma (IFN-γ), whereas the G protein generates Th2 cytokines (IL-4 and IL-10)(Jackson and Scott 1996). Even the *in vitro* recall response of RSV-primed lymph node cell response consists of T helper cells that are predominantly of the Th2 subset, secreting IL-4, and inducing an eosinophilic response in lungs of RSV-infected mice (Johnson, Johnson et al. 1998).

Based on reactivity with panels of monoclonal antibodies and their natural hosts, RSV is divided into human, bovine and caprine RSV. The human RSV is divided into group A and B, and group B HRSV is further divided into subgroup B1 and B2. The existence of two subgroups of BRSV has also been suggested by Baker et al. (1992), but its existence has not been widely accepted.



Although HRSV A and B strains differ in all 10 viral proteins, the G glycoprotein between HRSV A and B shows the greatest divergence between groups, with only 53% amino acid homology between prototype HRSV A and B viruses (Johnson, Olmsted et al. 1987). Group A and group B viruses cocirculate during epidemics, although one may predominate (Akerlind and Norrby 1986; Hendry, Burns et al. 1988; Mufson, Belshe et al. 1988; Hendry, Pierik et al. 1989). Group A HRSV infection may also cause more severe disease than group B HRSV infection (Taylor, Morrow et al. 1989; McConnochie, Hall et al. 1990; Walsh, McConnochie et al. 1997). The impact of antigenic diversity on RSV epidemiology is not completely understood, but it may partly explain the susceptibility to reinfection throughout life (Beem 1967; Henderson, Collier et al. 1979), and the yearly variation in severity of epidemics within communities (Collins, McIntosh et al. 1996). BRSV belongs to a different subgroup than HRSV A and B. It has a narrower host cell range (Stott and Taylor 1985) and only about 30% amino acide homology with subgroup A and B of HRSV. A closer relationship was found between bovine and caprine RSV (Trudel, Nadon et al. 1989b).

Protective immunity after natural infection

Immunity to RSV is mediated by antibody- and cell-mediated immune responses. Serum antibody acquired by infection as well as maternally derived antibodies, secretory antibody, and major histocompatibility complex class I (MHC-I)-restricted cytotoxic T lymphocyte (CTL) (Richardson, Yolken et al. 1978; Bangham, Cannon et al. 1985; Bangham, Openshaw et al. 1986; Watt, Zardis et al. 1986), may all contribute to immune protection. Immunity to RSV after natural infection is incomplete, and reinfection occurs throughout life (Beem 1967; Henderson, Collier et al. 1979). Healthy older children and young adults, however, are usually protected against RSV-associated LRI.

RSV replicates primarily in the respiratory epithelium. Therefore, serum neutralizing antibody does not prevent infection, although high levels of serum neutralizing antibody against RSV protect the lower respiratory tract (Prince, Horswood et al. 1983; Walsh, Schlesinger et al. 1984; Prince, Horswood et al. 1985). In agreement with this finding, RSV infection in the elderly correlates with low serum neutralizing antibody (Falsey 1998). Cotton rats that were given live RSV i.n. were resistant to rechallenge for up to 6 months, suggesting secretory antibody, but not serum antibody, correlated with protection of the upper respiratory tract (Mills, Van Kirk et al. 1971). In infants, the development of RSV-specific IgA in nasal secretions correlated temporally with disease clearance (McIntosh, Masters et al. 1978). Studies in

mice indicate that cell-mediated immunity is important in termination of RSV infection, but not in protection against infection (Cannon, Stott et al. 1987).

RSV vaccine development

The history of RSV vaccine development has paralleled that of progress of vaccinology in general. Initial immunoprophylaxes were made with inactivated RSV; subsequently, live attenuated intranasal and parenteral RSV vaccinations were tested. Later attempts include subunit vaccines, antiidiotypic antibodies, cDNA-derived vaccines, vector delivery systems, and synthetic peptide vaccines. So far, none of these strategies have proved efficacious or satisfactory for general use (Table 1).

Table 1. Past and present major attempts to develop RSV vaccines

Vaccine Formulation	Result	Why failed
Formalin-inactivated HRSV (FI-HRSV)	Not only failed to protect against the disease but also exacerbated clinical response	Formalin treatment leads to a Th2-biased response, low anti-F response
Ultraviolet light (UV)-inactivated BRSV	Both i.n. and i.m. vaccinations failed	Reduction or loss of antigenicity by UV light
Live attenuated temperature-sensitive mutants of HRSV	Field trials of these vaccines have been disappointing	Underattenuation; overattenuation; genetic instability.
Attenuated live BRSV (Rispoval®)	Contradiction	Unclear
Vaccinia-F	Complete resistant in lower respiratory tract, partial resistance in URT	
Vaccinia-G	Only lower respiratory tract was protected For both it was reported that there were pathologic changes in the lung after challenge	
Purified RSV F and G glycoproteins	Reportedly to be protective without enhancing lung pathology	
RSV ISCOMs	Efficient in stimulating VN antibody, CTL response as well as mucosal antibody response	

Model antigens

OVA

Ovalbumin is considered to be an inert antigen with low capacity to modulate immune response; for this reason it has been widely used as a model antigen for immunological studies. It is useful for studying the immunological properties of the adjuvant component with minimal influence of the antigen.

Gp120
The envelope protein of HIV-1 is a 160 kd precursor protein that is cleaved into the external envelope glycoprotein gp120 and the transmenbrane glycoprotein gp41. It is gp120 that is responsible for binding to the major cellular virus receptor, the CD4 molecules, which are present on T helper cells and macrophages (Dalgleish, Beverley et al. 1984; Klatzmann, Champagne et al. 1984). Moreover, gp120 is shown to be the main target for virus-neutralizing antibodies, thus attracting much of the vaccine research; it is biologically a very active protein (Chanh, Dreesman et al. 1986; Ho, Kaplan et al. 1988).

Cholera toxin (CT)

CT is a major enterotoxin produced by *Vibrio cholerae*, which consists of two subunits. The A-subunit (28 kd) is built up by two polypeptide chains, A1 (23 kd) and A2 (5 kd) linked by a disulfide bond. The B-subunit (58 kd) consists of five identical polypeptide chains (11.6 kd)(Fishman 1990) held together by non-covalent interactions, forming a ringlike configuration around a channel in which the A subunit centers.

The receptor for CT is the monosialoganglioside GM1 (Holmgren 1981), which is present on all nucleated cells. The binding occurs through the five B-subunits with very high affinity (0.1 to 1 nM) (Fishman 1990). It is the A subunit that activates the adenylate cyclase system leading to the enhancement of cAMP causing the toxic effect. CT is a powerful mucosal immunogen and a mucosal adjuvant, stimulating antigen-specific secretory IgA and serum IgG antibodies to antigens coadministered by the oral or nasal route (Elson and Ealding 1984; Lycke and Holmgren 1986). Some debate exists over the efficacy of cholera toxin B (CTB) as mucosal adjuvant. CTB has been reported to enhance the immune response to some mucosally administered antigens (Tamura, Samegai et al. 1988; Lee and Chen 1994; Hathaway, Partidos et al. 1995; Vadolas, Davies et al. 1995), while some investigators have shown that upon simple coadministration of CTB or administration of CTB chemically conjugated to another antigen, its adjuvant activity is weak and requires the addition of a small amount of CT, and the induction of oral tolerance by CTB has also been documented (Lycke and Holmgren 1986; Liang, Lamm et al. 1988; Czerkinsky, Russell et al. 1989; Wilson, Clarke et al. 1990; Tamura, Yamanaka et al. 1994). These claims indicate that although CT and CTB have shown considerable promise, there is still research to be carried out to overcome the toxicity problem of CT, and to clarify the adjuvant activity as well as the capacity to induce oral tolerance to coupled antigens by CTB (Czerkinsky, Sun et al. 1999). In parallel, other alternatives must to be explored.

Mucosal immunity

The mucosal immune system consists of lymphoid cells associated with mucosal surfaces throughout the body, including the oropharynx, the gastrointestinal tract, the lungs, genitourinary system and the mammary glands. It has been estimated that around 80% of the immune system is associated with mucosal surfaces (Czerkinsky and Holmgren 1995). These cells are accumulated in, or in transit between, various mucosa-associated lymphoid tissues (MALT), forming the largest mammalian lymphoid organ

system (Bienestock 1985). This reflects the relative importance of immune reactions against antigens and pathogens that initially occur primarily at mucosal surfaces.

The mucosal immune system

The current knowledge of the mucosal immunity is largely based on the studies of the lymphoid tissue of the gut-associated lymphoid tissues (GALT), and great similarities are also found in other MALTs. Thus bronchus-associated lymphoid tissues (BALT) shares many anatomical similarities with GALT, serving similar functions in the upper respiratory tract (URT) (Rudzik, Clancy et al. 1975; Bienenstock 1980). The GALT consists of the Peyer's patches (PP), the appendix, mesenteric lymph nodes (MLN) and solitary lymphoid nodules as well as dispersed lymphocytes in the intraepithelial lymphocytes (EL) and lamina propria (LP) of the intestine (Crotitoru and Bienenstock 1994). The most characteristic feature of the PP is the presence of a specialized follicle-associated epithelium containing M cells (named for their microfolds). These cells are interspersed among absorptive epithelial cells covering the dome of the PP on the luminal side of the gut. M cells are actively pinocytosing cells, and they transport much of what they take up to the underlying subepithelial lymphoid cells. This transport has led to the concept that the M cells provide a critical sampling process of the contents of the intestinal lumen, allowing the mucosal immune system to continuously sample the luminal contents. Bacteria, viruses, and protozoa have been shown to be taken up by M cells. Small molecules may also cross the M cell by fluid-phase endocytosis. There are structural parallels between PP and other lymphoid structures such as lymph nodes. Their nodules and germinal center are composed primarily of B cells. The germinal centers contain central-activated B cells and antigen-presenting cells (APC) and dentritic cells (DC), and there is a surrounding dense layer of resting B cells. Adjacent to the germinal centers are interfollicular zones that contain predominately $CD4^+$ T cells. B cells in the germinal center are enriched in IgA surface positive cells. There is evidence that cells in this site undergo immunoglobulin class switching to IgA phenotype. Cells activated in the PP do not mature into immunoglobulin secreting cells in here. They migrate to mesenteric lymph nodes, undergo further division, and home back to the lamina propria where terminal differentiation into IgA-secreting plasma cells occurs.

Significant numbers of $CD3^+$, $CD4^-$, $CD8^+$ T cells are also present in PP (London, Rubin et al. 1987; Hanson and and Brandtzaeg 1989), which can mount functional cytotoxic T lymphocyte response. Thus, all necessary immunocompetent cells, including $CD4^+$ Th cells, $CD8^+$ CTLs, $sIgA^+$ B cells, and accessory cells such as dendritic cells and macrophages are all present in IgA inductive sites.

The secretory IgA

The majority of immunoglobulin secreting cells in the MALT produce IgA. IgA synthesized in mucosal organs is primarily dimeric, whereas IgA synthesized in the bone marrow is predominantly monomeric and contributes to a major proportion of serum IgA.

Exogenous factors, such as T cell factors, and TGF-β promote the B-cell switch to IgA (Mestecky and McGhee 1987). PP is enriched in IgA

bearingcells, and the IgA switch may be occurring there. The factors provided by T cells to help the maturation of IgA^+ B cells into IgA secreting plasma cells are IL-2, IL-4, IL-5, IL-6 and IFN-γ. In addition, it seems likely that cell-contact between T and B cells may provide additional "cognate" interactions that augment IgA B cell differentiation. The terminal differentiation to IgA-committed B cells, however, does not occur in PP, but occurs after these cells circulate and "home" to the diffuse lamina propria compartment of the GI tract and other mucosal surfaces. LP contains high levels of various factors, such as cytokines, that promote terminal differentiation of B cells into IgA-secreting plasma cells.

Secretory IgA is a product of both gastrointestinal plasma cells and epithelial cells. IgA plasma cells in the gastrointestinal tract produce primarily dimeric IgA that has a covalently bound J chain. Dimeric or polymeric IgA has the ability to bind to the polymeric immunoglobulin receptor (secretory component) synthesized and supplemented to the IgA molecules in epithelial cells. The polymeric immunoglobulin receptor acts as the transport system for IgA by uptake from the basal membrane into endocytic vesicles. It transports IgA molecules to the apical lumenal membrane, where the polymeric receptor is cleaved, releasing IgA with a portion of the bound receptor (secretory piece) to the lumen of the gut, thus forming secretory IgA. The transport system is also found in salivary glands, mammary glands and biliary epithelial cells.

The properties of IgA are particularly suited to its function as a host defence molecule in the gastrointestinal tract. IgA is relatively resistant to proteolysis. It does not activate the complement system readily, thus prevents the marked activation of the complement system with inappropriate inflammatory consequences in the gastrointestinal tract. IgA has been shown to inhibit bacterial adherence to the epithelium, to inhibit macromolecule absorption, and to inhibit inflammation induced by antigen-antibody complexes with other immunoglobulin classes. IgA can neutralize viruses and enhance the nonimmunological host defence mechanisms provided by lactoperoxidase and lactoferrin. *In vitro* model systems have shown that IgA can mediate antibody-dependent cell-mediated toxicity. Although IgA is the predominant antibody isotype found in the mucosal surfaces, recent studies show that IgG is present in comparable amounts in mucosal secretions (Quiding-Jarbrink, Granström et al. 1995).

The common mucosal immune system

Following antigen presentation by accessory cells in the inductive site such as PP, B and T cells leave the PP in efferent lymphatic system and reach the systemic circulation through the thoracic duct. Circulating B cells then enter distant mucosal tissues, where they are preferentially retained. In these mucosal effector sites, B cells clonally expand and mature into IgA plasma cells (Mestecky and McGhee 1987; McGhee, Mestecky et al. 1989; McGhee, Mestecky et al. 1992). This cell distribution pathway from IgA inductive tissues (e.g. GALT and BALT) to IgA effector sites (e.g. LP regions of the intestine, the bronchi, the genitourinary tract and secretory glands) has been named the common mucosal immune system.

Mucosal immunization

Most human pathogens initiate their infectious processes at the mucosal surfaces, but most licensed vaccines today are usually administered parenterally. This is due, in part, to the difficulty in inducing effective mucosal immune responses, especially through non-replicating antigens. However, new and promising approaches are being developed to overcome the problem.

Mucosal immunization apparently has several advantages over parenteral immunization. 1. the efficacy of currently available vaccines can be enhanced by vaccination procedures to achieve both mucosal and systemic immunity. 2. Safety and minimization of adverse effects may be increased by vaccination strategies involving mucosal immunization. 3. Delivery of vaccines by oral or other mucosal routes reduces the need for personnel and equipment required for injections, and could facilitate administration of vaccines in a manner similar to prescription drugs. 4. Interference from maternally derived antibodies may be partially avoided because they seldom reach the mucosal surfaces. 5. Mucosal administration might increase vaccine efficacy in the elderly because mucosa-associated lymphoid system remains vigorous at a time, when systemic immunity declines in the elderly.

Studies carried out by Rosenthal et al. (1997), showed that i.n. immunization of female mice with AdgB8 (a recombinant adenovirus capable of expressing HSV glycoprotein B) induced anti-HSVgB IgA and IgG in vaginal washes, compared with intravaginal immunization with AdgB 8, which resulted in little or no anti-HSVgB IgA and only low levels of specific IgG in the vaginal washes. This indicates that although there is a compartmentalization in the common mucosal immune system, where the female reproductive organ is a poor inductive site of immune response, and nasal cavity is one of the most effective inductive sides for immune response. Therefore, i.n. administration for inducing immune response in the remote organ might be a better choice.

ISCOM technology

Some fifteen years ago, Morein and his colleagues first described the ISCOM concept (Morein, Sundquist et al. 1984) in searching an ideal antigen delivery system, which embodied the characteristics of

1. presenting antigens in a physically immunogenic form i.e., several copies in a submicroscopic particle to resemble an infectious agent.

2. optimizing the targeting of antigen and adjuvant to lymphatic organs and cells by enclosure of adjuvant and antigen in a stable, uniform particle; and

3. optimizing the immunomodulatory capacity by presenting the adjuvant and antigen components in the same particle, reducing the amount of antigen and adjuvant required for enhancing the immune response.

An ISCOM is 40-nm cage-like structure exhibiting icosahedral symmetry (Özel, Höglund et al. 1989) with a number of morphological subunits and is composed of a matrix containing *Quillaja* saponins, cholesterol and phospholipid at a molar ratio of about 1:1:1. The assembly of ISCOM is facilitated by hydrophobic interaction between these components and the antigen. The ISCOM antigen can be an envelope protein of a virus, a cellular membrane protein, or peptides containing hydrophobic domains or any antigen that has a hydrophobic domain. Non-amphipathic proteins, such as

gp120 of HIV, may be integrated into an ISCOM by structural modifications, such as partial denaturation of proteins with urea (Åkerblom, Nara et al. 1993),exposure to low pH (Pyle, Morein et al. 1989; Morein 1990; Åkerblom, Nara et al. 1993), or high temperature (Höglund, Dalsgaard et al. 1989) to expose internal hydrophobic regions within proteins. Another strategy is to covalently attach fatty acids' tail to soluble proteins allowing them to be incorporated into the ISCOMs (Ekström, Hu et al. 1999).

APC response to ISCOMs
ISCOMs have been shown to increase the MHC class II expression on antigen-presenting cells (APCs), resulting in enhanced antigen presentation by monocytes (Watson, Watson et al. 1992). Bergström-Mollaoglu et al. (1992) demonstrated that the frequency of cells expressing MHC class II molecules increased after *in vitro* antigen stimulation of spleen cells primed by influenza ISCOMs or HIV gp160 ISCOMs.

Studies on proinflammatory cytokine secretion by activated APC revealed that IL-1 (which plays an important role in initiation of immune response and inflammation as well as being a second signal for T-cell activation) in spleen (Villacres-Eriksson, Bergström-Mollaoglu et al. 1993), IL-6 (a key factor in the generation of CTL) measured in spleen and peritoneal cells and IL-12 (a cytokine promoting Th1 expansion) measured in serum (Behboudi, Morein et al. 1997; Villacres-Eriksson, Behboudi et al. 1997) were induced after immunization of mice with influenza ISCOMs.

Evidence of ISCOM-borne antigen being presented by different APCs was demonstrated by mixing T cells from mice primed with influenza ISCOMs and subsequently stimulated *in vitro* with splenic DC, B or peritoneal cells pulsed with the same ISCOMs. All populations of APC stimulated the T cells to proliferate and to produce IL-12 (Villacres-Eriksson, Behboudi et al. 1997).

T-cell response to ISCOMs
The purpose of using adjuvant is to improve the magnitude and the nature of the immune response. The latter purpose, that is, immune modulation, has received considerable attention recently. In 1986, Mosmann and colleagures described two types of $CD4^+$ T cell, termed Th1 and Th2, categorized by the cytokines they produced; that is Th1 cells secrete IL-2 and IFN-γ while Th2 cells produce IL-4, IL-5, IL-10, and IL-13, (Mosmann and Coffman 1989). In mice, Th1 responses result in activation of macrophages, production of antibody of the IgG2a isotype, DTH reactions and CTL response. Th2 responses give rise to eosinophilia, and production of antibody of the IgG1 and IgE isotypes (Morrison, Taylor et al. 1999). A Th1-biased response is desirable for combating intracellular parasites and viruses, whereas a Th2-like response is favourable for dealing with certain extracellular microorganism infections.

The potential of ISCOMs to activate murine Th cells to secrete Th1-like cytokines, such as IL-2 and IFN-γ has been well documented. Production of IL-2 and IFN-γ has been observed with a great variety of ISCOMs, including ISCOMs with influenza virus antigens (Fossum, Bergström et al. 1990; Villacres-Eriksson, Bergström-Mollaoglu et al. 1992; Lövgren-Bengtsson and Sjölander 1996; Sjölander, Lövgren-Bengtsson et al. 1997; Villacres-Eriksson,

Behboudi et al. 1997), OVA (Maloy, Donachie et al. 1995; Sjölander, van't Land et al. 1997), herpes simplex virus type I (HSV-1) glycoproteins (Hassan, Brewer et al. 1996), and Epstein-Barr virus (EBV) gp340 (Dotsika, Karagouni et al. 1997). These results indicate that ISCOMs are strong inducers of murine Th1 cells, which is in agreement with the observation that ISCOMs up-regulate IgG2a production and induce CTL response.

Besides having a potent Th1 cytokine profile, the capacity of ISCOMs to induce Th2-type cytokine production seems to be related to several factors, such as experimental system, antigen and the route of immunization. It appears that when measuring cytokine in the supernatant of cell culture after antigen stimulation *in vitro*, the IL-4 production is low (Maloy, Donachie et al. 1995; Villacres-Eriksson 1995; Lövgren-Bengtsson and Sjölander 1996; Sjölander, Lövgren-Bengtsson et al. 1997). However, when the T cell response to PSA-2 ISCOMs (protective Leishmania major parasite surface Ag-2 ISCOMs) was measured by ELISPOT, high numbers of IL-4 secreting cells were detected despite that the fact that the corresponding culture supernatant contained only tiny amounts of IL-4 (Sjölander, Baldwin et al. 1998), reflecting the consumption of IL-4. Moreover, OVA ISCOMs-activated T cells produce levels of IL-4 after restimulation comparable to those from cells primed with OVA in aluminium hydroxide, an adjuvant known to induce a Th2-like immune response (Sjölander, van't Land et al. 1997).

In conclusion, ISCOMs have induced a potent Th1 type response in a majority of the experiments carried out. In some cases, ISCOMs have also induced a concomitant Th2 response, resulting in what could be called a balanced Th1/Th2 response.

More importantly, ISCOMs have the capacity to deliver antigen to the cytosol (Villacres, Behboudi et al. 1998), which paves the way for MHC class I-restricted antigen presentation resulting in CTL response (Takahashi, Takeshita et al. 1990),

B-cell response to ISCOMs

The adjuvant properties of ISCOMs to stimulate high antibody responses have been well established in a great number of studies. The antibody responses induced by ISCOMs are usually 10 times higher than that induced by non-adjuvanted antigens in particular form, and are also high compared with other adjuvants. Subcutaneous immunization of mice with influenza protein (Cox, Coulter et al. 1997) could induce clear-cut antibody responses at antigen doses as low as 0.01 μg. ISCOMs containing OVA, gp340 of EBV or HSV-1 were highly immunogenic at doses ranging from 1 to 5 μg of protein (Dotsika, Karagouni et al. 1997; Sjölander, van't Land et al. 1997; Sjölander, Baldwin et al. 1998). The tiny amount of antigen required for induction of antibody response might reflect the advantage of incorporation of antigen and adjuvant in the same ISCOM particle, efficiently targeting APC. This advantage was indicated by the fact that immunization with influenza ISCOMs required 6- to 10-fold less Quil A to induce the same magnitude of antibody responses as influenza antigen coadministered with ISCOM Matrix (ISCOM without antigen) (Lövgren-Bengtsson and Sjölander 1996). Importantly, functional viral epitopes inducing neutralization were preserved in various viral antigens after incorporation into ISCOMs (Morein, Sundquist et al. 1984; Trudel, Nadon et al. 1989a; Höglund, Åkerblom et al. 1990).

Studies have also shown that a balanced antibody response encompassing all immunoglobulin isotypes and subclasses was induced by ISCOMs containing various antigens. In general, up-regulation of IgG2a was recorded, which is in agreement with the cytokine profile of Th cell response (for review, see Morein et al., 1998, and Maloy, Donachie et al. 1995).

Protective immunity induced by ISCOMs

Protective immunity has been induced against a variety of microorganisms including viruses, bacteria, mycoplasma and parasites. Table 2 lists examples of ISCOM-borne antigens having induced protective immunity including microorganisms for which other vaccines or experimental vaccines failed (for review, see Morein, Villacres-Eriksson et al. 1999).

Table 2. Protective immunity induced by ISCOMs containing various microbial antigens

Antigen	Animal	Disease
Haemagglutinin, neuramimdase, influenza virus	Mice	Pneumonia
Haemagglutinin measles virus	Mice	Encephalitis
Fusion protein, measles virus	Mice	Encephalitis
Haemagglutinin and fusion protein, phoid distemper virus	Seal	Lethal infection
Haemagglutinin and fusion protein, canine distemper virus	Dog	Pneumonia
G protein, rabies virus	Mice	Lethal infection, post-exposure immunization
Gp120, simian immunodeficiency virus	Monkey	Lethal infection
Gp125, HIV-2	Monkey	Viraemia
Gp120, HIV-1		
Envelope protein, bovine diarrhea virus envelope protein	Sheep	Abortion
Gp120 and P24 of HIV-1	Monkey	Viraemia
Gp70, feline leukemia virus	Cat	Viraemia
Gp360, Epstein-Barr virus	Tamarin monkey	Lethal tumour
Surface antigens, Toxoplasma gondi	Mice	Lethal infection
Immuno-affinity purified protein, Trypanosoma cruzi	Mice	Lethal infection

ISCOMs for mucosal immunization

The first attempt to induce mucosal immune response was carried out by Lövgren et al. (1990) with influenza virus ISCOMs in mice. Protective immunity was induced by one immunization measured by mortality, weight loss and virus isolation. Soon after Jones et al. (1988) showed that influenza virus ISCOMs induced, after i.n. administration, IgA in respiratory tract measured by ELISPOT and CTL response. Similar to CT and LT, ISCOMs were shown to prevent induction of immunological tolerance and to exert adjuvant activity in the digestive tract. Low but repeated oral doses with OVA

ISCOMs induce secretory IgA, CTL and systemic immune responses (Mowat and and Maloy 1994). Using fluorochrome-labelled ISCOMs containing the G protein of rabies virus, Claassen et al. (1995) showed that ISCOMs targeted PP more effectively than rabies virus particles. Another possible route for targeting to the lymphatic system in the gut is through the enterocytes, which may act as APCs (Santos, Lider et al. 1990).

After i.n. administration of the envelope proteins of influenza virus in ISCOMs (Morein, Villacres-Eriksson et al. 1999), a high serum antibody response is obtained, which is almost of the same order as that following parenteral immunization. A secretory IgA response is also efficiently evoked both at the local respiratory mucosa and distant (genital and intestinal tract) mucosa. In general, antigens derived from envelope viruses or cell membranes incorporated into ISCOMs retain their biological activity and conformation. Thus, the targeting property of virus glycoproteins are conserved, facilitating the penetration of mucus, efficiently evoking mucosal immune responses.

Aims of the study

The overall aim of this study is to characterize the mucosal antibody responses induced by ISCOMs delivered via intranasal mode of administration.

More specifically, the studies aimed

1. to prepare and characterize physico-biologically ISCOMs prepared from envelope proteins of respiratory syncytial virus;

2. to characterize mucosal and systemic antiboy responses induced by RSV ISCOMs after intranasal and subcutaneous modes of immunization;

3. to evaluate the potential of RSV ISCOMs as a mucosal adjuvant and delivery system for RSV antigen and passenger antigens;

4. to adapt and develop techniques for extraction of mucosal antibodies from the common mucosal immune system (i.e., locally in the upper and lower respiratory tracts and remotely in the genital and intestinal tracts) to be used in ELISA and virus neutralization (VN) tests; and

5. to combine the mucosal targeting and adjuvant properties of rCTB and ISCOMs.

Comments on methods

The protein, lipids and Quil A ratio for RSV ISCOM formation (paper I)

Based on earlier studies of preparation of ISCOMs from enveloped viruses, RSV ISCOMs were prepared by mixing protein, lipids and Quil A at a weight ratio of 1:0.1:5. There were no complete ISCOM particles in these early attempts. With increasing lipid proportion, the typical cage-like ISCOM particles gradually appeared. The optimal ratio for RSV ISCOM formation was 1 protein to 1 lipids to 5 Quil A. This study and others (Lövgren-Bengtsson and Sjölander 1996) indicate that the amount of lipids that follows the virus will differ from virus to virus, from cell culture system to cell culture system, and with the methods used for virus purification. Virus cultivated in eggs may contain more lipids to be brought into the ISCOM assembly process. Since the viral proteins are less influential for the ISCOM formation than lipids and Quil A, the best ratio for RSV ISCOM or ISCOM matrix (ISCOM without antigen) formation is 1:5 (Lipids to Quil A). In conclusion, the final mixture of components for optimal formation of ISCOMs should have a correct stochiometry (Lövgren-Bengtsson 1998).

Extraction of mucosal immunoglobulins with 2% saponin (paper II, III, IV, V)

The mucosal Ig extraction method was first introduced to us by Czerkinsky (personal communication). The method was then published (Bergquist, Lagergard et al. 1995) as the perfusion extraction method (PERFEXT). We have subsequently used the method to extract mucosal immunoglobulins (Igs) in the lungs, and in the upper respiratory, female reproduction and intestinal tracts. There are advantages to the extraction method compared with the traditionally used washing method. This method is technically less demanding, therefore, it gives consistent results. Also, extracted samples give slightly higher yields of mucosal Igs than samples collected by washing from the lungs and significantly higher Ig yields from the URT .

Studies carried out by Bergquist et al. (1995), Johansson et al. (1998) and us (Hu, Ekström et al. 1999) showed that contamination by the blood was insignificant and addition of enzyme inhibitors and preservatives might not be necessary (Hu et al., manuscript in preparation).

The replacement of 2% saponin with distilled water for the extraction of mucosal antibodies made the VN test feasible (paper IV)

Although mucosal antibodies extracted with 2% saponin in PBS are suitable for ELISA, the cell lytic effect of saponin prevents its use in virus neutralization (VN) tests. Therefore, a study aimed at finding a replacement to the saponin was conducted. After extraction of mucosal Igs with different solvents, the total and specific mucosal IgA and IgG antibodies were determined. Distilled water matched the performance of the standard 2% saponin procedure. More importantly, samples extracted with distilled water could be used successfully in the VN test without lysing cells.

The ratio of specific Ig/total Ig was used as the measurement of mucosal antibodies (paper III)

Unlike the serum antibodies, mucosal antibodies are subjected to a great deal of fluctuation due to biological cycles, stimulations or the solvents used for extraction or washing. The ratio of specific Ig to total Ig is perhaps the best way to approach achieving consistent results useful for comparisons and reliable conclusions as described previously by Russell et al. (1996) and by us (Hu, Ekström et al. 1999).

Non-competing system for antigens incorporation into ISCOMs (paper V)

Incorporation of two components, OVA and rCTB, into the same ISCOM particles using the standard procedure based on hydrophobic interaction will lead to: (1) competition between the two antigens, resulting in uncontrolled biased integration to the more hydrophobic component, and (2) difficulty in quantifying OVA and rCTB individually. In this study, lipidated OVA was incorporated by hydrophobic interaction into ISCOMs together with GM1, which is a receptor of lipid nature for CTB. Subsequently, rCTB was taken up by the ISCOMs through high affinity (0.1 to 1 nM) (Fishman 1990) ligand and receptor interaction. Thus, in this non-competing process, OVA and rCTB could readily be quantified separately. OVA was recovered to about 50% and rCTB approached 100% incorporation.

Results and discussion

Characterization of RSV ISCOMs (paper I)

RSV ISCOMs prepared from envelope proteins of respiratory syncytial virus bear all of the classic characteristics of ISCOMs and are highly immunogenic through both s.c. and i.n. administrations.

RSV ISCOMs prepared with different virus preparations from both group A and group B of HRSV were first characterized physical-biologically. The virus proteins, lipids and Quil A were shown to be integrated into the ISCOMs because they were recovered in the same fraction after sucrose gradient centrifugation. The sedimentation behavior is similar to thyroglobulin with a value of 19s. The electromicroscopy showed typical cage-like ISCOM structures with a diameter of about 40 nm, consisting of circular subunits of 12 nm in diameter (Morein 1990). The protein recovered in ISCOMs from the original whole virus proteins is constantly around 15% regardless of HRSV strains used. The protein to Quil A ratio is about 1:10 on a weight basis. Western blot analysis revealed that the ISCOMs contained mainly the F, and to a less extent, the G glycoproteins.

The immunological property of the ISCOMs was then evaluated in mice. The immune responses in mice after both s.c. and i.n. immunizations, and challenge infection showed that the RSV ISCOMs were highly immunogenic, inducing protective immunity to challenge infection. Most interesting is that ISCOMs given i.n. facilitated a strong anamnestic IgA response to challenge infection, which was not seen after s.c. immunization with killed virus or ISCOMs given s.c. or in non-vaccinated animals.

Intranasal administration of RSV ISCOMs induced both strong mucosal and systemic antibody responses (paper I, II. III)

The dose and induction

One microgram of RSV ISCOMs administered s.c. induced a strong serum antibody response, but no detectable mucosal response. Intranasally, this dose induced a low response. The 1-μg dose of ISCOMs administered twice i.n. induced, besides a mucosal antibody response, a similar serum antibody pattern as after two s.c. immunizations but at different levels as measured by ELISA. A fivefold increase of the i.n. dose induced a serum antibody response comparable to that of s.c. immunization. These dose differences observed between the different modes of administration are consistent with the claim made by Yetter et al. (1980) that only about 20% of the inoculum might finally reach the lower respiratory tract in anaesthetized mice, and this fraction of antigen is subjected to further degradation. Therefore, at least a fivefold increase of the dose was required when the i.n. mode of administration was used.

Immunization using un-anaesthetized mice could not deliver a sufficient amount of the antigen to the lungs with the low doses used; the number of immunizations used for influenza virus antigen or OVA resulted in low or undetectable local and systemic responses (work in progress). Therefore, the nasal mucosa was not a primary inductive site for ISCOMs or for liposomes

(Michale, Eldridge et al. 1994) action. This is also possibly more a result of too little antigen reaching URT than a low inductive capacity to induce immune response of the region. It is also unlikely that stimulation of the GALT by swallowed material was a contributing factor, since oral administration of the ISCOMs was less effective (Sjölander and Cox 1998). It is conceivable that the ISCOMs delivered intranasally under light anesthesia would preferentially reach the lower respiratory tract (Yetter, Lehrer et al. 1980). RSV ISCOMs delivered i.n. to mice under anaesthetic, however, induced comparatively high levels of specific IgA in URT. In this case, we do not really know if the induction took place in URT or in the lungs or in both organs.

It should be noted that the dose of 5-μg used for i.n. administration in our animal trial is just five time of that of s.c. (1μg) immunization, which is far more efficient than that of 200 μg filaments of haemophilus influenzae (FHA) used for i.n. versus 8 μg for s.c. (Shahin, Witvliet et al. 1990). A 10-μg dose was also tested in the present study (paper III) and did not increase mucosal and systemic antibody responses any further, indicating the ceiling had been reached by the 5-μg dose.

Thus, studies carried out in paper II, III and IV were basically with 1 μg for s.c. and 5 μg for i.n. modes of administration.

Systemic antibody responses induced by RSV ISCOMs
When comparable doses of RSV ISCOMs were used for i.n. and s.c modes of administration (i.e., 5 μg and 1 μg per mouse for i.n. and s.c. respectively), the systemic IgM and IgG responses reached higher levels in the animals immunized i.n.. The IgA and IgE levels in serum were transient after both the first and second i.n. immunizations. The IgG2a, IgG2b, IgG3 were roughly at the same levels between i.n. and s.c. immunizations at all bleeds. This might indicate that only slightly different systemic immune responses were evoked by i.n. and s.c. modes of immunization. On the other hand, the inactivated RSV induced the highest serum IgA and IgE levels, indicating the induction of a more Th2-like immune response.

Intranasal administration of RSV ISCOMs induced potent secretory IgA antibody responses
The most interesting feature of i.n. immunization with the ISCOMs was that a unique (not seen after s.c. immunization), strong (peaked close to 1/10 000) and long-lasting (up to 22 weeks after the second immunization, when the experiment was terminated) mucosal IgA response was detected locally in the URT and lungs, and remotely in the genital and intestinal tracts. The strong mucosal IgA responses show that the ISCOMs have the ability, as a non-replicating antigen delivery system, to replace live RSV for mucosal application. This mucosal adjuvant capacity has, so far, been observed only with CT and LT. RSV ISCOMs surpassed a live RSV in inducing mucosal IgA response, but apparently the live RSV does not induce a strong infection in mice.

The role played by sIgA as the first line of defense against infection has been well established, especially in the protection of URT. It is also likely to work with IgG in a pivotal role in the protection of the lungs. On the other hand, CTL response is widely considered as the means to destroy virus infected cells;

therefore, it arrives in a later stage and might be more related to the recovery (Cannon, Stott et al. 1987).

The capacity of RSV ISCOMs to induce virus neutralizing antibody (Trudel, Nadon et al. 1989a) and CTL (Trudel, Nadon et al. 1992) had been demonstrated before the current studies were conducted. However, the potential of RSV ISCOMs to evoke mucosal antibody responses by mucosal application was not investigated. The surprisingly high IgA response in the URT, the lungs, and the genital and the intestinal tracts induced by RSV ISCOMs compared with the other ISCOM formulations (Ekström, Hu et al. 1999) pointed to the mucosal targeting property of RSV envelope proteins. Such property has been noted in other proteins from various microorganisms as well, such as Salmonella, Escherichia coli, Mycobacterium, Yersinia enterocolitica, adenoviruses, polioviruses et al (Michale, Eldridge et al. 1994).

An important contributing factor to the immunoenhancing capacity of ISCOMs is the delivery of the antigen and the adjuvant (Quil A) in the same particle, which increased this capacity 6- to 10-fold compared to admixing antigens with ISCOM-Matrix (Lövgren-Bengtsson and Sjölander 1996). One promoting factor is the targeting of antigen and Quil A to the same APC. It should be noted that the immunomodulating property of the viral proteins (mainly F glycoprotein) in concert with Quil A need not necessarily contribute to a mucosal IgA responses because both components drive to Th1 type of response. On the contrary, the IgA response could be expected to benefit from Th2 type of cytokines, such as IL-4, IL-5 and TGF-ß. However, mucosal administration of OVA ISCOMs did stimulate IL-5 production (Maloy, Donachie et al. 1995). Furthermore, the strong mucosal IgA and transient serum IgA and IgE responses might indicate that a Th2 response was also present, underlining that the route of administration plays an important role in determining the mode of an immune response; i.e., i.n. administration promotes a Th2-biased response, whereas s.c. immunization elevates a more Th1-like profile. In this case, the outcome seems to be balanced judged by the Th1/Th2 quota.

The ability to induce a strong common mucosal antibody response through mucosal administration is the indication of mucosal stimulation by powerful antigens. RSV ISCOMs given i.n. induced strong mucosal antibody responses both at the local inductive sites of the URT and the lungs, and also induced high levels of mucosal antibody responses in the remote genital and intestinal tracts. The functional VN antibody was also detected at these remote mucosal sites with titers similar to those at the local inductive sites.

Intranasal is an effective route for the induction of a local and a common mucosal antibody responses

One interesting aspect of inducting a common mucosal immune response is the selection of a route for the administration. There is obviously a compartmentalization or subcompartmentalization regarding both homing and final differentiation of plasma cell precursors. For instance, whereas oral immunization may induce good antibody responses in the small intestine, in the ascending colon, and in some distant exocrine glands such as the mammary and salivary glands, it is relatively inefficient at evoking an IgA antibody response in the distal segments of the large intestines, in the tonsils, or in the female genital tract. Rectal immunization evokes strong local

antibody responses in the rectum, but little if any response in the small intestine and in the colon. Intranasal immunization induces better local antibody response, i.e., in the upper and the lower respiratory tract. Therefore, the ideal route for a mucosal immunization against RSV should logically be intranasal to maximize the local immune response in these regions. However, for the induction of a common mucosal immune response aiming at the female reproductive organs, the i.n. mode of administration seems to be the choice in view of the fact that the female reproductive organs are poor inductive sites of immune response.

There is clear evidence showing that the cleaner the site of induction, the more efficient it is in inducing a immune response. For instance, two doses of 100 μg each of filamentous haemagglutinin antigen (FHA) of B. *pertussis* were required to achieve immune responses when administration intranasally in mice, whereas systemic immunization with this antigen was obtained with an 8-μg dose (Shahin, Witvliet et al. 1990), and single-dose intranasal vaccination was more than 100 times as effective as oral vaccination in inducing antiviral IgA antibodies in respiratory tract and serum. Moreover, intranasal immunization with CTB-combined HA vaccine was 10-100 times as effective on a dose basis as oral administration in inducing similar antibodies in intestinal secretions (Walker 1994). In our experiments (not published) the mucosal and systemic immune responses of mice following oral administration of rCTB ISCOMs were inferior to those obtained by i.n. administration. Taken together, the i.n. route is one of the most effective routes in inducing both a local and a common mucosal antibody responses.

Functional aspects of IgA and IgG (Paper III)

Both i.n. and s.c. administrations with the ISCOMs induced functional VN antibody in serum and in the URT, the lungs, the genital and intestinal tracts indicating the neutralizing epitopes of the F protein were well preserved.

The closer correlation between VN and IgG antibodies compared with that of VN and IgA antibodies should not rule out the protective value of the IgA *in vivo* because the *in vitro* neutralization test carried out in the present study measures only one mechanism; i. e., that the pre-existing IgA or IgG binds to intact virus, protecting the cells from infection. There are two other levels of mucosal IgA functions. When IgA is passing through infected epithelial cells from the basal membrane to the apical site, the IgA may then form a complex intracellularly in the infected cell with newly synthesized virus proteins, preventing virus assembly or release. In the lamina propria, immune complexes containing virus or virus proteins and oligomeric IgA could be excreted via the polymeric immunoglobulin receptor (pIgR) across the epithelium into the mucosal lumen. These three types of mucosal IgA functions (Mazanec, Huang et al. 1996), plus the rapid turnover of the epithelial cells at the mucosal surfaces, constitute the mucosal environment of IgA, implicating effective antibody functions in the defence against mucosal infections. The *in vitro* neutralizing assay with a totally different cell type and structure cannot mimic the mucosal environment; therefore, the biological function of the massive amount of IgA induced by the ISCOMs needs to be further investigated.

The fact that IgA does not to activate the complement system may be important for evading this adverse immunological reactions caused by IgM

and IgG. The ISCOMs administered by the mucosal route did not evoke an aberrant immune response that may endanger the mucosal surfaces. It is well established that >80% of all antibodies produced in mucosal-associated tissues is the secretory IgA (sIgA) isotype (Mestecky and McGhee 1987; McGhee, Mestecky et al. 1989), which is also confirmed in the present study showing there is no correlation between serum and mucosal RSV specific IgA. In contrast, the statistical analysis indicates that a major proportion of the IgG antibody induced by RSV ISCOMs is likely to be transudated from the blood, which is in agreement with the observation of others (Rosenthal and Gallichan 1997).

ISCOMs for the delivery of passenger antigens (paper III, V)

Mucosal targeting properties of microorganisms are vitally important for invasion of their hosts. Such properties may reside in the surface proteins of viruses or in filamentous or in toxic bacterial products. It should be possible to use their mucosal targeting properties in vaccine, provided suitable formulations are constructed. For example, the much-desired HIV vaccine with the candidate vaccine antigen gp120, like the inert model antigen OVA, seems to lack such capacity and is therefore incapable of inducing efficient mucosal immune responses on its own.

To evaluate the combined mucosal targeting and adjuvant effect of rCTB and ISCOMs, various combinations of OVA, rCTB in ISCOMs or OVA and rCTB admixed with ISCOM-matrix were tested in mice. The rCTB was incorporated via GM1 receptor into the ISCOMs. rCTB ISCOMs or rCTB administered with ISCOM matrix administered intranasally enhanced by about 100-fold higher mucosal IgA responses to rCTB locally in the URT, lungs, and remotely in the genital and intestinal tracts, than rCTB alone. The inert antigen OVA in ISCOMs induced significantly higher mucosal IgA responses than did those induced by OVA coadministered with ISCOM matrix. Thus the presentation of antigen and adjuvant in the same particle is more important for a weaker antigen while the biologically active antigen rCTB can benefit efficiently from the adjuvant properties of ISCOMs, even when it is coadministered as a separate entity. The adjuvant and antigen properties of rCTB were further analysed with regard to synergism or antagonism. The synergism measured by IgA antibody response after i.n. immunization against the passenger antigen OVA could only been seen as increased IgA levels to OVA in the remote genital tract. However, an immunomodulatory effect of ISCOMs was seen in serum as an enhancement of the IgG2a response, recorded both after i.n. and s.c. immunizations. Remarkably, rCTB coadministered with ISCOM matrix (the matrix was actually OVA-ISCOMs) did not induce high serum IgG2a response, possibly due to a low dose of OVA being used.

It has been well documented that ISCOMs are powerful inducers of Th1 cell response with increased production of IFN-γ, IL-2 after parenteral administrations; also a comparatively Th1-biased immune response is induced by mucosal mode of immunization. A single feed of OVA in ISCOMs induced primary serum IgG and systemic DTH responses (Mowat and and Maloy 1994), showing the oral tolerance could be overcome by ISCOMs. On the other hand, it seems that ISCOMs rely on two mechanisms that allow access of ISCOM-borne antigens to the APCs: a selective one mediated by the antigen or a targeting molecule and a second, more general one, which allows the integration of the hydrophobic ISCOM structure into the plasma

membrane. ISCOMs containing OVA were taken up much less efficiently by the APCs than the influenza virus ISCOMs (Morein and and Lövgren-Bengtsson 1998) indicating the importance of incorporating mucosal targeting proteins into the ISCOMs. Combining mucosal targeting property of rCTB and mucosal adjuvant activity of ISCOMs might be much superior as an adjuvant and delivery system than either CTB or ISCOMs alone. The induction of an IgG2a to rCTB by its incorporation into ISCOMs compared with the lack of IgG2a response by rCTB alone, indicates the capacity of ISCOMs to switch a Th2-type response towards a Th1-like profile, but further experiments to delineate local and systemic T cell responses are required to show the immunomodulating capacity of ISCOMs.

In paper III, a combined gp120/RSV ISCOMs was given intranasally to mice. As expected, the sIgA response to gp120 was enhanced at most of mucosal sites examined, i.e., URT, lungs, and the genital tract. This indicates that the concept of incorporating mucosal targeting molecule and a passenger antigen in the same ISCOM particles is also valid for a biologically active antigen like gp120.

Concluding remarks

Extensive studies have been carried out with ISCOMs, bearing a variety of antigens to explore antibody- and cell-mediated immune responses, after parenteral modes of administration. This thesis extends the studies to encompass and to explore the properties of ISCOMs as delivery and adjuvant systems for mucosal i.n. administration.

RSV ISCOMs delivered intranasally induce strong, long-lasting mucosal antibody responses encompassing mucosal IgA and IgG in both the URT and the lungs, and also a systemic antibody response at an equal level to that after s.c. immunization. The induction of VN antibody response and protective immunity demonstrate the presentation of conformational antigen epitopes, and makes RSV ISCOM a promising RSV candidate vaccine for mucosal administration.

The mucosal targeting property of envelope proteins of RSV combined with the adjuvant activity of *Quillaja* saponin in the same ISCOM entity is a potential use of virus proteins in an ISCOM formulation for presentation of passenger antigens, lacking own capacity to induce sufficient immune responses by mucosal administration, as exemplified by the model antigen OVA and gp120 of HIV. The present studies also show great promises for ISCOMs as a delivery system using the i.n. mode of administration for induction of a common mucosal immune response to include remote mucosal target organs. This novel approach should be further explored for other antigens of clinical importance and to include other natural occurring mucosal targeting molecules, showing complementary properties to RSV envelope proteins.

The combination of mucosal adjuvant CTB or LTB with ISCOMs is another interesting approach for the development of mucosal delivery system. The Th2-biased immune response and the property of inducing systemic tolerance by CTB to the linked antigens could be overcome and also fine-tuned by addition of different amount of a Th1-biased adjuvant of *Quillaja* saponin in the ISCOMs, as evidenced in the present studies with an elevated IgG2a response and in other studies with production of Th1-type of cytokines. Thus, it is possible to tailor-make an immune response to suite the antigens involved, in view of what is desired for protective immunity.

The technology adapted in the present studies for the extraction of mucosal antibodies with saponin is proved applicable for virtually all mucosal sites available at necropsy. The use of distilled water to replace the saponin facilitates functional study of the mucosal antibodies extracted.

Further studies are underway to uncover the cytokine profile induced by i.n. administration of RSV ISCOMs, both before and after live virus challenge. The antibody-producing cells localized in the URT, in the lungs and in the spleen, including IgA-, IgG-, IgG1- and IgG2a-producing cells are being compared between speciments obtained after s.c. and i.n. modes of administration. The incorporation of detoxified LT (Douce, Giuliani et al. 1998) and CT (Yamamoto, Takeda et al. 1997) in the ISCOMs are being carried out and considerable attention is being given to cytokine

characteristics and DTH, for a better understanding of their mucosal adjuvant activities.

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Acknowledgements

This work has been carried out at the Department of Veterinary Microbiology, Section of Virology, Biomedical Center, Uppsala.

This long struggle to become a scientist rapidly comes to an end in the spring planting season. As I look forward to a fascinating future of Immunology and Vaccinology. I would like to express my sincere gratitude to the following people.

Thank you to:

my supervisor, Professor Bror Morein, for accepting me first as a visiting scholar, then as a PhD student, for introducing me to the exciting field of ISCOM technology, for teaching me the art of conducting scientific research, for stimulating discussions, for his constant encouragement and his visionary guidance, all of which help me greatly throughout the study;

my second supervisor, Associate Professor, Malik Merza, for teaching me how to carry out experiments and how to become an independent researcher;

my third supervisor, Associate Professor, Lennart Åkerblom, for his help and friendship;

Barbro Stensmo, the invaluable secretary, for her constant help both in private and research-related matters.

Associate Professor, Karin Lövgren-Bengtsson, for the valuable discussions;

Jill Ekström, for her collaboration and our fruitful work;

Associate Professor, Marianne Elvander, for her participation and contribution during the initial study; and

Magnus Simonsson, for his computer expertise.

Thank you, too, to my past and present colleagues and friends in Uppsala for friendship, including:

Izzdeldin Abusugra, Anna Bellman, Eva Emmoth, Sergey Gennadyevich, Sigrid Sjölander, Anders Sjölander, Diana Hansen, Tommy Linnè, Sandor Belák, Margareta Johansson, Jorge Moreno-López, Alia Yocoub, Marianne Nordenman, Sven-Åke Bergqvist, Ulf Gustafsson, Mikael Lundblad, Berndt Klingeborn, Gunilla Blomquist, Katarina Ranlund, Ann Nordengrahn, Leif Oxburgh, Claudia Rabino, Claudia Baule, Carlos Ros, Ulrika Allard, Gunilla Singelman, Anders Olofsson, Maria Villacrés-Eriksson, Martin Svenda, Bengt Rönnberg, Shariar Behboudi, Andras Ballagi-Pordany, Bo Sundqvist, Susanne Aldén, Irene Dergel, Kenneth Backström, Anna-Britta Pettersson, Annie Persson, Gunnel Svedlund, Thorleif Joelsson, Annika Eriksson, Torsten Fridberger, Anna Svensson, Ricardo Feinstein, Kristina Klintevall, Susanne Palacios de Feinstein, Frederik Widén, Marit Liljefors, Lennart Lindberg, Per Karlsson, Ingela Stake, Gull-Britt Olsson, Cesar Obando, Stefan Alenius, Maria

Persson, Stefan Vilcek, Modra Murovska, Mohammed Saleh Yassin, Pei Wang, Shu Wang, Yinghua Zhou, Wei Liu, Hongxin Zhao, Haitao Yang, Wen Pen, Songhua Hu, ChengMing Zheng et al.

Acta Universitatis Agriculturae Sueciae
presents doctoral theses from the faculty of agriculture, landscape architecture and horticulture, the faculty of forestry and the faculty of veterinary medicine at the Swedish University of Agricultural Sciences. Each faculty has its own subseries, Agraria, Silvestria and Veterinaria, respectively, with separate numbering.

ISSN 1401-6257
ISBN 91-576-5417-4