Starch Microparticles as an Oral Vaccine Adjuvant with Emphasis on the Differentiation of the Immune Response

BY
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Abstract

Polyacryl starch microparticles have been developed as an oral vaccine adjuvant capable of inducing strong local and systemic immune responses in mice. In this thesis, the starch microparticles were studied in order to increase basic understanding of their function. In particular, the thesis addressed aspects of the uptake of the particles and their presentation to the immune system after different routes of administration, in correlation with the differentiation of the induced immune response.

When using human serum albumin as a model antigen conjugated to the microparticles, it was found that the route of administration and the use of different combinations of routes, parenteral or oral, affect the profile (Th1/Th2 balance) of the induced immune response. It was also found that oral boosters are needed for the development of a local s-IgA response.

Ligated mouse intestinal loops in combination with confocal laser-scanning microscopy demonstrated that the uptake of the particles by the intestinal mucosa takes place over the follicle-associated epithelium (FAE) that covers the Peyer’s patches. The particles are also taken up in the villus epithelium when conjugated with rCTB, a ligand to the GM1 receptor. This qualitative difference in uptake did not affect the induced immune response. Thus, the addition of rCTB to the microparticles did not improve or influence the profile of the immune response. Chronic stress, known to alter the barrier function of the FAE, increased the cellular response but did not affect the humoral immune response.

Despite positive results in rodents, the particles were not able to boost a humoral immune response in man when tested with diphtheria toxin-cross reacting material (CRM197). Possible methods of improving the adjuvant effect in man are discussed.

Keywords: Vaccine adjuvant, Microparticles, Oral immunisation, Th1/Th2 differentiation, Mucosal immune response, Uptake, M cell

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List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>CT</td>
<td>cholera toxin</td>
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<td>CTB</td>
<td>cholera toxin B subunit</td>
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<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<td>CRM197</td>
<td>diphtheria toxin cross-reacting material 197</td>
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<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>DTH</td>
<td>delayed-type hypersensitivity</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>FAE</td>
<td>follicle-associated epithelium</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>GALT</td>
<td>gut-associated lymphoid tissue</td>
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<td>GM1</td>
<td>monosialoganglioside</td>
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<td>HSA</td>
<td>human serum albumin</td>
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<tr>
<td>HSAmP</td>
<td>HSA-conjugated starch microparticles</td>
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<tr>
<td>IEL</td>
<td>intraepithelial lymphocyte</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>IL</td>
<td>interleukin</td>
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<td>ISCOM</td>
<td>immunostimulating complex molecules</td>
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<tr>
<td>LT</td>
<td><em>E. coli</em> heat-labile enterotoxin</td>
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<tr>
<td>LTB</td>
<td>B subunit of <em>E. coli</em> heat-labile enterotoxin</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MLN</td>
<td>mesenteric lymph node</td>
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<td>NK</td>
<td>natural killer</td>
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<tr>
<td>OVA</td>
<td>ovalbumin</td>
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<td>PLG</td>
<td>polylactide-co-glycolide</td>
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<td>rCTB</td>
<td>recombinant cholera toxin B subunit</td>
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<td>rCTBmp</td>
<td>rCTB-conjugated starch microparticles</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>S.E.M</td>
<td>standard error of the mean</td>
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<tr>
<td>TCR</td>
<td>T-cell receptor</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>Th1</td>
<td>T helper cell of type 1</td>
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<tr>
<td>Th2</td>
<td>T helper cell of type 2</td>
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<tr>
<td>UEA</td>
<td><em>Ulex Europeaus</em> Agglutinin 1</td>
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1 Introduction

Conventional vaccines have been extremely successful in eradicating or significantly reducing the incidence of and morbidity associated with a large number of infectious diseases such as smallpox, polio and tuberculosis. Most of these conventional vaccines are based on live attenuated or inactivated whole pathogens that do not cause infection but are capable of inducing an immune response. They also include subunit vaccines containing detoxified toxins purified from toxin-secreting bacteria such as *Corynebacterium diphtheriae*. The aim of these vaccines is to induce antibodies against the pathogens or toxins and also to induce a cytotoxic response. Despite the success of this strategy, there are some major limitations. Firstly, there are some safety issues. Attenuated pathogens may cause infection in immunosuppressed individuals by reverting to a more virulent phenotype. Also, inactivated whole pathogens may cause unwanted side effects. Secondly, some pathogens are difficult or even impossible to culture, making the preparation of the vaccine problematic. Moreover, this strategy is not possible for vaccines against new types of vaccine targets such as allergy, autoimmune diseases and cancer. Therefore, together with growing regulatory pressure and advances in the biotechnology field, well-defined, safer molecular antigens have been developed.

However, these “molecular vaccines” are less immunogenic and require an adjuvant. Adjuvants (from Latin *adjuvare*, to help) are substances that, in combination with a specific antigen, produce a more robust immune response *in vivo* than if the antigen was administered alone. This broad definition of an adjuvant covers a variety of substances [1]. However, aluminium salts (alum) and MF59 (squalene o/w emulsion) are the only adjuvants licensed in Sweden for human use to date. Alum elicits a strong antibody response [2] but is not capable of inducing a cellular response, which is highly desirable for vaccines targeting viral diseases, infections linked to intracellular pathogens or cancer (therapeutic vaccines) [3]. These adjuvants are only intended for parenteral route, whereas the site of infection is often at the mucosal surfaces. It would thus be beneficial to develop an adjuvant for mucosal immunisation which would mimic the natural infection and activate an immune response at the site of infection. This thesis focuses on the use of starch microparticles as an oral adjuvant and investigates aspects that might affect the character of the induced immune response.
2 The immune system

The immune system is a complex network of differentiated immune cells and molecules that are tightly regulated to protect individuals from invasion by bacteria, viruses and other “foreign” materials. However, this brief review covers only those areas of the immune system of particular relevance to this thesis.

The immune system can be divided into the non-specific innate immune system and the specific acquired immune system. The innate immune system is the inborn system that restrains an infection until the acquired immune system can respond and clear it. The innate immune system consists of barrier functions at body surfaces (skin and mucous membranes), antibacterial substances (lysozyme, defensins) and immune cells such as phagocytes (monocytes/macrophages), polymorphonuclear cells (neutrophils, eosinophils and basophils) and natural killer (NK) cells. These cells recognise conserved molecular structures (pathogen-associated molecular patterns, PAMPs) from microbial pathogens, such as lipopolysaccharides (LPS) and polycytosine guanine (CpG) motifs, via pattern recognition receptors (PRRs) such as the toll-like receptors. Recognition triggers production of inflammatory signals that allow the innate immune system not only to respond directly, but also to instruct the adaptive immune system about the nature of the pathogenic invaders [4].

The adaptive immune system encompasses the antigen-specific immune response based on B and T lymphocytes. Key features of the adaptive immune system include antigen specificity, diversity, immunological memory and discrimination between self and non-self. There are two major branches of the adaptive immune response: humoral immunity and cell-mediated immunity; these work together against various invaders. The humoral immune response is mediated by B cells which, after recognition of invading microbes, mature and proliferate into antibody-secreting B cells (plasma cells). These antibodies bind to the invaders to prevent infection and facilitate elimination. Antibodies can also effectively neutralise toxins produced by pathogens such as C. diptheria and Clostridium tetani. However, a humoral immune response is not effective against intracellular pathogens [5].
The cellular immune response is mediated by different subtypes of T cells; the two major subtypes are T helper cells and cytotoxic T cells. Upon activation, T helper (CD4+) cells produce low molecular weight proteins known as cytokines which exert various effects, e.g. proliferation of B and T cells, on other immune cells; thus, CD4+ T cells regulate or 'help' other lymphocyte functions. Cytotoxic T (CD8+) cells, when activated, respond by killing infected cells or altered self-cells.

2.1 Antigen presentation

The T cell with its T-cell receptor (TCR) can only recognise antigenic peptides when they are presented on class I or II major histocompatibility complex (MHC) molecules (Figure 1). The function of class I MHC molecules, which are expressed on most nucleated cells, is restricted to the presentation of antigens to T cells that express CD8 on their surfaces, which will become cytotoxic T cells when activated. MHC class I molecules present endogenously produced antigens from for example normal cellular proteins, proteins from viruses that replicate in the cells or cancerous cells. These proteins are degraded into peptide fragments in the cytosol by the proteasome, transported to the endoplasmic reticulum, assembled with MHC class I molecules and subsequently presented on the cell surface.

MHC class II molecules are expressed on antigen-presenting cells (APC; e.g. macrophages, dendritic cells [DCs] and B cells) and present antigens to CD4-expressing T helper cells. Captured antigens (exogenous) are degraded into peptides in the endocytic pathway, assembled with MHC class II molecules transported from the Golgi complex and presented on the cell surface.

Figure 1. Schematic representation of antigen presentation. a) MHC class I molecules present endogenous antigens to CD8+ T cells which, after differentiation into effector cells, kill the antigen-presenting cell (APC). b) MHC class II molecules present exogenous antigens to CD4+ cells which, upon activation into effector cells, secrete various cytokines that, for example, activate macrophages or activate B cells to produce antibodies. TCR, T-cell receptor; MHC, major histocompatibility complex.
The activation of T cells requires several different signals. Firstly, the TCRs recognise and bind the antigen presented by class I or II MHC molecules on the APC. The co-receptor, CD4 or CD8, binds directly to the MHC molecules as a further boost to the signal for activation. Secondly, co-stimulatory signals that are nonspecific to the antigen are required. CD28 receptors, which are constitutively expressed on T cells, bind to B7.1 (CD80) and B7.2 (CD86) ligands induced by microbial products and inflammatory stimuli on APC. These cellular interactions stimulate the T cell to proliferate and produce cytokines essential for further differentiation of the immune response. However, if the APC lack the co-stimulatory molecules, T cells have been shown to become anergic (non-responding) [6].

2.1.1 Antigen-presenting cells

Macrophages, DCs and B cells are all professional APCs capable of capturing, processing and presenting antigens to T cells. The DCs are the major APCs and these cells display some unique properties. In the peripheral tissue, immature DCs reside as sentinels ready for signs of pathogen invasion. In their immature state they are characterised by a high capacity for capturing antigens [7]. The process of capture is undertaken by phagocytosis, macropinocytosis, and receptor-mediated endocytosis [8]. This enables the DC to capture and present a wide range of materials such as soluble antigens, particles, apoptotic cells and bacteria. Also, DCs seem to be able to distinguish between inert particles and bacteria and to alter their phagocytic capacity in response to this [9]. After microbial or inflammatory stimulation, the DCs start to mature, often while migrating to the T-cell area of the lymphoid organs; this involves changing from antigen-capturing mode to T-cell stimulation mode [10]. These migratory and functional properties are hallmarks of the DCs that have not been described for other APCs [11].

Cells from the dendritic family share phenotypic and functional properties; however, the expression of certain cell–surface markers is distinctly heterogeneous, dividing the DCs into various subsets. It is not clear whether the differences in surface expression represent different cell lineages or different states of maturation. However, the subsets differ in their location, phagocytic capacity and response to different stimuli [11, 12].

2.2 Differentiation of the immune response

The induction of both humoral and cellular immune responses depends on the activation of T helper cells, as these cells, through their secretion of cy-
tokines, are the conductors of the immune response. The general opinion is that there are two main types of T helper cells: type 1 (Th1) and type 2 (Th2); these are subdivided on the basis of the cytokines they produce [13] (Figure 2). Activated Th1 cells mainly produce interleukin (IL)-2 and interferon (IFN)-γ, which stimulate cell-mediated immunity such as delayed-type hypersensitivity (DTH) [14] and activation of macrophages, cytotoxic T cells and inflammatory responses. In addition, IFN-γ also stimulates immunoglobulin (Ig) G2a production by B cells [15]. Th2 cells produce cytokines (IL-4, IL-5, IL-6, IL-10, IL-13) that activate humoral immunity (IgE and IgG1), eosinophils and mast cells. The cytokines produced by the two T helper subtypes acts as autocrine growth factors amplifying the expansion of the respective subtype [16]. Moreover, the subtypes cross-regulate each other’s development, as IFN-γ inhibits Th2 expansion [17, 18] and IL-10 inhibits Th1 expansion [19, 20].

**Figure 2.** Schematic representation of the differentiation of T helper cells. The Th1 cells mediate the cellular immune response involved in destruction of intracellular pathogens (viruses, parasites and intracellular bacteria) and organ-specific autoimmunity. Th2 cells are antihelminthic and increase allergic reactions [21].

It is generally considered that the two subtypes develop from a common precursor; the most clearly defined factor determining development into one of the T helper cell subtypes is the cytokine microenvironment present during the initiation of the immune response. IL-12, produced mainly by macrophages and DCs, is the Th1-inducing cytokine, while IL-4 is important for the development of a Th2 response. The main cellular sources responsible for the initial burst of IL-4 are not completely understood. However, NK cells, mast cells, basophils, eosinophils and mature CD4+ T cells are good
candidates [21]. Other factors suggested to be of importance for the differentiation of Th1 and Th2 cells are the amount and nature of the antigen, the presence of adjuvants, the genetic background of the host, the duration and strength of the co-stimulatory signals, and the route of entry of the antigen/micro-organism [22-24]. Interestingly, DCs induce different T helper cell responses according to their distinct microenvironment. DCs from Peyer’s patches in the intestine and those from the respiratory tract prime a Th2 response, whereas DCs from the spleen induce a Th1 response [25, 26]. These observations may explain why the route of entry of the antigen/micro-organism affects the differentiation of the T helper cells.

2.3 Mucosal immune system

The mucosal surface of the human body (gastrointestinal, respiratory and urogenital tracts) has a combined area of at least 400 m² and, with the skin, separates the inner and outer environments of the body. This makes the mucosal surface the major site of entry of various pathogens. However, unlike skin, the mucous membranes are associated with the exchange of materials such as gases in the respiratory tract and nutrients in the gastrointestinal tract. Also, the gastrointestinal mucosa is in constant contact with commensal bacteria that colonise the mucosa in a symbiotic relationship. Thus, the immune system associated with the mucosal surfaces requires tight regulation so as to offer protection from pathogens while simultaneously allowing the absorption of nutrients.

The mucosal immune system consists of different types of organised mucosal-associated lymphoid tissues (O-MALT). Structurally, these tissues range from loose clusters of lymphoid cells scattered in the mucosal lining to highly organised lymphoid structures such as the tonsils, appendix and Peyer’s patches. These structures function as inductive sites for the mucosal immune response; together with the effector sites, they form a common mucosal immune system [27, 28].

2.3.1 Induction site of gut associated lymphoid tissue

In the gut-associated lymphoid tissue (GALT), the main inductive sites are the Peyer’s patches and the main effector site is the lamina propria [29] (Figure 3). Peyer’s patches are clusters of lymphoid follicles, each covered by a unique epithelium known as the follicle-associated epithelium (FAE). This epithelium differs from the general epithelium in that it has reduced levels of membrane-associated hydrolases, no polymeric Ig receptors, and no
or only few goblet cells and endocrine cells [30, 31]. These factors lead to less mucus production, which facilitates closer contact with the luminary antigens, and minimised digestion of the antigens. Most importantly, the FAE exhibits M cells specialised in antigen sampling. The apical surfaces of M cells lack highly developed brush borders and a thick glycocalyx. In addition, the basolateral surfaces of M cells are invaginated to form a “pocket” containing B and T lymphocytes, macrophages and occasional DCs. The short distance between the apical and basolateral membranes of the cells favours transepithelial transport. Since M cells have low levels of lysosomes, they are thought to transport the antigens more or less intact to the underlying lymphoid tissue. M cells transport not only soluble peptide antigens but also macromolecules, particles and even entire micro-organisms [32-34].

After uptake through the M cells (Figure 3), the antigens are processed and presented by APCs to trigger an immune response. Immature DCs underneath the FAE migrate after capturing antigen to T-cell enriched interfollicular regions of the Peyer’s patches [35, 36]. The microenvironment in the Peyer’s patches is more likely than organs such as the spleen to induce a Th2 type of response. DCs isolated from Peyer’s patches appear to have a particular propensity for producing IL-10 and priming T cells to produce IL-4 and IL-10 (Th2 cytokines) and IFN-γ, whereas spleen DCs are primed predominantly for IFN-γ secretion [26].

Three different subtypes of DCs, based on their surface markers CD11b and CD8α, have been found in the Peyer’s patches. These cells differ according to their location and in their response to stimuli [35-38]. Thus, DCs that secrete IL-10 and induce a Th2 response are restricted to myeloid subsets (CD11b+). These cells also appear to mediate suppressive T-cell responses by secreting high levels of IL-10 and possibly transforming growth factor (TGF)-β upon encountering innocuous antigens (food antigens). In contrast, the lymphoid (CD8α+) and double negative (CD11b−/CD8α−) DCs both secrete IL-12 and induce Th1 cells [36, 38].

DCs also offer an alternative pathway for antigen uptake across the epithelium (Figure 3). In response to bacterial components, such as flagellin from Salmonella, epithelial cells in the intestine produce chemokines that attract immune cells, particularly DCs, into the gut mucosa [39]. These DCs send out dendrites between the epithelial cells into the gut lumen where they sample microbial antigens directly from the lumen; they then migrate to the lymphoid follicles where they present the antigens to B and T cells [40]. Absorptive enterocytes can also take up antigens and present them to intraepithelial cells. However, it has been proposed that they induce local oral tolerance rather than activate naïve T cells, as they constitutively express classical and non-classical MHC molecules, but they lack co-stimulatory
signals and secrete immunosuppressive cytokines, including IL-10 and TGF-β [41].

Figure 3. Schematic representation of proposed mechanisms for induction of an immune response at the intestinal mucosa. The magnification of the follicle-associated epithelium (FAE) shows an M cell with pathogens or particles entering from the gut lumen. DC, dendritic cells; encircled B, B cells; encircled T, T cells; IELs, intraepithelial lymphocytes; MLN, mesenteric lymph node [29, 42].

Antigen-sensitised T and B cells in the Peyer’s patches or mesenteric lymph nodes (MLN) migrate into the bloodstream via the thoracic duct and thence to their effector site, the lamina propria. The lymphocytes preferentially migrate back to the mucosal tissue where they were initially activated (homing) [43]. This homing occurs by the binding of specific surface molecules on the lymphocytes to molecules expressed in the mucosal tissue. The homing receptors that are expressed on their surfaces thus reflect the mucosal site at which the cells were activated. After mucosal immunisation, nearly all IgA and IgG antibody-secreting cells express α4β7 integrin, the mucosal homing receptor, whereas only a few of these cells express L-selectin, the peripheral homing receptor [44-46].
2.3.2 Effector site of gut associated lymphoid tissue

IgA antibodies play a major role in the protection of mucosal surfaces. These antibodies are secreted into the gut lumen where they bind to the pathogens, thus limiting their adherence to the epithelium and reducing colonisation rates [47].

The microenvironment in the germinal centres of the Peyer’s patches, especially the presence of TGF-β [48, 49], is instrumental in stimulating B cells to express surface IgA [50, 51]. These IgA+ B cells migrate to and enter the lamina propria, where terminal differentiation into IgA-producing plasma cells occurs. Maturation into plasma cells is enhanced by cytokines such as IL-5, IL-6 and IL-10 [52-55]; IL-6 appears to be particularly important in this respect [56]. These cytokines are distributed throughout the lamina propria, which supports the production of IgA [57, 58].

B cells in the lamina propria produce dimeric IgA joined by a peptide called the J-chain; this dimer binds to polymeric Ig receptors on the apical side of epithelial cells. After binding, the receptor-IgA complex is transported through the epithelial cell into the lumen. The polymeric Ig receptors are enzymatically cleaved but part of the receptor, the secretory component, remains bound to the IgA dimer. The secretory component masks sites susceptible to proteases and thereby stabilises the secretory IgA molecule (s-IgA) in the harsh environment of the mucosal secretions [59].

Another important “first line” of the mucosal surface defence system is the presence of intraepithelial lymphocytes (IELs) (Figure 3). These lymphocytes are interspersed within the epithelial cells and are found mainly in the villi of the small intestine. IELs are predominantly T cells and most of them are CD8+ cells [60]. These are thought to be important cytotoxic cells that can eliminate virus-infected epithelial cells [61].
In the field of vaccine research, it would be desirable to find vaccination strategies that are capable of inducing an effective immune response against pathogens at both systemic and mucosal levels. Parenteral immunisation does not generally induce a mucosal immune response in the form of s-IgA. Rather, positioning of the antigens at the mucosal surface is believed to be necessary for induction of a successful mucosal immune response [62, 63]. Further, immunisation at one mucosal surface can induce an immune response at distant mucosal surfaces [27, 28]. The explanation for this phenomenon is the common mucosal immune system which governs the interplay between the induction site and the effector site, as discussed earlier. However, the immune response at the mucosal effector sites is not uniform. Generally, immune responses are stronger at nearby mucosal effector sites, or at sites related in lymphoid drainage [64]. It is important to understand the compartmentalisation of the common mucosa before deciding on the route of immunisation in rational vaccine formulation research. The oral and nasal routes of immunisation are the most extensively studied for mucosal immunisation. Other mucosal sites such as those reached by tracheal, rectal and vaginal routes could be chosen under certain circumstances.

The focus of this thesis is the oral delivery of antigens to mucosal sites. This route of administration offers several advantages in addition to the immunological ones: it is easy to administer, has a low cost, and has potential to increase patient comfort and compliance. On the other hand, the oral route is also associated with some challenges: i) the hostile environment in the gut, ii) the poor uptake of vaccines over the intestinal epithelium and iii) the generally poor immunogenicity of orally delivered antigens. Thus, oral vaccines will require not only effective delivery across epithelial barriers, but will also require the use of strategies that trigger a protective immune response in the absence of undesired inflammatory reactions. The fact that there are only a few commercial oral vaccines on the market may be a reflection of these difficulties. Live, attenuated whole-cell oral vaccines against bacterial infections such as those caused by *S. typhi* and *Vibrio cholerae* have been registered [65]. Whole-cell vaccines, however, as discussed earlier, are associated with safety problems.
3.1 Mucosal adjuvants

The mechanisms of action for most adjuvants are still poorly understood, as immunisation often activates a cascade of reactions. During recent years, however, basic immunological research, the need for secure well-defined and effective vaccines, and the search for novel adjuvants for mucosal vaccination have lead to an interest in theoretical and mechanistic studies of adjuvants and vaccines. Subsequently, mucosal adjuvants can now be broadly classified into two categories according to their actions: immune potentiators and antigen delivery systems [66].

The immune potentiators are compounds that directly activate immune cells through specific receptors and/or cytokine pathways [66]; these include the toxin-based, cytokine-based and innate immunity-associated adjuvants. Examples from this category are cholera toxin (CT) and *E.coli* heat-labile enterotoxin (LT), which are the most potent oral adjuvants recognised to date. In particular, CT and LT effectively elicit a strong mucosal immune response but also greatly enhance systemic immunity to co-administered protein antigens [67]. Structurally, these toxins consist of two subunits: the toxic A subunit, which activates the adenylate cyclase system, and a non-toxic pentameric B subunit (known as CTB and LTB, respectively), which is responsible for binding to the monosialoganglioside GM1 receptors present on all nucleated cells. The use of the toxins in humans are, however, limited by their high toxicity [68]. In order to overcome this problem, site-directed mutagenesis has been performed on the toxins. This generates CT and LT mutants which have reduced toxicity but which retain significant adjuvant activity in animals when given by the nasal route and also, although to a lesser extent, by the oral route [69].

Antigen delivery systems are intended to specifically locate antigens at induction sites and target them to key immune cells such as APCs. The starch microparticles studied in this thesis belong to this category; these are described in more detail in the next section. However, a short summary of some examples of other vaccine delivery systems researched for oral vaccination is presented below.

*ISCOMs* (immunostimulating complex molecules) are about 40 nm cage-like structures that form spontaneously when mixing saponin Quil-A, which also has immunostimulatory properties [70], with cholesterol [71]. The formation of the ISCOM structure and the incorporation of antigens are mainly mediated by hydrophobic interactions. Oral immunisation with ISCOMs containing the model antigen ovalbumin (OVA) induces a wide range of mucosal and systemic immune responses [72]. In addition, ISCOMs are generally
considered to be an effective adjuvant for stimulation of a cytotoxic T lymphocytes (CTL) [73].

**Liposomes** are uni-and multilaminar structures of phospholipid bilayers that close up to form vesicles. The size of the liposomes varies from 50 nm to several micrometers. Hydrophilic antigens can be incorporated inside the liposomal structure and amphiphilic antigens can be associated in the bilayers [74]. Conventional liposomes are sensitive to degradation in the gastrointestinal tract. However, by optimising the phospholipid content, incorporating derivates of polysaccharides or cross-linking the lipids in the liposome membrane, stable liposomes can be prepared for oral immunisation [75].

**Polylactide-co-glycolide (PLG) microparticles** are biodegradable microparticles prepared using different emulsion techniques [76]. For oral immunisation, the antigens are entrapped within the polymer particle and thus protected from degradation in the gastrointestinal tract. PLG microparticles induce both systemic and mucosal humoral responses as well as CTL to the entrapped antigen after oral immunisation [77].

### 3.1.1 Polyacryl starch microparticles

Polyacryl starch microparticles were developed by Prof. Sjöholm’s research group; detailed information is available from several reviews [78-82].

Polyacryl starch microparticles are prepared from hydrolysed starch (Maltodextrin, MD6, Mw 5000), which is acryloylated by allowing glycidyl acrylate to react with the starch. This occurs mainly at the primary hydroxyl groups in position 6 of the glucose units of the starch and the degree of derivatisation, defined as the number of acryloyl groups per glucose residue [83], usually lies between 0.13 and 0.14 for the starch used in this thesis.

The particles are formed by radical polymerisation of acrylic groups using ammonium peroxodisulfate and N,N,N’,N’-tetramethylethylene diamine (TEMED) as initiators in a water-in-oil emulsion [84, 85] (Figure 4). The size of the particles is determined using a laser diffractometer (LS 230; Coulter Counter); the particles used in this thesis had a diameter of about 2-3 µm based on number distribution.

The antigens are covalently conjugated to the microparticles using Bethell’s carbodiimide method [86]. The free hydroxyl groups on the glucose residues of the starch are activated with carbonyldiimidazol (CDI) and reacted with free amino groups on the antigen to be associated. The particles have a highly porous structure and the conjugated antigens may subsequently be
found throughout the particles [87]. The conjugation of the antigens to the particles offers protection from degradation in the gastrointestinal tract and the cross links between hydrocarbons formed during preparation of the particles slows down the degradation of starch by hydrolases.

Figure 4. Preparation of starch microparticles and conjugation of antigens (courtesy of Niclas Rydell). CDI, carbonyldiimidazol; TEMED, N,N,N',N'-tetramethyl-ethylenediamine.

After intravenous injection, the particles are distributed throughout the reticuloendothelial system [85], finally ending up in the lysosomal vacuomes of the Kupffer cells in the liver [88, 89]. Moreover, starch microparticles have a weak stimulatory effect on macrophages, which stimulates IL-1 [90] and H$_2$O$_2$ [91] production. The starch microparticles have adjuvant properties after oral and parenteral administration in mice. Oral immunisation with the model antigen human serum albumin (HSA) conjugated to the microparticles induced strong local and systemic immune responses in mice [92]. A similar response and protection from infection were also seen with conjugated antigens from *Mycobacterium tuberculosis* and *S. enteritidis* [93, 94]. However, the particles themselves are not immunogenic [95].
4 Aims of the thesis

The overall objectives of this thesis were to study different aspects of the uptake of starch microparticles and their presentation to the immune system in correlation with differences in the induced immune response (Th1/Th2 balance).

*The specific aims were to:*

- Investigate the effects of changing the route of administration on the differentiation of the immune response.

- Study the binding and uptake in the mouse intestine of two antigens with different binding properties after conjugation to starch microparticles.

- Compare the immune response induced after immunisation with these antigen-conjugated starch microparticles with different binding properties.

- Study the consequences of stress-induced alterations of the mucosal epithelium of the intestine on oral immunisation with starch microparticles.

- Evaluate the use of starch microparticles as an oral adjuvant in man.
5 Results and discussion

5.1 The route of administration influences the immune response

When designing a vaccine strategy for optimal protection it is important to know how the differentiation (Th1/Th2 balance) of the immune response is affected by factors such as the choice of adjuvant, the route of administration and the dose or number of doses. The aim of paper I in this thesis was to characterise the immune response induced after oral and parenteral immunisation with starch microparticles as an adjuvant in immunisation schemes in which the route of administration was changed from oral to parenteral and vice versa. HSA was chosen as a fairly neutral model antigen in this respect i.e. one that is unlikely to send out specific danger signals, as seen with pathogen components which can polarise the immune response towards a Th1 or Th2 response [96]. Three sets of immunisation studies were performed with HSA conjugated to microparticles (HSAm) in mice. The experimental processes are outlined in Figures 5a, b and c.

All groups immunised with HSAm responded with a strong HSA-specific IgM+IgG response with essentially the same final titres. However, in experiment 1, the antibody response in the group that received only oral doses was slower in onset.
5.1.1 The influence on the Th1/Th2 balance

The subclass profile of the humoral IgG response, i.e. HSA-specific IgG1, IgG2a and IgG2b expressed as a ratio (IgG1/IgG2a+IgG2b), was used as a measure of the Th1/Th2 balance. All groups showed a mixed response, with IgG1 dominating (Table 1). The route of administration affected the Th1/Th2 balance. In experiment 2, comparing oral and intramuscular immunisations, it was found that by the end of the immunisation programme the primary oral immunisation increased the influence of the Th2 response whereas oral booster doses lowered the ratios, indicating a higher Th1 influence. Also, a trend toward a higher DTH response in the group boosted orally was observed, which indicates a higher Th1 response [14].

Oral and subcutaneous immunisations (experiment 1) had similar effects on the Th1/Th2 balance, as indicated by the subclass ratio (Table 1) and cytokine analyses. This apparent difference between the effects of subcutaneous and intramuscular administration in experiments 1 and 2 was confirmed in experiment 3. The subclass ratio was higher after subcutaneous immunisation than after intramuscular immunisation (Table 1). In support of this, the IgE response was higher in the group immunised subcutaneously than in the intramuscular group, indicating a higher Th2 response since the IgE response.
is co-regulated with IgG1 via the Th2/IL-4 pathway [97]. Thus, the response to subcutaneous immunisation with HSAmp is more Th2 oriented than the response to intramuscular immunisation.

Table 1. The subclass ratio in serum after oral (or), intramuscular (im) or subcutaneous (sc) immunisation with HSAmp according to Figure 5a-c.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Immunisation</th>
<th>Mean subclass ratio ± S.E.M&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 55</td>
<td>Day 76</td>
</tr>
<tr>
<td>1</td>
<td>or-or-or-or&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.1 ± 9.9 (n=6)</td>
</tr>
<tr>
<td></td>
<td>sc-sc-sc-sc&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.0 ± 2.3 (n=5)</td>
</tr>
<tr>
<td></td>
<td>sc-sc-or-or&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18.6 ± 5.0 (n=6)</td>
</tr>
<tr>
<td>2</td>
<td>im-im-im</td>
<td>82.2 ± 23.2 (n=6)</td>
</tr>
<tr>
<td></td>
<td>im-or-or&lt;sup&gt;e&lt;/sup&gt;</td>
<td>24.8 ± 4.0 (n=8)</td>
</tr>
<tr>
<td></td>
<td>or-or-or&lt;sup&gt;e&lt;/sup&gt;</td>
<td>147.6 ± 48.1 (n=7)</td>
</tr>
<tr>
<td></td>
<td>or-im-im&lt;sup&gt;e&lt;/sup&gt;</td>
<td>480.8 ± 213.3 (n=7)</td>
</tr>
<tr>
<td>3</td>
<td>im-im-im&lt;sup&gt;e&lt;/sup&gt;</td>
<td>7.7 ± 2.0 (n=6)</td>
</tr>
<tr>
<td></td>
<td>sc-sc-sc&lt;sup&gt;e&lt;/sup&gt;</td>
<td>56.2 ± 18.3 (n=6)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values represent arithmetic means (± S.E.M) of serum specific ratios of IgG1/(IgG2a+IgG2b) at the end of the immunisation programme. <sup>b</sup> One value was an outlier and was excluded. <sup>c</sup> The ratio was significantly higher in the or-or-or group than in the im-or-or group (P<0.05). Primary oral immunisation resulted in a higher ratio (P<0.0001) than primary intramuscular immunisation, while the ratio was lower with an oral booster (P>0.05) than with an intramuscular booster. <sup>d</sup> The ratio was significantly higher in the or-im-im group than in the im-im-im (P<0.005) or im-or-or (P<0.001) groups. <sup>e</sup> The ratio was significantly higher in the sc-sc-sc group than in the im-im-im group (experiment 3) (P<0.0001). Statistical analyses were performed using logarithmically transformed values; for details see Section 2 in Paper I

These results show that the profile of the immune response obtained with starch microparticles as an adjuvant is dependent on the route of administration. In general, it seems that the immunological profile with oral immunisation is closer to that with subcutaneous than with intramuscular immunisation. The mechanisms governing the activation of T helper subsets appear to be quite complex and are not completely understood. As mentioned previously, it has been established that the differentiation of T helper cells into Th1 or Th2 phenotypes is largely controlled by the action of cytokines, where a Th1 or Th2 response is promoted by IL-12 and IL-4, respectively [21]. Thus, differentiation of the T cells is ultimately dependent on factors influencing which cytokines are present during antigen presentation, such as the type of APC and the distinct microenvironment in which the antigen presentation takes place. It is therefore not surprising that the route of administration affects the differentiation of the T cells. After oral administration, the antigen will most likely be presented in the Peyer’s patches or MLNs. Moreover, with intramuscular immunisation in the mouse hind leg,
APC would drain to local lymph nodes distinct from those in the subcutaneous tissue in the scruff of the neck. Thus, it is possible that antigens delivered by different routes of immunisation would encounter different types of APC which could lead to differences in antigen processing and presentation in the distinct microenvironment of the different lymph nodes.

The IgG subclasses in serum showed a mixed Th1 and Th2 response with a greater Th2 influence in all groups. On the other hand, the DTH assay indicated a strong Th1 influence in all groups tested. This dual Th1/Th2 response may have occurred because of the ability of HSA to give rise to different peptides upon degradation, with subsequent distinct abilities to induce either a Th1 or Th2 response. Several studies have demonstrated that different routes of immunisation can induce different patterns of T and B cell epitope recognition [98, 99]. Todryk et al. demonstrated that the route of immunisation (subcutaneous, intraperitoneal, oral) of a streptococcal antigen affected both T and B cell epitope recognition, when the effect of extracellular degradation in the different compartments was taken into consideration [100]. In our study, the route of administration probably affected the degradation pattern of the HSAmp, which could perhaps be one of the reasons for the differences seen in the T helper cell profile.

5.1.2 The influence on the local s-IgA response

The most obvious difference in outcome among the dosage regimens studied was the development of local s-IgA. Only mice that received oral booster doses with HSAMP had a significant s-IgA response and the mice receiving all immunisations orally had the highest s-IgA response (Figure 6). Corroborative results were seen in experiment 2. Only low amounts of s-IgA were detected after subcutaneous and intramuscular immunisation, which did not increase over time. The lack of synergistic effects of combining parenteral and oral immunisation in both the local s-IgA and systemic IgM+IgG responses was surprising considering that several reports have shown that using different routes of administration can synergistically broaden and quantitatively improve the immune response [101-103].
Figure 6. HSA-specific IgA in mouse faeces after the third booster according to Figure 5a. Values are given as means in µg/mg total IgA (± S.E.M). The group immunised orally (or-or-or-or) responded with significantly higher quotas than the other groups over time (P<0.05 and < 0.0001, respectively).

5.2 Recombinant cholera toxin B subunit (rCTB) conjugated to the microparticles

The non-toxic B subunits of CT and LT, CTB and LTB, have both been used as carriers to target soluble antigens to the mucosal surfaces so as to induce an immune response [104-107]. While the B subunits possess immunomodulating properties, the mechanisms for this capacity have not been fully elucidated. Nonetheless, the B subunits have been shown to (i) increase antigen presentation by APCs by promoting expression of MHC class II and co-stimulatory signals [108-112], (ii) increase CD4+ T cell activation [113] and (iii) induce apoptosis of CD8+ T cells [114].

In papers II and III, the effects of conjugating rCTB to the starch microparticles were evaluated. Both the targeting function to the intestinal epithelium and the immunomodulating properties were addressed.

5.2.1 Uptake of the microparticles over the intestinal epithelium

It is apparent that starch microparticles can deliver antigens to the APC in the GALT in such a way that an immune response is induced. Therefore the antigen-particle complex or fragments thereof must somehow pass through the intestinal epithelium. However, basic understanding of the mechanisms involved in the uptake of the starch microparticles in the gastrointestinal tract and the subsequent development of the immune response is poor. The
aim of paper II was, therefore, to study the uptake of the antigen-conjugated starch microparticles by the intestinal mucosa using two different antigens, HSA and rCTB. HSA was chosen as a neutral antigen with no known specific binding properties and rCTB was chosen for its binding properties to the GM1 receptors.

It seemed preferable to study the uptake of the particles in vivo after intragastric feeding, as is the case with immunisation. However, when doing so, only a few particles were found in the lumen of the intestine and none were found in the tissue. Therefore, we decided to study the uptake of the particles after injecting them into mouse intestinal loops. The intestinal loops, each containing one Peyer’s patch, were excised and fixed in ice-cold 95% ethanol after exposure to the particles. Entire specimens were mounted, exposed to fluorescence-labelled reagents staining the cytoskeleton, the particles and/or M cells, and examined in a confocal laser-scanning microscope (CLSM); further details are provided in paper II.

Four potential routes for particle uptake from the gastrointestinal lumen have been identified: (1) via the M cells, (2) via transcellular routes involving enterocytes, (3) by paracellular means (reviewed in e.g. [115, 116]) or (4) by the aid of DCs [40]. The main pathway for particle uptake is considered to be through the M cells, as these cells possess special features facilitating uptake of particulate matter, as described earlier. In our study, HSAmp was taken up over the FAE (Figure 7a), most likely by the M cells. rCTB-conjugated microparticles (rCTBmp), on the other hand, were taken up over both the FAE (Figure 7b) and the villus epithelium (Figure 7c). It was not possible to make

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**Figure 7.** Starch microparticles in intestinal mucosa after 40 ± 5 min exposure of particle suspension in mice intestinal loops. a) HSA-conjugated microparticle in a follicle. rCTB-conjugated microparticles in b) a follicle and c) a villus. The specimens were stained after fixation in ice-cold ethanol with rhodamine phalloidine (red) and fluorescein isothiocyanate (FITC)- conjugated anti-HSA or anti-rCTB antibodies (green).

**Figure 8.** a) A follicle, with surrounding villi, in a Peyer’s patch from a mouse intestinal loop. The specimen was stained with rhodamine phalloidine (red) and FITC-conjugated UEA-1 (green), which binds preferentially to mouse M cells and also the mucus of goblet cells or the granules of Paneth cells. b) Alexa Fluor 647-labelled rCTB-conjugated microparticles (blue) in or in close contact with M cells (green) of a follicle after administration into a mouse intestinal loop.
Figure 7.

Figure 8.
a quantitative determination of the total number of different particles taken up in, for example, a follicle of a Peyer’s patch using whole mounted specimens. However, a clear qualitative difference was found. Thus, the conjugated antigen did influence the uptake of the particles. In order to confirm that the M cells were responsible for the uptake of the particles, M cells were labelled with fluorescence-labelled lectin Ulex Europaeus Agglutinin 1 (UEA-1) [117] (Figure 8a). Labelled rCTBmp were found in or attached to M cells of the follicles as demonstrated in Figure 8b, while no microparticles were found in or on epithelial cells.

The uptake of microparticles in the FAE is not surprising and correlates well with studies done on other particle systems. For example, in studies of rabbit intestinal loops, it was found that PLG particles ranging in size from 1 to 4 µm were taken up by the M cells, although the samples applied in this study included both large and small particles [118]. This suggests that the starch microparticles used in our study are a suitable size (about 2 µm in diameter) for M cell uptake and antigen delivery to the GALT.

Two possible theories can be put forward for the uptake of rCTBmp in the villus epithelium. The uptake of the microparticles may have been due to rCTBs binding to GM1 receptors, thus facilitating the uptake process, or to DCs recruited to the intestine penetrating the epithelium and sampling the rCTBmp directly from the lumen. However, it seems unlikely that rCTBmp and not HSAmp would be able to attract DCs to the intestinal lining and facilitate uptake as bacteria do. Also, in support of the first theory, enzyme-linked immunosorbent assay (ELISA) analyses showed that non-labelled rCTB is present on the surface of the particles and this can bind to GM1 receptors in the wells of the ELISA plates, whereas labelling with the dye considerably lowered the efficiency of the receptor interaction (Figure 9).

![Figure 9](image.png)

*Figure 9. Displacement in ELISA with rCTB-conjugated microparticles (rCTBmp) and biotin-labelled CTB. The rCTBmp or Alexa Fluor 647-labelled rCTBmp (Alexa rCTBmp) were added in different concentrations to plates coated with GM1 receptors. Biotin-labelled CTB was added to competitively displace the microparticles. The values are given as arithmetic means for triplicates ± SD.*
Thus, our results indicate that rCTB conjugated to the starch microparticles binds to the GM1 receptor in vitro and that CTB also mediates the binding of starch microparticles to the receptor in vivo. These results might be regarded as being in conflict with the results obtained by Frey et al., who showed that polystyrene particles in the µm range coated with CTB failed to adhere to either enterocytes or M cells in vivo owing to the barrier function of the intestinal cell glycocalyx [119]. However, the microparticles used here are made of starch with a porous structure and a very rough surface with protrusions [87] that may have facilitated an interaction between rCTB and the GM1 receptors.

5.2.2 Effects on the immune response after oral immunisation

As pointed out earlier, the microenvironment could influence the profile of the immune response and, therefore, the qualitative difference in uptake of the microparticles between the villus epithelium and the FAE (Figure 7) after oral administration could have had consequences for the character of the induced immune response. Moreover, one could speculate that varying mechanisms of uptake and interaction with the epithelium could also affect the differential inducement of subepithelial cytokine pathways and subsequent antigen processing. This was recently shown by Grdic et al. [120], who studied the immune response induced by OVA plus two different oral adjuvants which worked in completely different ways: the ISCOM carrier system and CT. While ISCOMs are probably taken up by non-specific mechanisms, CT is bound to the GM1 receptor. Both adjuvants improved the immune response but it was clearly shown that while OVA-ISCOM combinations induce the production of IL-12, the effects of CT were not dependent on this cytokine [120].

In order to study the possible consequences of these differences in mucosal uptake, rCTB was conjugated with HSA on the same particles (HSA/rCTBmp) as well as on separate particles given concomitantly (HSAmp + rCTBmp). The induced immune responses were compared with the response induced by immunisation with either HSAm or rCTBmp according to table 2. However, there were no differences between the immunised groups after oral immunisation, either quantitatively as assayed by IgM+IgG levels, the s-IgA response and the DTH response, or qualitatively by studying the IgG subclass profile. Thus, the differences in uptake and the presence of rCTB did not influence the immune response to HSA after oral immunisation. Our findings were unexpected; we had anticipated that the addition of rCTB to the HSAm would increase the number of binding events and therefore increase the uptake through both the Peyer’s patches and the villus epithelium, resulting in increases in the local and systemic
immune response. Some conclusions from this study may, however, be proposed.

Table 2. Immunisation protocol of various formulations of antigen-conjugated microparticles in mice

<table>
<thead>
<tr>
<th>Group/treatment</th>
<th>Immunisation route</th>
<th>Dose (mg mp)</th>
<th>HSA (µg/mg mp)</th>
<th>rCTB (µg/mg mp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>62, 62, 62, 81</td>
<td></td>
</tr>
<tr>
<td>HSAmp or 1</td>
<td></td>
<td>1.5</td>
<td>- - - -</td>
<td>34, 34, 34, 24</td>
</tr>
<tr>
<td>rCTBmp or 1</td>
<td></td>
<td>1.5</td>
<td>65, 65, 53, 53</td>
<td>28, 28, 25, 25</td>
</tr>
<tr>
<td>HSA/rCTBmp or 1</td>
<td></td>
<td>1.5 + 1.5</td>
<td>62, 62, 62, 62</td>
<td>34, 34, 24, 24</td>
</tr>
<tr>
<td>HSAmp or 0.5</td>
<td>s.c.</td>
<td>0.5</td>
<td>68, 68, 68</td>
<td></td>
</tr>
<tr>
<td>rCTBmp or 0.5</td>
<td>s.c.</td>
<td>0.5</td>
<td>- - - -</td>
<td>24, 24, 24 -</td>
</tr>
<tr>
<td>HSA/rCTBmp or 0.5</td>
<td>s.c.</td>
<td>0.5</td>
<td>39, 39, 39 -</td>
<td>25, 25, 25 -</td>
</tr>
<tr>
<td>HSAmp + rCTBmp or 0.5</td>
<td>s.c.</td>
<td>0.5 + 0.5</td>
<td>68, 68, 68 -</td>
<td>24, 24, 24 -</td>
</tr>
</tbody>
</table>

* The or immunisations were given in series of 3 consecutive days; s.c immunisations were given on the first day in each respective series. Each immunisation was given three weeks apart. mp, microparticles; or, oral route; s.c., subcutaneous route; HSAmp, HSA-conjugated mp; rCTBmp, rCTB-conjugated mp; HSA/rCTBmp, HSA and rCTB-conjugated mp; HSAmp+rCTBmp, mixture of HSAmp and rCTBmp given concomitantly.

Particles taken up in the villus epithelium may be processed and presented to adjacent lymphocytes: antigens absorbed apically in intestinal epithelial cell lines are processed and presented on the basal surface to CD4+ T cells [121]. However, as mentioned before, enterocytes express MHC class II but do not normally express co-stimulatory molecules required for T cell activation [122, 123] and it has been suggested, therefore, that they induce oral tolerance rather than an immune response. Alternatively, the antigen-particle complex or fragments thereof may be transported across the epithelial cells and taken up by DCs in the lamina propria, which subsequently migrate to the MLNs where antigen presentation would take place. However, since the immune response was not improved, suppressed or qualitatively altered, it is more likely that uptake through the villus resulted in local suppression of the immune response in the villi lamina propria, an effect possibly mediated by down regulating T cells secreting IL-10 and/or TGF-β [124]. Alternatively, the antigen-particle complex may have been hydrolysed and degraded within the lysosomal compartments of the enterocytes, thus losing the immunoreactive epitopes and becoming unable to induce an immune response.

Nevertheless, one would have expected an increase in the immune response as a consequence of increased uptake in the Peyer’s patches. This has been shown with other systems; for example, conjugation of rCTB to liposomes [125] and bilosomes [126] improved the immune response after oral immu-
organisation, probably by targeted delivery to the GM1 receptor on the M cells. Our study shows that even if the addition of rCTB to the particles did increase their binding to the GM1 receptors, the immune response to HSA was not improved. Although the amount of particles delivered to the intestine is an important factor for the immune response, the dose-response curve is not very steep [92]. A 10-fold increase in the dose of the particles only slightly increased the antigen-specific humoral response. Thus, targeting the particles to GM1 receptors appears not to be sufficient to promote a detectable increase in the immune response.

5.2.3 Effects on the immune response after s.c. immunisation

The immunomodulating properties of CTB were also investigated after subcutaneous immunisation, studying sets of particle formulations similar to those used in the oral experiments (Table 2). As with oral immunisation, the addition of rCTB to the HSA-conjugated particles failed to improve the immune response. Although the adjuvant effect of CTB is not fully understood, binding to the GM1 receptor seems to be a key factor, and conjugating CTB rather than just mixing it with the antigen appears to be preferable in this respect [106]. While conjugated rCTB still binds to the GM1 receptor (Figure 9), it is possible that the signalling events are somehow affected by conjugation to the particles, so that the immunomodulatory signals are inhibited. This hypothesis is supported by recent studies which showed that although mutated LTB and CTB bound to GM1, subsequent immunomodulatory properties were absent [127, 128]. This tends to support our finding of a lack of improvement of the immune response to HSAmp by the addition of rCTB after subcutaneous immunisation, despite binding of rCTB to GM1.

However, some changes could be seen. When studying the rCTB-specific response, it was found that the presence of HSA on the same particles lowered the IgM+IgG response (Figure 10) and the DTH response (Table 3). These effects were not seen when the antigens were given on separate particles. Also, it was found that the HSA-specific subclass ratio was lowered when rCTB was present on separate particles (HSAmp + rCTBmp) but not when conjugated together on the same particles (Table 4). We propose, therefore, that HSA is more Th2-directing than rCTB, and the more dominant antigen when HSA and rCTB are taken up, processed and presented by the same APC. The decreased subclass ratio seen when the antigens were conjugated to separate particles is possibly a “bystander” effect of the microenvironment induced by rCTB. On the other hand, rCTB is known to induce a mixed Th1/Th2 response with both IgG1 and IgG2a antibodies [129, 130], but the response is obviously skewed more to Th1 with rCTB than with HSA. Hence, it seems that the observed differences after subcutaneous im-
munisation with HSA- and rCTB-conjugated particles are more an effect of the presence of the two different antigens rather than of rCTB binding to GM1 receptors and its immunomodulating properties.

Figure 10. rCTB-specific IgM+IgG response after subcutaneous immunisation (s.c) according to Table 2. Mean antibody titres are given as –log₂ (dilution x 10) + S.E.M. * The mean antibody titre was statistically significantly lower (P < 0.01) in the HSA/rCTBmp group than in the rCTBmp group on day 56.

Table 3. rCTB-specific delayed-type hypersensitivity (DTH)⁶ response after subcutaneous immunisation (s.c) according to table 2.

<table>
<thead>
<tr>
<th>Group/ treatment</th>
<th>% Increase in ear thickness⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>rCTBmp s.c.</td>
<td>37.9 ± 3.8 (n=5/6)</td>
</tr>
<tr>
<td>HSA/rCTBmp s.c.</td>
<td>12.5 ± 4.5* (n=6/6)</td>
</tr>
<tr>
<td>HSAmp + rCTBmp s.c</td>
<td>32.1 ± 6.6 (n=6/6)</td>
</tr>
<tr>
<td>Naïve</td>
<td>4.1 ± 4.3 (n=6/6)</td>
</tr>
</tbody>
</table>

⁶ The DTH response was measured as the increase in mouse ear thickness 72 h after injection of 10 µL rCTB in the ear two weeks after the last booster. *Values represent means ± S.E.M. *The mean DTH response was statistically significantly lower in the HSA/rCTBmp group than in the rCTBmp group (P < 0.01).

Table 4. HSA-specific subclass ratio on day 56 after subcutaneous immunisation (s.c.) according to table 2.

<table>
<thead>
<tr>
<th>Group/ treatment</th>
<th>Subclass ratio²</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSAmp s.c.</td>
<td>20.2 ± 5.7 (n=6/6)</td>
</tr>
<tr>
<td>HSA/rCTBmp s.c.</td>
<td>13.4 ± 2.8 (n=12/12)</td>
</tr>
<tr>
<td>HSAmp + rCTBmp s.c</td>
<td>6.7 ± 1.5 (n=12/12)</td>
</tr>
</tbody>
</table>

² Values represent means ± S.E.M. of serum HSA-specific ratios of IgG1/(IgG2a+IgG2b) two weeks after the last booster. * The mean ratio was statistically significantly lower in the HSAMP + rCTBmp group than in the HSAMP group (P < 0.05).
5.3 Effects of chronic stress on oral immunisation

That stress affects both systemic and mucosal immune responses after parenteral immunisation has been well established in a number of different species [131-138]. However, the effects of stress on oral immunisation are much less well known. An additional factor when it comes to oral immunisation compared to parenteral immunisation is the effect of stress on the intestinal epithelium. For example, exposure of rats to repetitive stress results in increased intestinal permeability, bacterial attachment and mast cell hyperplasia, and initiation of inflammation [139, 140]. Interestingly, chronic stress in rats also causes modulation of the barrier function of the FAE, allowing increased uptake of luminal antigens and bacteria [141]. In paper IV, we hypothesised that this increased uptake of luminal antigens could lead to increased antigen transport, subsequently inducing an abnormal immune response during oral vaccination. As HSAMP are taken up mainly via the FAE (Figure 7) and are similar in size to bacteria, it was considered a good model vaccine for studying this hypothesis. However, since the stress model has previously been evaluated in rats, this study was also performed in Wistar rats instead of the BALB/c mice used in the other studies. The experimental process is outlined in Figure 11.

![Figure 11. Experimental outline for the stress and immunisation process. Rats were submitted to daily water avoidance stress for two periods of ten consecutive days (stress) or kept in their home cage as controls (non stress). On the last three consecutive days in each session, the rats were immunised orally with HSA-conjugated microparticles (HSAMP) or physiological saline as control.](image)

During the two stress periods, the rats showed a reduction in body weight gain and an increased defaecation rate. The stress effects on the mucosal
barrier function were evaluated in a modified Ussing chamber [142]. Intestinal specimens from the rats were stripped from external muscles and myenteric plexus, and segments of villus epithelium and FAE were mounted in the chambers. Permeability studies using $^{51}$Cr-EDTA and horseradish peroxidase were carried out and the electrophysiological parameters were monitored both as a measure of barrier function and tissue viability. The effects of two stress periods (during primary and booster immunisation) on the intestinal physiology and mucosal permeability in our study were in accordance with the earlier study [141] where only one stress period was employed. Details can be found in Paper IV and in Velin et al. [141].

The mucosal immune response in the rats was affected by the chronic stress. When using immunohistochemistry to study the distribution of CD4+ cells, CD8+ cells and DCs in the follicles of Peyer’s patches, the expression of CD8+ was lower in the inter-follicular regions of stressed immunised rats than in the same regions of non-immunised controls. Interestingly, in stressed immunised rats, DCs were found both surrounding and inside the follicles whereas, in non-immunised control rats, DCs were found only surrounding and not inside the follicles. The CD4+ cells were found in the follicles of all rats and there were no differences in expression among any of the groups. The s-IgA response was analysed but no HSA-specific s-IgA was detected. Therefore, we are unable to say whether stress, as studied in our system, affects antigen-specific s-IgA levels. However, we found higher levels of total s-IgA in the faeces of stressed versus non-stressed rats. This higher level of intestinal total s-IgA could be a consequence of non-specific stress-induced barrier dysfunction and an inflammatory response.

Table 5. Flow cytometry analysis of rat CD4+ and CD8+ splenocytes a

<table>
<thead>
<tr>
<th>Group</th>
<th>CD4+ % of gate</th>
<th>CD8+ % of gate</th>
</tr>
</thead>
<tbody>
<tr>
<td>I, stress and non-immunisation</td>
<td>35.8 ± 2.2</td>
<td>14.6 ± 1.3</td>
</tr>
<tr>
<td>II, non-stress and non-immunisation</td>
<td>29.0 ± 2.0</td>
<td>19.7 ± 1.6</td>
</tr>
<tr>
<td>III, stress and immunisation</td>
<td>49.7 ± 3.4</td>
<td>14.1 ± 1.4</td>
</tr>
<tr>
<td>IV, non-stress and immunisation</td>
<td>44.3 ± 2.8</td>
<td>18.8 ± 1.9</td>
</tr>
</tbody>
</table>

a Splenocytes were prepared from spleens collected after the last stress session and labelled for CD4 or CD8 expression. b The groups were treated according to Figure 11. Means ± S.E.M. n=3; immunisation significantly increased the percentages of CD4+ cells (P<0.001) and stress significantly decreased the percentage of CD8+ cells (P<0.05).

The effects of stress on the systemic immune response were also addressed; the humoral HSA-specific IgM and IgG responses, the cellular DTH response and the proportions of CD4+ and CD8+ splenocytes were assayed. Several studies have shown a negative correlation between both acute [135,
and chronic stress [133, 136, 137] events and the systemic antibody response after parenteral immunisation. In our study, with chronic stress and oral immunisation, there were no changes in the systemic antibody response. This may be due to differences in the development of an immune response after oral and parenteral immunisation. Although the humoral response of the rats was unaffected by stress in this study, the cellular response was altered. The proportion of CD8+ splenocytes was significantly decreased in the stressed rats. The CD4+ splenocytes were increased as a consequence of the immunisation but were not affected by stress (Table 5). Moreover, the main finding in this study was that stress also significantly increased the antigen-specific DTH response (Figure 12). This suggests the presence of an exaggerated cell-mediated immune response after oral immunisation in stressed rats. Our findings support those of a recent study showing that psychological stress before immunisation exerted an adjuvant effect on skin DCs, which increased their migration and enhanced the DTH response after parenteral immunisation [143]. Other studies have also shown a reduction in the absolute number of T lymphocytes [144, 145], as well as in the CD8+ subpopulation [146, 147], in blood following stress in rodents, representing a redistribution of the lymphocytes from blood to organs such as bone marrow, lymph nodes and skin as a result of acute stress [148]. It has been proposed that trafficking of CD8+ lymphocytes to the skin is part of the enhanced cell-mediated response to acute stress [143]. However, previous studies of the effects of chronic stress on the cell-mediated immune response give conflicting results [149], which seem to depend on the duration and type of stress employed. We saw a clear increase in the DTH response after oral immunisation, suggesting that there may be differences in the effects of stress on the cell-mediated response between oral and parenteral immunisation routes; the stress-induced effects on the FAE could therefore be of importance. The enhanced immune response seen in the cellular arm but not in the humoral arm of the immune response to HSA could be an effect of differences in the Th1 and Th2 response. However, the Th1/Th2 profile, based on the IgG subclass profile in serum, did not show any influence of stress. Thus, the differences seen between the cellular and humoral arms might instead reflect the different compartments of the immune system: skin versus blood.
Figure 12. The delayed-type hypersensitivity (DTH) response was measured as the increase in rat ear thickness after injection of 10 µL of HSA in the ear two weeks after the booster immunisation. The ear thickness was measured before and 24, 48, and 72 h after injection. Group I, stress and non-immunisation; Group II, non-stress and non-immunisation; Group III, stress and immunisation; Group IV, non-stress and immunisation. Bars represent means ± S.E.M. n=2 in groups I and II and n=4 in groups III and IV; *P<0.05 for Group III vs. Group IV.

In conclusion, oral immunisation is obviously affected by chronic stress. This could be an effect of stress-induced increase in the uptake of antigens in the FAE resulting in induction of increased numbers of DCs within the follicles and redistribution of CD8+ lymphocytes.

5.4 The microparticles as oral adjuvant in man

In paper V, the starch microparticles were evaluated as an oral vaccine adjuvant in man. We decided that the microparticles might be best tested as a booster diphtheria vaccine. There are several reasons for this view. Most importantly, the effect of diphtheria vaccination is easily tested by following the diphtheria toxin-neutralising antibodies in a Vero-cell assay [150]. A value above 0.10 International Units per ml serum is considered as protective [151]. Thus, a significant increase in antibody titre would offer an adequate indication that the microparticles would function as an adjuvant as they did in mice. Moreover, the booster design was considered to be a more straightforward method, as a primary immune response might be more difficult to trigger. Also, diphtheria is a good choice from a commercial point of view. Experience suggests that herd immunity in a large proportion of the adults in western society is not currently sufficient to protect the population if a diphtheria epidemic spread from countries where the infection is still endemic [152]. The need for adult vaccination is therefore well documented. In addition, the risk of infection for the volunteers during the trial would be low.
Diphtheria toxin cross-reacting material (CRM197) was found to be the best candidate antigen to use in this study [153]. CRM197, unlike diphtheria toxin, is a natural non-toxic antigen which does not require formaldehyde detoxification and can be obtained at close to 100% purity [152]. It is also well tolerated in humans and is already used commercially as a carrier protein for carbohydrate antigens, for example from *Haemophilus influenzae* type b [154]. However, mild formaldehyde treatment was necessary in order to increase its oral stability and immunogenicity [153, 155].

The vaccine was tested in healthy pharmacy student volunteers at Uppsala University who had been vaccinated against diphtheria in the Swedish national vaccination programme and who had not received any booster vaccination in the last 5 years. Of 39 volunteers, 20 healthy volunteers with the lowest background levels of diphtheria toxin-neutralising antibodies (analysed in a Vero-cell assay) were chosen. The volunteers, in a fasting state, were divided into two groups and received 6 mg microparticles (conjugated with 210 µg CRM197) or 10 mg microparticles (350 µg CRM197) per dose on days 0-2 and 21-23. Blood samples were taken on days 21, 41 and 3 months after the first immunisation and analysed for antigen-specific IgG with ELISA and diphtheria toxin-neutralising antibodies in Vero-cell assay. Despite standardised ELISA and Vero-cell analyses and careful statistical treatment of the data, no increase in titres was detected. Thus, our study clearly shows that the starch microparticles did not function as an oral adjuvant in man.

The reason for the good results in mice but not in man obviously lies in the anatomical and physiological differences between mice and man. Factors that could differently affect the outcome of an oral vaccination in different species include transit time, dilution effects and possible differences in the density of the Peyer’s patches. Thus, it is necessary to find a new formulation of the starch microparticles, one which is more stable than that used in the trial but still able to deliver the antigen in such a way that an adequate immune response can be induced.

It is not only the stability of the particles that is important, it is also critical that the particles are taken up in sufficient amounts. Because of experimental difficulties, there are no available data on particle uptake after oral administration in man, so current understanding is based on experience from animal studies. There are conflicting reports on the proportion of orally administered microparticles that is taken up over the intestinal epithelium [115, 156, 157]. In many cases, hydrophobic polystyrene particles have been studied; because these adhere far better than less hydrophobic particles to the M cells, the results may be overestimated compared with biodegradable microparticles. In paper II, although a large amount of particles were injected into the
intestinal loop, only a few particles were detected in the epithelium. No quantitative data were obtained, but one could conclude that the uptake was scarce. Nevertheless, in these mice, a sufficient amount of the particles reached the immunocompetent cells to trigger an immune response. In man, the larger dilution may have further hampered the low uptake.

The uptake of antigens through the M cells is believed to be crucial for induction of a protective mucosal immune response after oral immunisation. This was also our experience in paper III, where rCTB conjugated to the particles improved the uptake in the villus epithelium but not the antigen-specific immune response. Efforts are being made to find a ligand specific for human M cells for targeting and increasing the particle uptake [158]. However, the formulation still has to reach the Peyer’s patches and the M cells with an intact antigen.

Moreover, even if the frequency of M cells in the Peyer’s patches is the same (about 10%) in man and mouse [159], it is unclear if there are enough patches in the proximal part of the human ileum to achieve the desired response. In man, Peyer’s patches are denser in the distal part of the ileum than in the upper part, where most of the particles are assumed to be still intact [160]. This suggests that a more stable particle could be more likely to reach the patches in the distal part of the ileum.

The dose of the microparticles also requires consideration. The mice were given 3 mg per dose and the volunteers in our study were given 6 or 10 mg per dose. Thus, it might be possible to provoke an immune response by increasing the dose of the vaccine, but this would probably not be economically feasible on a large scale.

5.4.1 Possible means for improving the adjuvant effect of the microparticles in man

It is possible to prepare a more stable particle by increasing the acryloylation of the starch. A higher degree of cross-links in the starch molecules would result in denser, and therefore more stable and less degradable, particles and vice versa. However, if the particles are made more stable, they might not be degradable in the APCs and, as a consequence, the adjuvant activity would be lost. It is clear that the particles used currently are taken up by APCs, degraded, processed and capable of inducing a strong immune response in mice. This intracellular processing is thought not to be different between mice and man. Therefore it seems preferable to keep the present composition of the particles.
One theoretical possibility for improving the formulation would be to make larger particles of the same composition that may survive the transit through the gastrointestinal tract better, resulting in particles of a suitable size in the distal part of the ileum where uptake might be more favourable. The difficulties with this approach would be to predict the size of the particles needed and the manner of fragmentation and degradation in the gastrointestinal tract. The most feasible alternatives would thus be enteric-coated particles or an enteric capsule formulation. The microparticles can be freeze-dried and a dry formulation suitable for enteric capsules could be developed. This would be the most attractive way to improve oral adjuvant activity of the starch microparticles in man.
6 Concluding remarks

In this thesis, different aspects of the use of starch microparticles as an oral adjuvant were studied in order to improve our basic understanding of their uptake and presentation to the immune system. From these studies, it is concluded that:

- The route of administration of the microparticles for immunisation (oral, subcutaneous or intramuscular) affects the profile (Th1/Th2-balance) of the induced immune response. This could be an effect of differences in the microenvironment (cytokines and APCs) present where the antigen presentation takes place. Also, only immunisation regimens including oral boosters elicited a significant s-IgA response. Thus, by using different combinations of routes of administration it might be possible to optimise the character of the immune response induced for each individual vaccine.

- After oral immunisation, the microparticles are taken up mainly over the FAE that covers the Peyer’s patches. However, when rCTB was conjugated to the microparticles, the particles were also taken up in the villus epithelium. This was an effect probably mediated by rCTB binding to the GM1 receptors on the enterocytes. Nonetheless, the qualitative difference in uptake did not improve or influence the profile of the induced immune response. Thus, the uptake in villi did not improve the immune response. This may have been a consequence of extensive degradation of the antigen-particle complex in the enterocytes or, alternatively, of the induction of a local suppressive response.

- Chronic stress, known to alter the barrier function of the FAE, increased the cellular response after oral immunisation but did not affect the humoral immune response. Suggested mechanisms for this increase include increased antigen uptake in the FAE, increased induction of DCs within the lymphoid follicles, and redistribution of CD8+ lymphocytes.

- Despite positive results in rodents, the particles were not able to boost the humoral immune response in man. This is possibly a consequence of the longer gut transit time and dilution effects in man together with possible differences in the distribution of Peyer’s patches between the species. Thus, the particles may have been degraded before they reached the distal
part of the ileum where the density of Peyer’s patches in man is high. This problem could feasibly be overcome by administering the vaccine in an enteric capsule after freeze-drying the microparticles.
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/Linda
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